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HEAT-INDUCED ALTERATIONS IN THE CELL NUCLEUS

Relation to hyperthermic cell killing and radiosensitization

Harm H. Kampinga

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RIJKSUNIVERSITEIT GRONINGEN

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Relation to hyperthermic cell killing and radiosensitization

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Alles wordt berekend
niets blijft onbekend
zoek niets

Overal komt narigheid van
nergens is vrede
wees nergens

Iedereen wil bewijzen
iedereen wil iets zien
Niemand heeft vertrouwen
niemand durft geloven
wees niemand

(Gebaseerd op: "De Openbaring"
van Freek de Jonge)

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Ionizing radiation

1.1.1 Types of ionizing radiation

The absorption of radiation energy in biological material may lead to excitation (raising an electron in an atom or molecule to a higher energy level, without electron ejection) or ionization (ejection of one or more electrons) of an atom.
Ionizing radiation releases a large amount of energy (> 33eV) per ionizing event and can be divided into two categories:

1) electromagnetic radiation (X-rays, gamma-rays)
2) radiation particles (electrons, protons, α-particles, neutrons, negative pi-mesones, heavy, charged ions)

Electromagnetic radiation is sparsely ionizing (low linear energy transfer (low LET)), while most particulate radiations are densely (high LET) ionizing(1).
The majority of radiation damage to biological molecules occurs indirectly by water-derived radicals. Direct energy absorption of ionizing radiation by biomolecules represents only a minor portion of the radiation damage.

1.1.2 Radiation dose

The radiation dose can be defined as the amount of energy deposition in the irradiated matter. The unit for radiation dose is the Gray (Gy) which is defined as the absorption of 1 Joule (J) per kilogram of tissue. In the general description of radiation effects the absorbed dose (in Gy), multiplied by the quality factor (QF) of the specific type of irradiation, is used. This "dose equivalent" allows for one to gauge the relative effectiveness of a particular type of radiation and is expressed in Sieverts (Sv). For γ-and X-rays the quality factor, by definition, is 1 and thus Gy and Sv are interchangeable for these types of radiation. For neutrons, for example, the

situation is different; these are about 10 times more effective than X-rays (QF = 10) and thus 1 Gy of neutrons represents 10 Sv (2)

1.1.3 Clinical application

Since the discovery of X-rays in 1895 by Wilhelm Conrad Roentgen, the use of radiation in medicine has developed enormously. X-rays now have become one of the most powerful tools in diagnostic medicine (2). The use of radiation in the treatment of diseases has also developed early after the discovery of X-rays and, especially, after the Second World War (2). Radiation therapy is based on the ability of ionizing radiation to kill cells and/or stop their ability to proliferate and thus, in theory, can be used to stop the uncontrolled division of malignant cells. Its applicability is, however, restricted and it is certainly not the cure-all for cancer; often it can only be used in a palliative way. At the moment about 50% of all cancer patients will receive some kind of radiation therapy; 50% of these are treated for palliation. Of those who are treated in order to be cured only about 50% will be cured indeed. Thus only 12.5% of all cancer patients (based on data from the United States) are cured by radiation therapy (2). During the last 10 years, radiotherapy has evolved greatly and the development of better radiation equipment (e.g. neutrons produced by a cyclotron) may hopefully lead to improvement of cancer cure. Other methods to improve the clinical response of tumors to radiation consist of combinations of radiation with drugs and/or hyperthermia. Especially the latter will be discussed in more detail in this thesis.

1.2 Cellular and molecular radiobiology: a short introduction

1.2.1. Radiation survival curves

In order to mathematically describe the effect of a certain radiation dose on the reproductive capacity (survival) of cells the term "target" has to be introduced. A target may consist of an essential function of the cell necessary for its survival (3). Several models can be used to describe radiation survival curves.

The most simple model assumes that only one vital target has to be hit for a cell to be killed and that this killing can be accomplished by one hit only. This single-hit model ($S = e^{-D/D_o}$; where S is cell survival, D is the dose and D_0 the dose resulting in 1/e (37%) cell survival) does not describe most cell survival curves after low LET, that display a shoulder part in the low dose range when survival is plotted logarithmically versus radiation dose (linearly)(4).

The multi-target single-hit model ($S = 1 - (1 - e^{-D/D_o})^n$) can be applied to shouldered survival curves (5). For the high dose range the model can be expressed as: $S = ne^{-D/D_o}$, where n is the extrapolation number, representing the theoretical number of targets to be hit to cause lethality. As a measure for the width of the survival curve, often the "quasi threshold

dose" (D_q) (apparent dose that for a linear lnS - dose response curve produces no cell kill) is used. This parameter can be calculated by the formula: $D_q = \ln n \times D_o$. The characteristic parameters of the multi-target model (D_o, D_q and n) are represented in figure 1A.
Another model that is more and more used in the field of clinical radiation biology is the so-called linear-quadratic (LQ) model (6,7), given by the expression: $S = e^{-(\alpha D + \beta D2)}$.
In this model α is the rate constant for a single hit process, linear with dose, and β is the rate constant for a double-hit process, its probability quadratically increasing with dose. A survival curve according to the LQ-model is visualized in figure 1B.
The multi-target-single-hit model is used often in cellular and molecular radiation biology and is, for practical reasons also, used to describe the survival curves in this thesis.

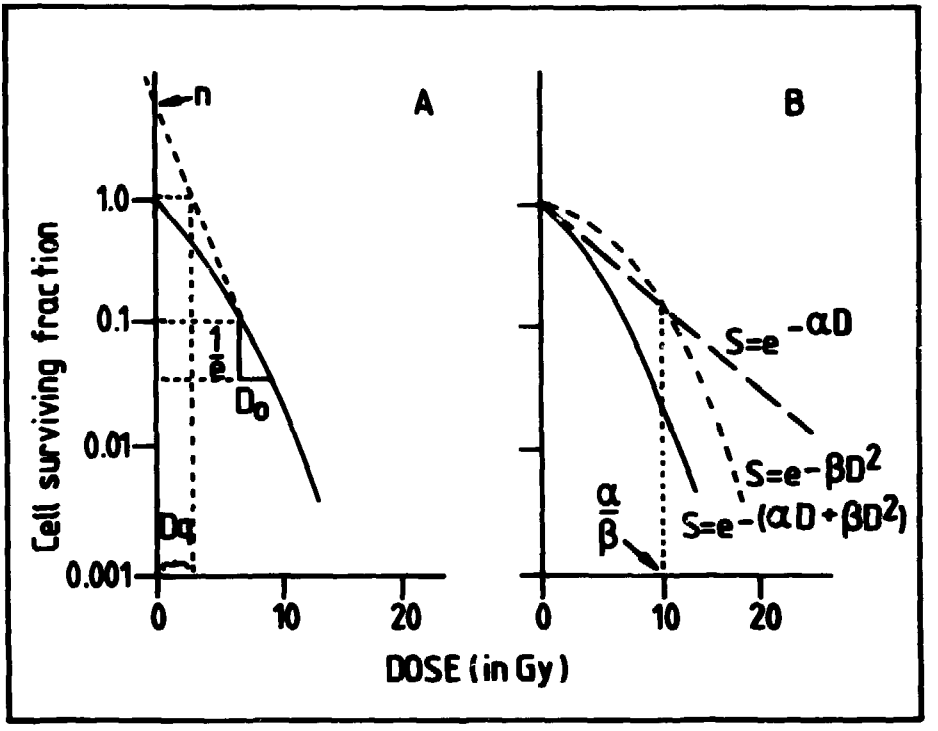

Figure 1: Radiation survival curves.
A: Visualization of the survival curve using the multi-target single-hit model. The parameters D_o, D_q and n are indicated in the figure.
B: Visualization of the survival curve using the linear-quadratic model. The curve can be separated into a linear ($e^{-\alpha D}$) and quadratic ($e^{-\beta D2}$) component. The α/β ratio is the dose (in Gy) where the contribution of the linear component equals that of the quadratic component in determining cell survival.

1.2.2 Molecular targets for radiation-induced cell killing

Two types of radiation-induced cell death may be distinguished in mammalian cells (8): reproductive death and interphase death. The mechanism of cell death is different for these two types (9,10). Radiation damage will take place in all classes of molecules including DNA (see 1.4), RNA (11), proteins (12), carbohydrates (13) and lipids (9,10,14). For dividing cells intact DNA is essential for successful mitosis, and the loss of a cell's ability to undergo (unlimited) cell division is called reproductive cell death. Reproductive ability is generally measured as the capacity of a cell to produce a colony of at least 50 cells (clonogenic ability). At biologically relevant doses ionizing radiation has been shown to cause damage to the DNA (see 1.4) and under various conditions modifications of cellular radiation sensitivity can be correlated to changes in the induction of chromosomal abberations in proliferating cells (15-18). Further details are presented in section 1.4.6. Interphase death can be defined as the impairment of cellular metabolism followed by disintegration of a cell before entering mitosis (8,9). Interphase death is usually measured as the loss of the cell's ability to exclude a dye (e.g. trypan blue) or its inability to maintain a normal ion-balance (e.g. K^+-loss). In dividing cells this only occurs at much higher doses (20-400 Gy) than those necessary to cause reproductive death (0-20 Gy)(9,10). In non-dividing cells (e.g. lymphocytes) interphase death can already occur after relatively low doses. Membranes are thought to be the targets involved in interphase death (9,10).

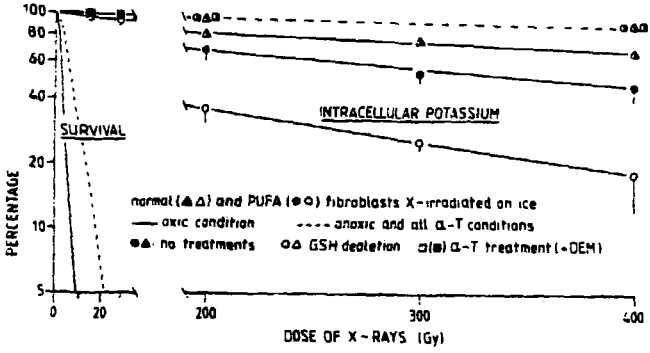

Figure 2: Comparison of the effect of X-irradiation on clonogenic ability (survival) and potassium content (interphase death) in normal and PUFA cells. (see text for details). [Taken from reference 9, with permission]

Figure 2 illustrates the difference between radiation-induced reproductive death (expressed as survival) and radiation-induced interphase death (K^+-loss) in mouse fibroblast LM cells. Modification of cellular membranes in terms of

their content of polyunsaturated fatty acids (PUFA) has no effect on the clonogenic ability of cells (survival) but has a clear effect on interphase death (K^+-loss). Also protectors of PUFA such as α-tocopherol (α-T) and GSH are only effective in preventing interphase death. In these experiments, GSH depletion is accomplished by treating the cells with diethylmeleate (DEM). For further information about differences between interphase and reproductive cell death see recent reviews by Konings (9,10).
In the experiments described in this thesis dividing cells and radiation doses < 20 Gy were used throughout; radiation-induced cell killing will thus concern reproductive cell death.

1.2.3 Radiation and cell cycle effects

The sensitivity to ionizing radiation varies with the cell cycle stage. Cells in or close to mitosis (M) are most sensitive, while cells in S-phase are most radioresistant (19). Furthermore, radiation causes a delay in cell cycle progression, with cells accumulating in G_2 (20) and a transient inhibition of DNA synthesis (21).

1.2.4 Cellular repair phenomena

The "shoulder" of a radiation survival curve (see section 1.2.1) is often interpreted as derived from repair of sublethal damage. When doses of low LET radiation (that are well above the shoulder region) are separated in time, cell survival increases with increasing time interval. The reappearance of the shoulder in the second dose response curve is called sublethal damage repair (SLDR)(3,22). High LET radiation is known to be unfavorable to allow such repair of radiation damage and high LET radiation (therefore) does not result in large-shouldered radiation survival curves. Another cellular repair phenomenon is seen when cells are held under such post-irradiation conditions that they cannot proliferate but still can metabolize (and perform repair). This type of recovery is operationally defined as potentially lethal damage repair (PLDR). If survival is depressed as compared to the "normal situation" by modifications of the post-irradiation conditions, the enhanced radiosensitivity is often interpreted as enhanced expression of potentially lethal damage (23). For further explanation of SLDR and PLDR see current textbooks (e.g., references 1,2).

1.3 DNA topology: just folding or more?

The first evidence that DNA, deoxyribonucleic acid, carries the genetic information of a living cell was given in 1944 by Avery et al. (24). Its structure was then discovered by Watson and Crick in 1953 (25). DNA is a polymer of nucleotides. The nucleotides consists of bases (adenine, guanine (purines) and cytosine, thymine (pyrimidines)) linked to a phosphorylated deoxyribose. The bases are linked to each other via phosphodiester bonds. The "genetic code" is based on the sequence of the bases. Two DNA strands

are coiled around each other's axis and form a double helical structure with the sugar-phosphate backbone on the outside and the bases on the inside. The two chains are held together by hydrogen bonds between pairs of bases, adenine always pairing with thymine and guanine always with cytosine. The precise sequence of the bases along the DNA chain carries the genetic information (26,27).

1.3.1 Folding of eukaryotic DNA

The amount of DNA reaches up to a contour of several centimeters per metaphase chromosome in the human cell (26). Forty-six of these chromosomes are packed in a human cell nucleus which is only a few microns in diameter. This shows the need for an enormous degree of DNA-packing, that has to be very dynamic in order to allow all kinds of DNA-associated-processes. The various levels of DNA packing are given in figure 3.
The first level of DNA packing is into nucleosomes; 146 basepairs of DNA are wrapped around an octamere consisting of two molecules each of the low molecular weight histone proteins H2A (14.5 kD), H2B (13.8 kD), H3 (15.3 kD) and H4 (11.3 kD). The joint between two such "core"-particles is the so-called "linker"-DNA (20-100 bp) which might be associated with histone H1 (21 kD) (27-29). The next level of DNA packing is the winding of these nucleosomes into a supercoiled helix (6 nucleosomes per turn): a solenoid (29-31). The third level of DNA folding in both interphase and metaphase appears to be the folding of the solenoids into loops or domains (32-34). These loops (10-180 kbp = 3-60 μm, with an average of 63 kbp = 21 μm for all studies: 35) are believed to be anchored to a non-histone protein network called "protein- scaffold" (metaphase: 33) or "nuclear matrix" (interphase: 36). The DNA-loops (in vitro) are highly supercoiled (37,38), their topological constraint being provided through anchorage to the nuclear matrix or -scaffold.
The architecture of the nucleus is of a staggering complexity. Given the fact that many DNA-associated processes such as replication, transcription, and repair have to be carried out with accuracy and fidelity, and sometimes involve rather large structural rearrangements (both spatial and temporal, e.g., in mitosis), one is compelled to think of the nuclear matrix as a dynamic, malleable structure (35). It is conceivable that the interplay between DNA and matrix divides up the nucleus into functional, structured domains. Whether the nuclear matrix is such a malleable structure, how it can be isolated, what its composition is and how it relates to DNA-associated-processes, will be discussed below. A schematic representation of the cell nucleus with the DNA attached to the fibrillar internal network of the matrix is given in figure 4 which is based on the data above and a review by Verheijen et al. (39 and references therein; see also 40-42).

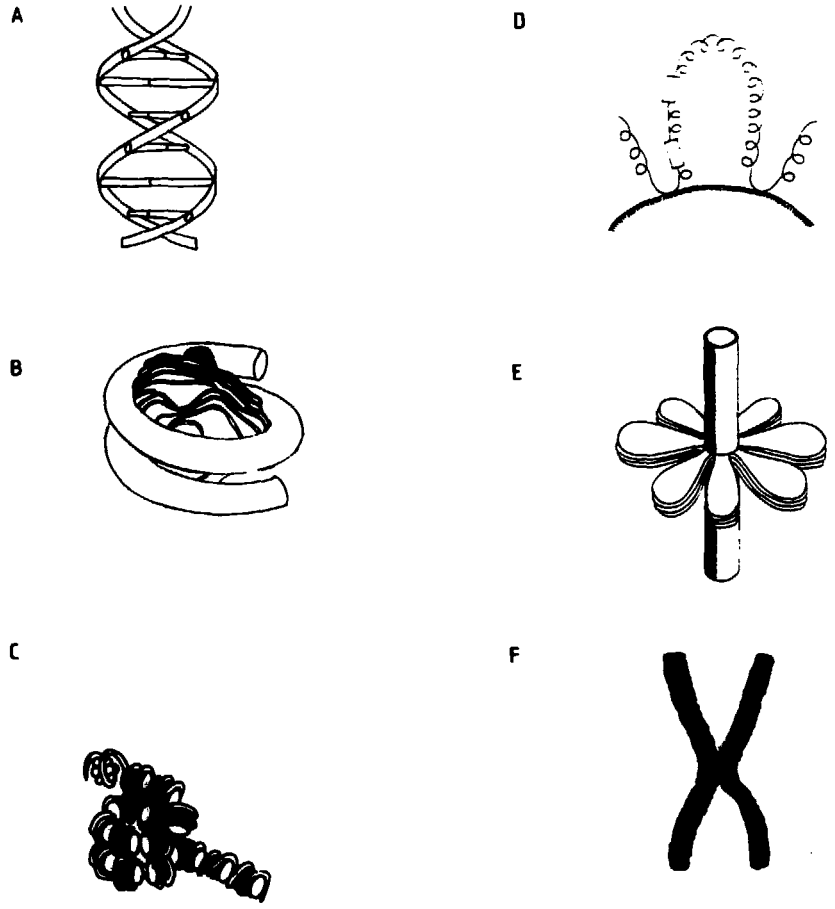

Figure 3: Organization of eukaryotic DNA: Linear DNA (A) is folded into nucleosomes (B) that are ordered into solenoids (C). The solenoids are wound into superhelical DNA loops attached to a nuclear matrix (D) in radial arrays called mini-bands (E) which form chromosomes (F).

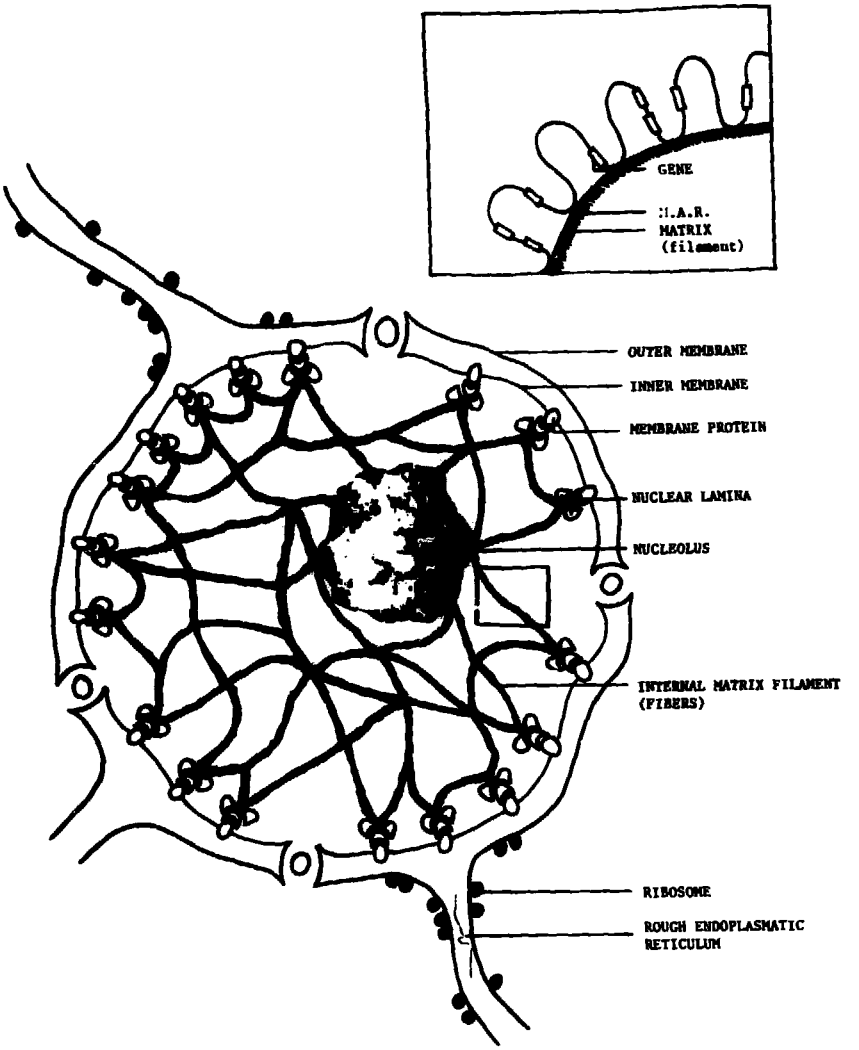

Figure 4: Schematic representation of the cell nucleus (after Verheijen et al: 39). The cell nucleus is surrounded by the nuclear envelope (inner and outer nuclear membrane, nuclear pores and nuclear lamina). The outer membrane appears to be continuous with the rough endoplasmatic reticulum and is covered with ribosomes. The internal matrix filaments are anchored to the inner membrane through the nuclear lamina which may be attached to the inner membrane through integral nuclear membrane proteins (40). In the insert, it is shown how the DNA is attached to nuclear matrix in (highly supercoiled) loops. The (permanent) attachment sites are drawn; they are called "Matrix Associated Regions (MARs)"(see 1.3.2).

1.3.2 Nuclear matrix: isolation, composition and ultrastructural aspects

The nuclear matrix is a structure that can be isolated after 1-2M salt extraction and nuclease digestion of isolated nuclei. In interphase rat-liver nuclei, it is a three dimensional network and has the same size as the corresponding nuclei. It consists mainly of proteins (98%), some RNA (1%), phospholipids (1%) and some residual DNA (43). Similar structures were found in other mammalian cells (44,45), in Drosophila cells (46) and cells from many other species (42). Current insight in the protein-composition of the nuclear matrix is still rather poor and the function(s) of most of its proteins is not clear as of yet. Perhaps the most well known and characterized proteins of the nuclear matrix are the nuclear lamina and nuclear pore complexes; the nuclear lamina are dominant proteins in matrices isolated from mammalian cells (47,48). The differences in protein composition of the matrix that are observed in many studies may be inherent to differences in cell types investigated and/or isolation procedures used (compare e.g., references 49-52). Some studies suggest that nuclear matrix proteins reflect the cell type origin (53) and/or the differentiation state of cells (54).

The nuclear protein matrix consists of three major ultrastructures: nuclear pore complexes plus nuclear lamina, residual nucleoli, and an internal matrix framework (c.f. reference 36). About 2-5% of the nuclear DNA remains bound to the matrix, even after exhaustive nuclease digestion (36). Release of this DNA only occurs under relative harsh conditions, such as treatment of the matrix with a polar detergent or 5M urea (plus 2M salt treatment). This suggests that there is a strong interaction of this DNA and the nuclear matrix; the sites at which this interaction occurs have been named "permanent attachment sites" or "MARs" (matrix-associated regions) (49). The residual DNA appears to be associated with the internal matrix component (55). It is thought that the internal matrix fiber (figure 3E) is anchored to the nuclear lamina and the latter is presumably coupled to the inner nuclear membrane by integral membrane proteins (figure 4; 40). It is noteworthy that matrix-like structures ("nucleoids" (37)) can be obtained upon treating cell nuclei with high concentrations of NaCl, but without nucleases, in which as much as 90% of the DNA is retained, whereas most (or all) histones are released. The protein composition of these structures is quite similar to that of preparations obtained upon high salt treatment plus complete nuclease digestion (compare 36,43,49; see Berezney 42).

Sites of attachment of DNA to the nuclear matrix

Most likely, there are 2 types of DNA attachment sites at the nuclear matrix: stable ("permanent") and dynamic attachment sites (56). The dynamic sites can be found everywhere and their may be several of these sites within one loop; at these sites, binding of the DNA to matrix would be relatively labile (56). Although the precise nature of the permanent attachment point (Matrix Associated Region (MAR)) has not been revealed as yet, some ideas have developed over the last years. We do know that the interaction must be tight, since it has to restrict or prevent the transfer of superhelical energy

from loop to loop. Furthermore the interaction must be preserved throughout mitosis. So, per loop two of these attachment sites can be found at both ends. Interestingly, Lewis et al (57) found a 170 kD protein in both interphase and metaphase scaffolds, which was suggested to be topoisomerase II (topo II). Since this enzyme is involved in the control of DNA super-helicity by catalyzing breaking and rejoining reactions, by means of covalent enzyme-DNA interaction (58 and see 1.4.4), it would be a good candidate for constituting the attachment point. Later it was proven indeed that topo II is an component of both the nuclear matrix (59,60) and the mataphase scaffold (61,62). This so-called scaffold protein I (ScI) was shown by immunostaining to be localized at the base of the DNA loops (62). The DNA involved in the interaction with the nuclear matrix consists of an AT-rich spacer (a non-transcribed region) of 657 bp between the genes coding for histones H1 and H3; heat-shock-protein(HSP)-gene interaction was found to be at a 960 bp, AT-rich region upstream of the regulatory sequence (63). The MARs were found to be enriched (63,64-68) in sequences enriched in the topo II cleavage consensus sequence (69). Since these sites are close to regulatory sites in the DNA, MARs may act as positive and/or negative regulators e.g. by targeting enhancers to the nuclear matrix or via regulation of torsional stress by topo II (67). Interestingly, it was found that the topoisomerase II inhibitors preferentially act on newly replicated DNA (70) that is localized at the nuclear matrix (see 1.3.3). These inhibitors, by the formation of cleavable complexes (see Chapter 6), can cause the release of DNA fragments with sizes comparable to the sizes of DNA loops (61,62).
In Table 1 the characteristics of both stable and dynamic attachment sites are summarized (56)

Stable attachment sites	Dynamic attachment sites
two per loop	multiple sites per loop
stable (tightly bound to proteins)	relatively labile binding
clustered with: - DNA replication origins - enhancers - topoisomerase II sites	found everywhere

Table 2: Characteristics of DNA-matrix attachment sites at the nuclear matrix (after Bodnar (56))

Functions of the nuclear matrix

The matrix has been implicated to be involved in DNA replication, transcription and maybe also in DNA repair (these 3 putative functions will be discussed in detail in 1.3.3-1.3.5). In addition, its involvement in RNA processing (intranuclear transport and splicing) (71-73), steroid receptor

binding (74) and various other processes (see 42) has been suggested. There are even some interesting speculations on the role of the nuclear matrix in carcinogenesis (75).

1.3.3 The nuclear matrix and DNA replication

In analogy to bacterial replication, where processes such as replication seem to involve an attachment to the cell-membrane (76), it was suggested that the membrane of the nucleus of eukaryotic cells provided attachment points for replication (77,78). However it was shown that the membrane itself is not involved in DNA replication, but that replicon initiation rather occurs throughout the entire nucleus (79-80). Besides, the binding of the DNA to skeleton structures during metaphase (no nuclear lamina) (81) suggests no direct interaction between DNA (and its metabolism) and the nuclear membrane. Several studies have now revealed that the nuclear matrix is the site of DNA replication. In both interphase nuclei and metaphase chromosomes the DNA appears to be arranged in loops with a size roughly matching that of replicons (82,83 and see Berezney (84) for other references).
Most convincing evidence for the role of the nuclear matrix in DNA replication comes from studies with radioactive thymine incorporated into replicative DNA. The experiments indicate that DNA replication initiates close to the basis of the DNA loops and extends from there (48,82,85-94). Using autoradiography, Vogelstein et al. (38) directly visualized the movement of nascent DNA from the matrix into the DNA loop; these types of analysis also revealed that replication starts at the nuclear matrix by a "reeling-through" mechanism (38,89,95; see also figure 5). Other evidence for the role of the matrix in DNA-replication came from electron microscopic analysis of restriction endonuclease (EcoR1) resistant DNA fragments in nucleoids; a 3-6 fold enrichment of branched (replicating) DNA molecules was found attached to these structures (96). Interestingly, newly replicated DNA was found to be associated with topoisomerase II (97), the enzyme that is thought to be a part of the nuclear matrix positioned at the basis of the DNA loops (see above). Characterization of human DNA sequences formed at the onset of DNA synthesis revealed an AT-rich region with consensus binding sites for topoisomerase II (98), comparable to the MARs.

Replisomes at the nuclear matrix

It has been suggested that eukaryotic DNA replication requires several factors combined in a replicational complex called replitase or replisome (27,99-101). Besides the eukaryotic replicative enzyme, DNA polymerase α, this replisome might include: DNA-primase, diadenosine tetraphosphate binding protein, 3-5'-exonucleases, DNA methylase, ribonuclease-H, DNA-dependent ATPase, topoisomerases, enzymes for precursor nucleotide biosynthesis, primer recognition proteins and maybe many others (see 102 and references therein). If replication occurs at the nuclear matrix, than these enzymes would have to show a replication dependent association with the nuclear matrix. Less than 2% of DNA polymerase α activity was

recovered in matrices from normal liver cells, whereas more than 15% of the total cellular activity was recovered from actively replicating regenerating liver (103-106). This association was also found using a monoclonal antibody for DNA polymerase α (107). Later Wood and Collins (108) showed that also DNA primase is bound to the nuclear matrix; this binding correlated with the biphasic nature of DNA replication and it was found that in synchronized HeLa cells primase was present in the matrix complexed to DNA polymerase α (109). Tubo and Berezney (102) showed that there is a cell cycle dependent association with the matrix of various enzymes involved in replication: primase, 3'-5' exonuclease, methylase, and ribonuclease-H were found to be associated in a pre-replicative manner with the matrix, correlating with the prereplicative association of replisomes in which primase might play an initial role in binding to the origins of replication. Polymerase α was shown to associate with the matrix at the onset of S-phase. No DNA ligase was found matrix-bound while only a minor portion of DNA polymerase β was matrix-associated. The latter enzyme only showed a slight increase pre- and late-replicatively, suggesting a role of the enzyme in genome screening (102,104). The dynamic, cell cycle related association of these enzymes with the nuclear matrix provides additional evidence that the matrix is dynamically involved in replication.

In vitro DNA synthesis at the nuclear matrix

Finally, the ability of the nuclear matrix to carry out in vitro DNA synthesis (103) which for at least 50% involves continuation of Okazaki fragments in replication forks initiated in whole cells (106) and to incorporate ^{3}H-AMP (primase activity: see reference 108) further substantiates the evidence for a role of the nuclear matrix in DNA replication.

Mechanism of matrix-associated replication

The precise mechanism by which the DNA is replicated at the matrix has still to be elucidated. In synchronized cells it was shown that replication pulses in early S-phase could not be chased away into the DNA loops. This suggested that regions at or close to replication origins (= normal attachment sites) are permanently associated with the matrix (see figure 5B; redrawn from reference 95). During initiation of DNA synthesis, these origins duplicate (fig. 5B) and the replication fork becomes attached at positions in between neighboring origin binding sites and parental DNA loops are then reeled through the fork attachment sites (Fig 5C,D; 87-89,95,110). With the termination of replication the matrix filaments are duplicated and separation of the daughter molecules occurs, followed by a formation/separation of the matrix into chromosome scaffolds (Fig 5D,E; 81). After mitosis the interphase DNA-matrix is restored.

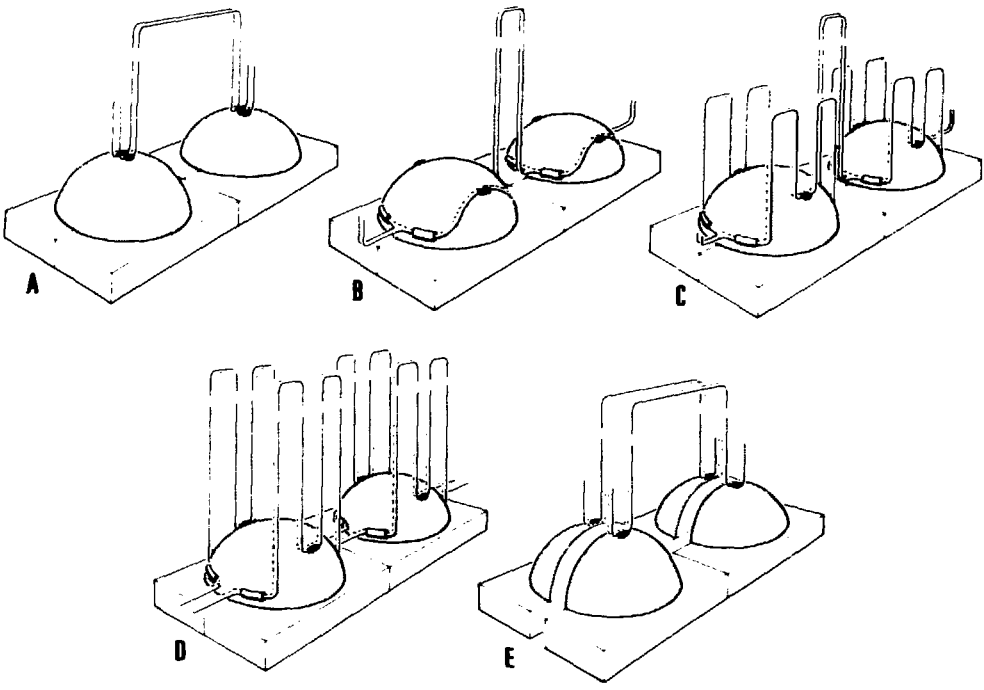

Figure 5: Schematic representation *of nuclear matrix associated DNA replication. For details see text. [taken from v.d. Velden and Wanka (95), with permission]*

1.3.4 The nuclear matrix and transcription

Uridine pulse incorporation studies

Several lines of evidence suggest that -apart from DNA replication- also transcription (and RNA processing) occurs at the nuclear matrix. Newly synthesized RNA, measured as pulse incorporation of ^{3}H-uridine, is bound to the nuclear matrix (42,111). Also, autoradiographic analysis of pulse- labeled RNA showed that newly transcribed RNA is matrix-associated (112,113). After a pulse- chase the ^{3}H-Uridine labelled regions remain matrix-associated (42,111). This, together with the finding that hnRNA is matrix-bound in the residual nucleolar structure (42), suggests that after transcription, the matrix functionally assembles RNA for further processing (e.g. splicing). Quite surprisingly, it has been found that the nuclear matrix is highly sensitive to RNase and falls apart when digested with this enzyme (114). This suggests that RNA plays a role in maintaining matrix structure.

Transcriptional complex at the nuclear matrix

It has been found recently (115) that RNA polymerases I and II may not be nuclear-matrix-bound proteins. Association or non-association, however, appears to be very dependent on the isolation procedure used; it cannot be excluded that the transcription complex is transiently and weakly bound to the nuclear matrix (60).

Active genes and the nuclear matrix

An enrichment of actively transcribed genes at the matrix was found by most investigators (116-123) but not by all (124). It seems that not transcriptional activity does not uniquely determines an association between gene(s) and matrix. Heat shock genes were found matrix-associated both before (low activity) and after heat shock (high activity). The actin gene was also nuclear matrix associated regardless of its transcriptional activity (120). Histone H2A and H1 genes are matrix-attached independent of their different transcriptional activity during the different cell cycle stages (116). An interesting model on how the matrix might be involved in transcription was recently published by Bodnar (56); in this model, gene expression is divided into three distinct steps:

1. uncoiling, which changes the chromatin structure of a loop, most probably through changes in supercoiling (regulated by topo II at the MARs?)
2. extension of an uncoiled loop, so that DNA sequences become accessible for "dynamic attachment" to the matrix.
3. promotor recognition by nuclear matrix-bound transcription factors, completing the process, preceding the actual start of transcription.

Interconnection between replication and transcription at the nuclear matrix

Since transcription and replication seem matrix-associated processes there might be interconnections between the two. One interesting speculation made by Small and Vogelstein (120) is that the location of active genes (near the matrix-attachment points) may explain why these are specifically replicated during the early part of the S-phase as was shown by Taylor (125). In this respect also the evolutionary relationship between origins of replication and origins of transcription is noteworthy (126).

1.3.5 The nuclear matrix and DNA repair

Repair pulse incorporation studies

The data on the existence of intranuclear localization of DNA repair patches have been apparently contradictory so far. Mullenders and coworkers did not observe a preferential association of a 10 minute repair pulse label with the nuclear matrix after irradiation with a high dose UV (30J/m2) of human fibroblasts VH-16, when replication was inhibited with HU (+/- ARA-C) (94,127). However, using a similar experimental set-up they did find nuclear matrix associated repair in XP-C cells (128) which was interpreted as

reflecting a preferential repair in active genes that are matrix associated (1.3.4). Using similar high doses of UV as well as high doses (1000 Gy) of radiation Gaziev et al (129) observed preferential pulse label incorporation in matrix-associated DNA of ZAH liver cells; they used HU to block replicative synthesis. In an autoradiographic study of unsynchronized HeLa cells, McCready and Cook (130) measured DNA repair synthesis after 15 and 40J/m2 UV-irradiation. No inhibitors were necessary, since S-phase cells (replication-labelled halo's) could be easily picked up and only non-S-phase cells were analyzed for repair. They found preferential repair at the matrix which was most prominent if the cells had been exposed to a relatively low UV-dose. Furthermore, the repair pulse label could not always be chased away. The experimental approach was rather poor and the number of measurements per condition were rather low, so that no definite conclusions can be drawn from this study. Studies by Harless and Hewitt (131), using autoradiography of DNA-halo's in VA13 human fibroblast cells, revealed that nuclear matrix associated repair was dose dependent (82% after 2.5J/m2 to 50% after 30J/m2 of UV). In recent studies, Mullenders et al. (132,133) also found matrix-associated repair after biologically relevant, low doses of UV (5J/m2), which was interpreted as to reflect the preferential repair of matrix-associated active genes (134-146). The same suggestion was given in interpreting the observation of matrix-associated repair of XP-C cells 128). These cells show limited repair after UV-irradiation, occurring non-randomly, with repair sites clustered in localized regions of the genome (147); these sites were suggested to be transcriptionally active sites (148). It was also found that Cockayne Syndrome Fibroblasts, that are partially deficient in preferential repair of active genes (149), do not show preferential repair of matrix associated DNA (133).

Possible mechanisms of matrix associated repair

The data cited above leave us with the following possibilities:

1. The matrix is not at all involved in repair. Since this was only suggested for very high but biologically irrelevant doses of radiation, this seems not very likely. Rather, the data indicate that there may be two systems for repair (one for "loop" DNA and one for matrix-associated DNA); these repair systems may be called upon in a different way and may become saturated at different dose-levels.
2. The repair of radiation-induced damage is matrix-mediated: repair is performed by enzymes that are constituents of the nuclear matrix as was suggested by Chiu et al (137). Supportive for this idea is the presence of enzymes at the nuclear matrix such as DNA polymerase 3'-5' exonuclease and DNA methylase (102); these enzymes are involved in DNA repair, apart from playing a role in replication.
3. The matrix-association of repair is a reflection of repair of the matrix-associated active genes; this fits in with the data obtained in XP-C cells (128) that preferentially repair active sequences (148). It also could explain why Cockayne's-Syndrome cells show preferential

repair of loop DNA and seem unable to repair sequences involved in active transcription (133). This also suggests that there might be two different repair systems: one for active and one for inactive genes.

Why should active genes be repaired more rapidly? Apart from the fact that the repair enzymes might be localized in the proximity of active genes, it could simply be a result of an increased accessibility of active DNA to repair enzymes, as was suggested by some investigators (139-141). This points to a possible relation between transcriptional activity and repair efficiency. The findings of Okumoto and Bohr (140) indeed suggest such a relation: they showed that preferential repair of the CHO methallothionein gene only took place after the gene had been transcriptionally activated. On the other hand, it was shown that the efficiency of damage removal was higher in the transcribed than in the non-transcribed strand of the DHFR gene (150) indicating that preferential repair in active genes is not simply because of a more open configuration of transcribed genes, but rather a process directly coupled to the transcription process itself, with a possible involvement of the RNA polymerase complex. This again strongly couples transcription and repair. This coupling is probably of biological significance: maintenance of a constant level of good-quality, essential transcripts could be critical for cells to survive.

1.3.6 The nuclear matrix: an artefact?

It is often argued that the matrix is an artificial structure formed by high salt induced aggregation of proteins. However, ultrastructural analyses (45,72,151; see 1.3.2) clearly indicate that the isolated structure is not simply a random aggregation of preexisting, soluble components (84). It is generally known that high salt usually prevents protein aggregation rather than inducing it. In addition, nuclear matrix-like structures were isolated under low salt conditions (152,153).

The association of DNA with the matrix is the same after using high- and low salt isolation methods (116). The idea that the association of newly replicated DNA with the matrix is a high salt artefact (154) was contradicted by reconstitution experiments that showed that nascent DNA did not associate in an aggregative way with the nuclear matrix under high salt conditions (82,85,92). In addition, matrix-associated DNA replication was also found in matrices isolated under isotonic conditions (90). Similarly, it was shown that the association of enzymes -involved in replication- with the nuclear matrix was not a high-salt induced artifact; reconstitution of solubilized enzymes with isolated matrices under high salt conditions proved to be unsuccessful (109).

According to Razin et al. (155) the association of active genes with the matrix is due an artificial randomization caused by the (high salt) isolation techniques used by some investigators. This was contradicted again by data from experiments in which isotonic isolation techniques were used. The data show that transcriptionally active sequences are also matrix-attached under

these isotonic conditions (156). Also, the association of newly synthesized RNA with the matrix could be established after isolation of matrices under isotonic (non-high-salt) conditions (111).

It must be concluded that the data available so far suggest that the DNA folding into loop domains attached to a dynamic and malleable nuclear matrix, functions as an organized entity in which DNA-associated processes can be controlled.

1.4 Radiation and DNA damage

Direct or indirect (via water radicals) radiation damage to the DNA occurs in the irradiated cell and a variety of lesions can be detected in the DNA. These types of damage, their detection in eukaryotic cells and the possible relation of their induction and rate and/or extent of repair to cell survival will be discussed.

1.4.1 Types of DNA lesions

The most common types of lesions in DNA found after ionizing radiation are:

a) base damage (bd)

A great variety of lesions to the bases in the DNA can be found (157). Many (>20) radiation products of thymine can be detected (158), including hydroperoxides (about 50% of all thymine lesions) such as the 5'-6' dihydroxydihydrothymine (t')-type damage as detected by Hariharan and Cerutti (159). Other bases are also damaged by ionizing irradiation, but studies, especially on purine damage, are scarce (159).

b) singlc strand breaks (ssb)

These are breaks in the phosphodiester bonds of the DNA backbone. Breaks can be "frank" (containing clean 3'-OH and 5'-PO_4 ends) or "dirty" (without 3'-OH or 5'-PO_4 end groups, or with end groups containing additional damage)

c) double strand breaks (dsb)

This is a strand break in both, complementary DNA strands of the double helix. With increasing radiation doses (>100 Gy) more dsbs will be detected than can be expected from a (curve)linear dose-response relationship, probably due to the induction of two (or more) single strand breaks in close proximity on the opposite strand.

d) alkali labile sites (als)

This type of damage is named after the way it is detected (in alkali). It concerns the sugar moiety or the bases in the DNA and will finally result in formation of ssbs or dsbs (when opposite to other als or to ssbs) upon treatment with alkali.

e) apurinic-apyrimidinic sites (ap-sites)

Loss of a base from the DNA results in a lesion called ap-site, which

is, to some extent, alkali labile (at pH values >12) and thus may be detected as a strand break.

f) sugar damage

This damage to the desoxyribose is often accompanied by base loss (rendering an ap-site), or it results in strand breaks.

g) DNA-DNA crosslinks

These are covalent bonds between bases in the DNA within the same strand (intrastrand crosslinks) or between bases of one strand and those of the complementary strand (interstrand crosslinks)

h) DNA-protein crosslinks (dpc)

Radiation may produce crosslinks between the DNA and nuclear (matrix) proteins.

In principle, any of these types of lesions may be lethal as such (160 + references herein) but the extent of the contribution of each of them to cell death after ionizing irradiation is not understood as of yet.

1.4.2. Detection methods for radiation induced DNA damage

Several methods have been developed to detect DNA damage in eukaryotic cells after ionizing radiation. Which type of damage is exactly detected, is often not fully understood. Some of the assays and the types of DNA damage presumably detected are given in table 2.

DNA damage assay	bd	ssb	dsb	als	ap	sugar	dpc
endonuclease sens. sites	+	-	-	?	?	-	-
t'type damage assay	+	-	-	-	-	-	-
alk. sucr. grad.	?	+	?	+	?	-	-
alk. unw. pH>12	some	+	+	+	some	some	-
alk. elution pH>12	some	+	+	+	some	some	-
pH=9.6	?	?	+	some	-	?	-
neutral elution pH=7	-	-	+	-	-	-	-
neutral sucrose gradient pH=7 sedimentation	-	-	+	-	-	-	-
filter binding assay[1]	some	+	+	+	some	some	+
nucleoid sedimentation	unclear; certainly ssbs are detected						
halo assay	unclear; certainly ssbs are detected						

Table 2. Methods for detection of DNA damage

+ = detected; - = not detected; ? = not known

1: This assay is used for detection of dpc's; corrections are made for calculations of the amount of non-dpc's.

The principles of these assays, described in Table 2 are given below.

a) Endonuclease sensitive sites.

The protein extract of micrococcus luteus (161) is added to lysed cells. DNA next to the base damage will thus be cleaved by endonucleases in the M.luteus extract, causing DNA breaks (162). These can than be detected as additional DNA breaks over the radiation-induced DNA breaks and alkali-labile sites (163) e.g., using alkaline elution (164).

b) Specific base damage, e.g., t'type base damage

For the detection of the most prominent damage to thymine (i.e., the formation of 5'6'-dihydroxydihydrothymine; over 50%), a selective, reductive assay, yielding 2-methyl glycerol and the corresponding urea derivative, was devised (159,165). The amount of 2-methyl glycerol formed is a measure for the amount of t'type damage.

c) Alkaline sucrose gradient.

Cells are lysed and the DNA strands are separated in alkali. Thereafter, the molecular weight distribution is determined by alkaline sucrose gradient centrifugation (166).

c) Alkaline unwinding assay.

Cells are lysed in an alkaline (pH>12) buffer containing approximately 1 M NaCl. DNA breaks (and alkali-labile sites) will serve as starting points for untwisting during the strand separation in the alkaline solution. After pH-neutralization and a short ultrasonic treatment, the resulting single- and double-stranded DNA fragments are separated by means of hydroxylapatite column chromatography (167,168)

e) Alkaline elution.

Cells are loaded onto a filter and lysed with a buffer consisting of 2 M NaCl, EDTA, SDS (and/or proteinase K) (pH 9). The lysed cells are then eluted with an NaOH-EDTA buffer at pH>12. At the sites of damage (see table 2) DNA will be fragmented and elute at an enhanced rate (169). The elution step is often (also) carried out at pH 9.6 (170), presumably leading to the detection of dsbs only. However, a higher rate of elution after X-irradiation at pH 9.6 than at pH 7.2 (neutral elution, detecting dsbs only?) has been observed (170,171). This was interpreted as pH 9.6 being more effective in removing protein from the DNA or removing other interfering cellular material than pH 7.2 (170), and not because of the detection of other non-dsb damage; pH 9.6 is well below the critical pH 11.6 at which DNA begins to denature. If this interpretation is correct, then the assay seems very unreliable, because it depends on DNA-protein interaction as well as on DNA dsb-formation and a good comparison between different experimental conditions and cell lines (with possibly different DNA-protein configurations) cannot be made.

f) Neutral elution.

This assay is identical to the one described above, except that the final elution step is performed at physiological pH (approx. 7). The sensitivity is rather low (damage is only detected upon radiation doses above 50 Gy). It is thought that only dsbs are detected by this method (170).

g) Neutral sedimentation.

This technique is also used for detection of dsbs only. In the original method (172), cells are lysed in detergent on top of a neutral sucrose gradient and the freed DNA is sedimented through the gradient to get an estimate on its relative molecular mass. Its applicability to the analysis of DNA damage in eukaryotic cells is however limited; only after exposure of cells to very high doses (100 Gy <D <2000 Gy) any damage can be detected. The method was upgraded by Blocher (173). He separated the DNA from other components by heating the cells in a solution of pronase and detergents after doses as low as 5 Gy. However, a post-irradiation treatment with 10 Gy just prior to cell lysis was necessary to get proper sedimentation profiles.

h) Filter binding assay.

This is a modification of the alkaline elution technique, in which the protein digestion step has been altered (174). There are several versions of the procedure. The principle is that most cellular protein will bind to the filter and will not elute, whereas the DNA will elute; the percentage of DNA bound to this protein (minus background binding) is then a measure for the number of protein-DNA crosslinks.

i) Nucleoid sedimentation.

Nucleoids can be prepared by gently lysing cells in the presence of a detergent and high salt at pH 8.0; they consist of supercoiled loops, their topological constraint being provided for by attachment to the residual nuclear structure (nuclear matrix). After centrifugation in sucrose gradients plus high salt (under these circumstances histones will be released from the DNA), damaged DNA will be uncoiled (loop extension) and sediment slowly whereas intact DNA will be highly supercoiled and sediment rapidly (175). The method is only applicable to cells exposed to low doses (max approx. 5Gy; 176,177); the assay also depends on the total mass of the particle (177). Furthermore, the particles do not appear to sediment independently; when irradiated and unirradiated particles are mixed and subsequently sedimented through the gradient, they appear not to sediment independently. Rather a smear of DNA is found and the sedimentation distance is determined by that of the slowest sedimenting particles (unpublished results). The exact type of damage detected is unknown, but it must involve a strand break or a conversion (biochemically or physically) into a strand break, since only strand breaks can lead to the unwinding of supercoiled DNA loops.

j) Halo assay.

This assay is -in principle- identical to the nucleoid sedimentation assay. However, individual nucleoids are directly visualized using a fluorescent, intercalating dye, propidium iodide. In this method centrifugation is avoided and thus no centrifugation-related artifacts are to be encountered. The assay is an extension of the methods developed by Vogelstein et al (38), as modified by Roti Roti and Wright (178), and is described in detail in Chapters 5 and 10.

Most of the assays discussed above detect more than one type of damage and/or can only be used after irradiation of cells with supralethal doses. After discussing the steps in DNA repair and the enzymes involved (1.4.3-1.4.5), the relevance of DNA damage induction and repair for radiation-induced cell death will be briefly evaluated. Rather than relating specific types of damage to cell death, the relation between damage induction and repair (as gauged using some of the individual assays) with cell survival will be discussed (1.4.6).

1.4.3. Steps in DNA repair

Depending on the type of damage inflicted, cells will use different repair procedures. In general, the cellular responses to DNA damage can be divided into 3 categories (206):

1. Reversal of DNA damage
2. Excision of DNA damage
3. Tolerance of DNA damage (replicative bypass of template damage with gap formation or translesion DNA synthesis). Since this does not concern DNA repair, it is out of the scope of this thesis and will not be discussed.

Both reversal and excision of DNA damage in eukaryotic cells are preceded by a coiling degree relaxation step performed by topoisomerases; this step is probably necessary to increase the accessibility of the damaged sites to repair enzymes (180-182). After the repair process has been carried out the same enzymes seem to be involved in the rewinding of the DNA. The involvement of topoisomerases, especially topoisomerase II, in DNA repair will be discussed in 1.4.4. The sequence of events in repair is summarized in figure 6.

1. Reversal of DNA damage

Four types of reversal of damage can be distinguished. The first type consists of a photoreversal of the damage. A light-dependent monomerization of pyrimidine dimers occurs with or without the involvement of an enzyme; the enzymatic photoreactivation (183) seems to be the most important (179). The evidence for the presence of photoreactivation in mammalian cells is still controversial. The second type of reversal involves a direct ligation of "frank" DNA strand breaks containing 3'-OH and 5'-PO_4 end groups. Less than 30% of all strand breaks induced by γ- or X-rays are frank strand breaks (184). Two distinct forms of DNA ligase have been identified in mammalian cells, ligase I and II (179,185,186), with molecular weights of 175 and 80 kD, respectively. The third type of reversal concerns the repair of a base loss (apurinic/apyrimidinic site: ap-site). It has been suggested (179,187) that a so-called DNA purine-insertase can directly insert a base in an ap-site. Ap-sites can also be repaired with an excision type of repair (see below). The existence of a mammalian insertase is still not proven, but indirect evidence obtained in certain XP strains (that do not have ap-

excision type of repair, but can repair depurinated DNA efficiently) is supportive for the presence of an insertase-like activity in mammalian cells (188). The fourth type of reversal process concerns the methyltransferase-mediated reversal of O^6-guanine alkylated products; this damage is particulary induced by monofunctional agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-methyl-N-nitrosourea (MNU), and methanesulfonate (MMS)(206). The presence of a methyltransferase activity in mammalian cells is evident (179,189).

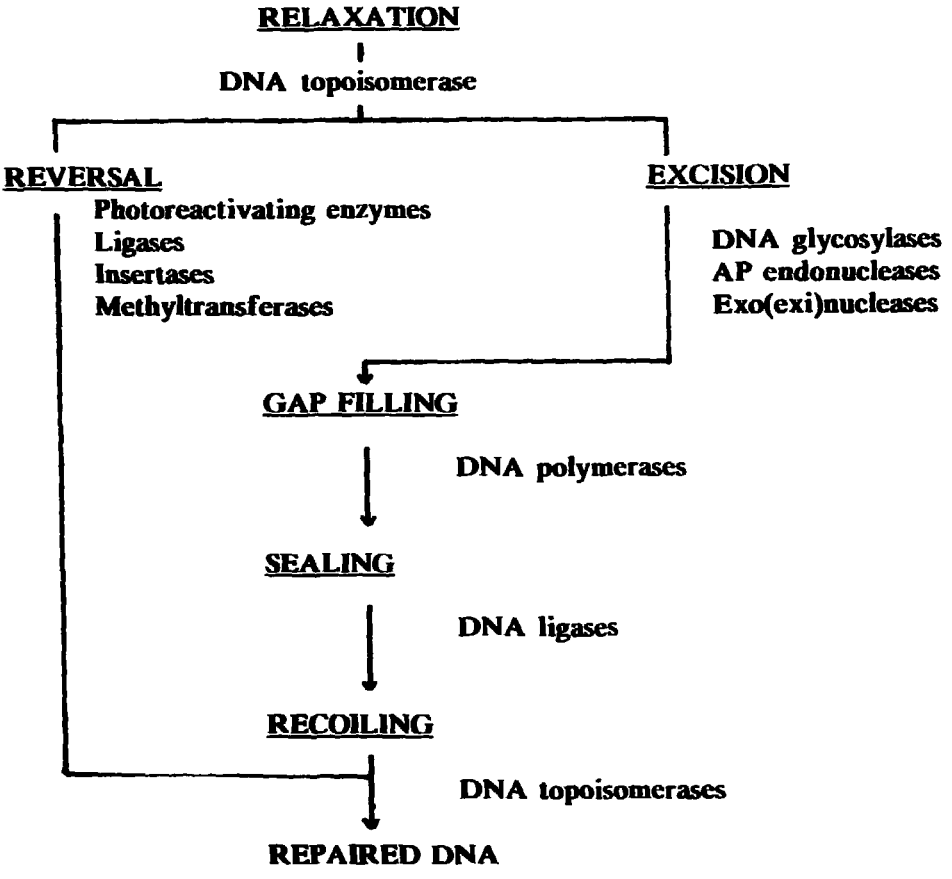

Figure 6: Schematic representation of the sequential steps in DNA repair and the enzymes involved.

2. Excision of DNA damage:

Except for the process of DNA damage reversal all DNA repair schemes so far investigated are forms of excision repair (190). Most research on excision repair concerns work in which UV-irradiation was applied, and much of the basic work was done on bacteria. This repair pathway in bacteria is not

necessarily the same as the one in eukaryotic cells (in which DNA organization into chromatin is a complicating factor). The relative contribution of excision repair in mending DNA damage might be different between prokaryotes and eukaryotes. Among eukaryotes, the relative contribution of excision repair might differ for different types of irradiation (rodents manifest a lesser extent of repair at isosurvival levels after UV, but not X-rays, than human cells do: see 191,192). However, the general outline of the repair process in eukaryotic cells is supposed to be rather similar to that of prokaryotes. Excision of DNA damage can be roughly divided into 2 categories (179,191, 193,194):

a) base excision repair:

Base excision is mediated by DNA glycosylases (see figure 7A). Glycosylases can catalyze the excision of a base, leaving an ap-site in the DNA. This site can be either repaired through insertase activity or by the action of 5'- and 3'- AP endonucleases that subsequently will excise the 5'-deoxyribose-phosphate residue, leaving a gap in the affected strand. Provided that a suitable 3'-OH terminus is present, gap-filling is done by DNA polymerases and after the last insertion strand sealing is accomplished through the action of DNA ligase.

b) nucleotide excision repair:

Nucleotide excision can be carried out along various pathways (see figure 7B). After DNA glycosylase-mediated base excision, the ap-site can (also) be attacked by a 3'-5' exonuclease, which will lead to the excision of an oligonucleotide. Another glycosylase activity (specific for dimers) can hydrolyze the 5' glycosyl bond in the dimer. Subsequent processing of the damage by 3'AP-endonuclease, 5' AP-endonuclease (or 3'-5' exonuclease) and 5'-3' exonuclease will result in the excision of an oligonucleotide plus a desoxyribose-phosphate moiety. Alternatively, (bulky) damage in the DNA can be attacked by a 5'-endonuclease (single nicking) plus a 5'-3' exonuclease that will release an oligonucleotide and a mononucleotide from the DNA. The final possibility is the direct excision of an oligonucleotide by an exinuclease (well known as the uvrABC gene product of E. coli). This polypeptide complex has both endo- and exonuclease activity and makes two (instead of one) breaks in the DNA on both sides of the damaged site. In all cases the resulting gaps can be filled in by the action of DNA polymerase and the chain(s) resealed by DNA ligase.

AP-endonucleases have been isolated from sources varying from bacterial to human (179); the enzyme found in eukaryotes has a molecular weight of 27-38 kD (179). The presence of an exinuclease activity comparable to the uvrABC enzyme of E. coli is not yet established for higher eukaryotes. Some products of the RAD genes in yeast seem to resemble such a complex (179). A number of both 5'-3' and 3'-5' exonucleases have been described in

eukaryotic cells (179,190,195). The specific characteristics of DNA polymerases (as well as of DNA topoisomerase II) of eukaryotic cells are explained in more detail below because of their relevance for the experiments described in this thesis.

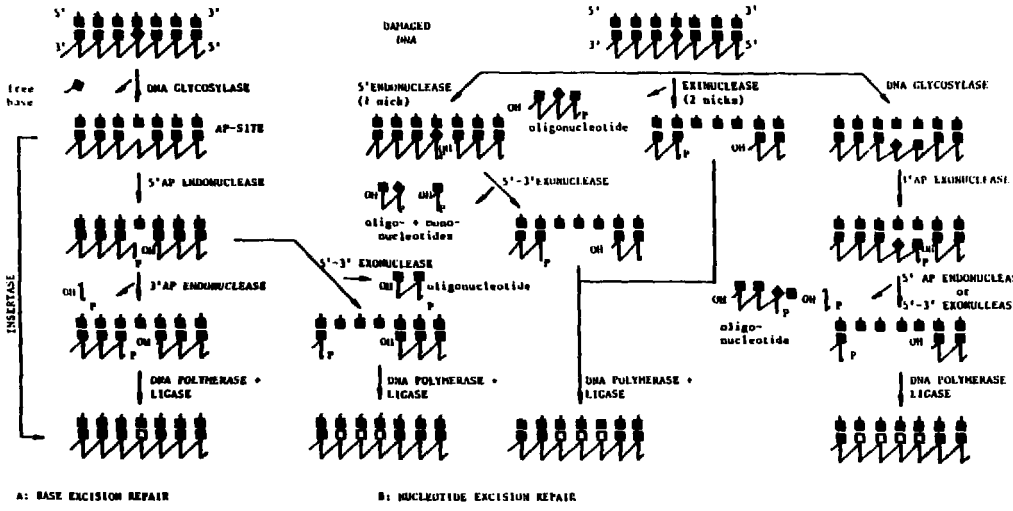

Figure 7: Schematic representation of possible pathways of excision repair A: base excision repair; B: nucleotide excision repair. See the text for further details.

1.4.4 The role of DNA topoisomerase II in the repair of damaged DNA

DNA topoisomerases are enzymes that control and modify the state of topological coiling of DNA by a transient breakage-resealing cycle of DNA strands. Enzymes that act by catalyzing transient double strand breaks are named topoisomerases II, while those that make only a single strand break are named topoisomerase I (58,196,197). The type I topoisomerase (topo I) are found to be associated with the nuclear chromatin, in a specific subset of nuclease hypersensitive sites within the active transcription units (58,197). The eukaryotic topoisomerase I has a molecular weight in the range of 60-75

kD (196). The enzyme has been implicated to be involved in the reassembly of the four histones into nucleosomes in the replication of DNA (196,198) and may have a function in transcription (58). Type II topoisomerase (topo II) is a homodimeric enzyme that has been isolated from various eukaryotes. The purified enzyme consists of a single subunit with a molecular weight in the range of 150-180 kD. In contrast to topoisomerase I, the enzyme needs ATP for its catalytic function (58,193,199) Topoisomerase II has been identified as a major scaffold protein and is present in the nuclear matrix (58; see 1.3). It seems to be involved in DNA replication. Newly replicated DNA was found to be associated with topoisomerase II (97). Partial hepatectomy led to increases in the cellular activity of topo II in regenerating rat liver cells (200) and topo II was shown to undergo significant cell cycle dependent alterations in both amount and stability (201). The enzyme is thought to be involved in the dissociation of the intertwined progeny DNA molecules. Furthermore, the enzyme seems to be involved in the segregation of complementary DNA strands (58,202,203). In this respect it is of interest that topoisomerase II activity is part of the replisome or replitase multienzyme complex (100,101). Topoisomerase II might also be involved in transcription causing the necessary uncoiling step (56,558,198; see 1.3.4). The precise role of DNA topoisomerase II in the repair of damaged DNA has yet to be established. Suggestions for a possible role of topo II in repair come from data AT cells, that are known to be hypersensitive to ionizing irradiation; their enhanced sensitivity was accompanied by an up to 10-fold reduction in cellular topo II activity (204,205). Increased cellular topoisomerase II activities were reported in cells that had been made resistant to nitrogen-mustard. The latter also indicates an involvement of topo II in repair (206). Other indications for a role of topo II in repair come from studies done with novobiocin; this inhibitor is known to -interalia- affect the activity of topoisomerase II (207). Novobiocin was found to inhibit the DNA relaxation and/or condensation step in repair of UV-induced DNA damage (180,182,207-213). In the case of ionizing radiation, the strand breaks themselves presumably will facilitate the unwinding process (207) and (therefore?) some investigators might have failed to notice an effect of novobiocin on DNA repair after ionizing irradiation (207,214). However, more recently, novobiocin was shown to inhibit the repair of DNA lesions induced by γ-rays (215) and to cause an increase in γ-ray-induced chromosomal aberrations (216). Also the repair of potentially lethal damage seemed to be inhibited by novobiocin (217). Thus, maybe if not in unwinding the DNA, topo II might be involved in the recoiling of DNA after the repair of ionizing radiation damage. However, it must be kept in mind that novobiocin is distinctly <u>not</u> specifically acting on topoisomerase II; it also causes swelling of mitochondria and serious disruption of energy metabolism (182), and various other side effects have been reported (218-220). The interpretation of data obtained from experiments, in which novobiocin was used, should therefore be subject to some caution.

1.4.5. The role of DNA polymerases in the repair of damaged DNA

DNA-dependent DNA polymerases are enzymes that catalyze the sequential incorporation of dNTPs (dATP, dCTP, dGTP, dTTP) in a template-directed manner in the 5'-3' direction of the synthesized strand. This DNA polymerase-catalyzed reaction needs a primer RNA/DNA to be initiated (27,99, 179,194) (see figure 8).

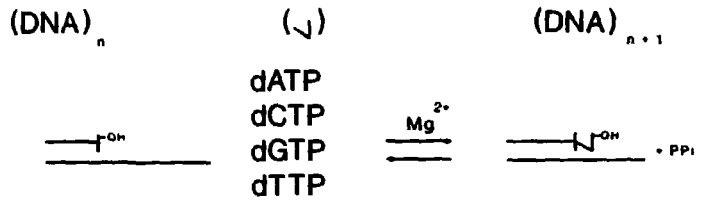

Figure 8: Outline of the mechanism of catalysis of DNA synthesis by a DNA polymerase.

Four groups of DNA polymerases can be distinguished: DNA polymerase -α,-β, -γ and -δ (194,221-225). Very detailed information on these enzymes can be found in recent reviews and books (27,99,179,194,223). Some specific properties and suggested function(s) of these enzymes are given in Table 3; no information on DNA polymerase δ is given here. Although the first description of the existence of a polymerase δ exists from 1976 (226), it is only very recently that more detailed analysis of this enzyme has started and that it was established that this enzyme was distinct from DNA polymerase α (225). It is nowadays suggested that it might be involved in both replication, in concert with DNA polymerase α, (225,227,228) and DNA repair of UV-induced DNA damage (229,230). The role of DNA polymerase α and β in DNA repair is given in more detail below. The need for DNA resynthesis in DNA repair (excision repair: 1.4.3) implies a need for DNA polymerase activity. Table 4 lists some properties of DNA polymerases that might (or might not) of an advantage in repair. In the search for the role of the various polymerases in repair synthesis, attempts in correlating intracellular levels of the enzymes with repair activity have not been successful. No correlations have been established so far between hereditary repair deficiencies and altered levels of cellular polymerases (194,231). Also, unlike in replication, the induction of repair does not seem to induce an increase in cellular levels of DNA polymerase activities. However, in studies with e.g., nondividing neuron cells or growth-arrested (quiescent) cells, in which DNA polymerase β comprises > 99% of all polymerase activity, an increase in Unscheduled DNA Synthesis (UDS) was found after damage induction by some (but not all) DNA damaging agents, suggesting that (in these systems) DNA polymerase β can act as the (only) synthetic repair polymerase (194,232).

PROPERTIES	DNA POLYMERASES		
	α	β	γ
Rel. act. (dividing cells)[1]	70-90%	10-15%	1-10%
Intracellular location	Nucleus	Nucleus	Mitochondria/Nucleus
Changes act. during cell cycle	yes	no	minimal
Native size (kDaltons)	200-600 multimeric	40 monomeric	185-315 multimeric
Catalytic polypeptide	150-190	40	47
Preferred primer template	Gapped DNA	Gapped DNA	Oligo(dT) poly(rA)
Preferred metal activator	Mg^{2+}	Mg^{2+}/Mn^{2+}	Mn^{2+}
Fidelity (nucleotide)	1/40000	1/4000	1/8000
Major modifiers:			
Salt (> 100 mM)	- -	+	+
Aphidicolin (10-20 μg/ml)	- -	no effect	no effect
N-ethylmaleimide (0.5-10 mM)	- - -	-/no effect	no effect
ddTTP: (ddTTP/dTTP (0.5-5))	no effect	- -	- -
Arabinosyl-CTP	- -	-	-
Novobiocin (0.2-0.6 mg/ml)	- -	no effect	-/no effect
Proposed function(s):			
replication of nuclear DNA	yes	no	no
replic. of mitochondrial DNA	no	no	yes
repair after UV irradiation	most likely yes	yes	no?
repair after ionizing irradiation	most likely yes	yes	no?

Table 3: Major distinguishing properties of animal cell DNA polymerases
The data are derived from references 27,99,194,222,223
+=inhibition -=stimulation
1. DNA polymerase δ activity was not included for calculation of percentage activity in cycling cells

The use of isolated mutants in the study of the relation: repair/ DNA polymerases

So far, we are aware of only one set of studies performed with mutants. It concerns a temperature-sensitive aphidicolin-resistant variant cell line isolated by Chang et al. (233). These studies show that the cells have a higher UV sensitivity at the restricted temperature than the wildtype cells (233); the sensitivity for ionizing radiation seemed unaffected (234) which either indicates that apc-sensitive polymerase is not involved in repair after ionizing radiation or that the fidelity of the mutant polymerase at the restricted temperature has decreased, having more effect on the long-patch repair after UV-irradiation than on the short-patch repair after ionizing irradiation (194).

DNA polymerase ∝		DNA polymerase β	
advantage	disadvantage	advantage	disadvantage
gap size preference		gap size preference	
	no strand displ.[1]	strand displacement[1]	
	processive action	distributive action	
	no associated nuclease act.[2]		no associated nuclease act.[2]
	not reactive with nicks or single base gaps[3]	reactive with nicks or single base gaps[3]	

Table 4: Properties of purified DNA polymerase ∝ and β that might or might not be an advantage to their functioning as repair enzymes (data from Loeb and Fry (194) and references therein)

1. Only a (dis)advantage when resynthesis would precede exonucleolytic activity

2. Only true for highly purified enzymes

3. Only of importance for reversal of damage or base excision repair.

Defined in vitro repair systems

When mimicking the induction of alkali-labile and ap-lesions by endonucleolytic excision of 1-4 nucleotides in naked DNA, Bose et al. (235) found that isolated polymerase ∝ from human lymphoblastoid cells could fill these gaps, in contrast to what was found by Mosbaugh and Linn (236,237) who suggested that HeLa polymerase ∝ fills gaps of 20-63 nucleotides but not gaps smaller than 15 nucleotides, whereas polymerase β can fill gaps as small as one nucleotide (base excision repair: 236,237); the latter authors also showed that polymerase β can perform limited strand displacement in reconstructed in vitro systems.

Inhibitor studies

Although inhibitors of DNA repair enzymes have been widely used to obtain information on the role of specific enzymes in the repair process, it has to be borne in mind that inhibitor studies have potential drawbacks. Inhibitors are often not as specific as one would like them to be and interchangeability of functions of the various polymerases might lead to unjustified conclusions about the involvement of certain polymerases when inhibitors are used in the assays.

DNA polymerase ∝ activity can be specifically inhibited by the tetracyclic diterpene tetraol, aphidicolin (APC; see table 2). This agent inhibits DNA polymerase ∝ in a competitive way with respect to dCTP, in a non-competitive way with respect to other dNTPs, and non-competitively with respect to DNA (238,239). On the role of aphidicolin-sensitive polymerase (∝) in repair processes conflicting reports exist. Some investigators (240,241) found no effect of APC on DNA repair synthesis after UV-irradiation while others

found significant inhibition of the DNA repair processes (242-253). Also, the repair of X-ray-induced damage was often affected by aphidicolin (254-256: this thesis, Chapter 9) but again not always (257). Furthermore, aphidicolin was, sometimes, found be to able to act synergistically with X-rays to induce chromosomal abberations (258-260) while -on the other hand- it was found not to potentiate radiation-induced cell killing (261), an effect that might depend on the concentration used (255).

DNA polymerase β can be specifically inhibited by ddTTP. Since ddTTP will not cross the cell membrane, permeabilized cells or other in vitro systems have to be used. Depending on cell type and dose of inhibitor used, ddTTP may or may not inhibit repair after UV-irradiation (247,262) or γ-irradiation (262,263). In another approach intact cells were incubated with ddThymidine, which can penetrate the cell. It is assumed that ddThymidine is than phosphorylated inside the cell and subsequently can perform its inhibitory action on DNA polymerase β. Using this system Tyrrell et al. (248) found inhibition of UV-induced repair synthesis.

The inconsistency between the various reports might be explained in several ways. At first it was often found that less aphidicolin was necessary to inhibit replication (certainly a DNA polymerase α dependent process: see refs above) and isolated polymerase α than to inhibit repair (250,255,264; Chapter 10). Thus at those concentrations of APC that do inhibit replication, some residual polymerase α might still be available for repair synthesis. It is possible that DNA polymerase α might perform replication and repair in association with different enzyme complexes (e.g. replitase) which may vary in function and APC-sensitivity. Different cell lines might need different APC concentrations before repair inhibition is observed; van Zeeland et al. (265) noticed different effects of the same concentrations of aphidicolin on radiation-induced repair synthesis and chromosomal aberrations in fibroblasts and HeLa cells. Different intracellular concentrations of DNA polymerases and dNTPs (also between confluent and exponentially growing cells) could very well explain some of the apparent controversies (194,208,243). The analysis of repair patch-size in presence of APC, showing in shorter patches (266) or a reduced number of patches, that were found to be created through strand-displacement (β activity: 267) might lead to the suggestion that polymerases act together in the repair process, or even substitute for each other. Other evidence also indicates that DNA polymerase α might act jointly or sequentially with DNA polymerase β in the repolymerization upon UV-irradiation (247,249); DNA polymerases may substitute for each other in repair but (polymerase β seems not involved in replication: see 194 or 223 for review) not replication synthesis (213). It is also possible that the fraction of repair synthesis mediated by either DNA polymerase α or β is dependent on the nature of the DNA damage (250,262); it is suggested that β polymerase could perform base excision, whereas nucleotide excision is more likely to be performed by an APC-sensitive polymerase. Finally, in vitro repair studies done by Dresler and Lieberman (251) suggest that there is a

radiation dose dependent involvement of DNA polymerases in repair: at low doses β- and at high doses α-polymerase predominates.
The data from experiments with the inhibitor aphidicolin have to be interpreted with some caution, since DNA polymerase δ shows similar sensitivity to this inhibitor (251). Also, the relative insensitivity of both DNA polymerase -α and -δ to ddTTP and arabinosylnucleotides is the same and therefore the data cannot be considered as conclusive in distinguishing the role of α and/or δ polymerase in repair. The finding that α polymerase is over 100 times more sensitive to butylphenyl-dGTP than is polymerase δ (268) and that UV-induced DNA repair synthesis and DNA polymerase δ show similar resistance to this inhibitor (230,269) might even indicate that DNA polymerase δ is and DNA polymerase α is not involved in repair of UV-induced damage; the association of accessory exonuclease activity with DNA polymerase δ (225 and references therein) makes this even more likely.

1.4.6. Relation of radiation-induced DNA damage to cell killing

When different cellular compartments are selectively treated with ionizing radiation, it turns out that the cell nucleus is the most radiosensitive organelle. Increasing amounts of DNA damage were observed with increasing irradiation doses given to the nucleus. One of the oldest arguments for DNA being directly involved in radiation induced cell death is the gross correlation between cellular DNA content in different organisms and radiosensitivity. Incorporation of BrdUrd into the DNA of living cells increases radiosensitivity, while e.g., incorporation of radiosensitive fatty acids into cellular lipids did not change clonogenic ability after radiation (see reference 9). Labelling of RNA or DNA with tritiated radioactive precursors of high specific activities resulted in a 5-fold greater cell killing for DNA labelling over RNA labelling (11). Under many circumstances, cell killing by ionizing radiation appears to correlate with the induction of chromosomal aberrations (15-18). From the evidence of these and other experiments it is accepted that DNA damage is the main cause for radiation-induced reproductive death. What type of damage to the DNA molecule has to be considered as being critical is not yet established. It is obvious that repair of these critical lesions is of ultimate importance for cellular survival.

Causal relations for different types of DNA damage induction and repair with radiation killing have been investigated by using different experimental conditions within one cell line such as different radiation qualities and states of oxygenation, chemical radiation modifiers (scavengers, thiols, sensitizers), hyperthermia, and comparing cells from different cell cycle stage. Also cell lines with different radiosensitivity and, more recently, radioresistant or-sensitive mutants were compared. Table 5 gives a survey of the various assays available for determination of DNA damage induction and repair. A general discussion on this is given below.

1. Endonuclease sensitive sites (ess)

Especially the finding that no protection was found for induction and repair, by the radiation protector cysteamine, does not support the idea that ESS are the primary cause of radiation-induced cell killing. Also, no difference in damage induction and repair between the radiosensitive AT cells and it wildtype counterparts was observed.

2. Specific base damage (t'type-bd)

The reduced induction of bd (while no effect on repair) with increased LET (resulting in enhanced radiation-toxicity) suggests that t'type damage is not a major lethal lesion. This type of damage can only be detected in eukaryotes after relatively high doses (>50 Gy).

3. Filter binding assay (DNA-protein crosslinks (dpc))

It is very difficult to estimate the biological relevance of dpc's. In general, high doses are required to see their induction. This induction e.g., shows an "inversed" OER-effect (more damage induction under anoxic than oxic radiation conditions). Furthermore dpc's can continue to exist over relatively long periods of time whereas in the same period cellular repair (survival) is already complete (192).

4. Nucleoid sedimentation- and halo-assay

Both assays are able to detect damage induction after radiation doses as low as 0.5-1 Gy (37,178). So far, the number of existing data is insufficient to allow an evaluation on the biological relevance of the damage detected by these assays. The fact that various types of DNA damage are detected by this assay (as long as they will lead to breaks) has both advantages and disadvantages. The assays do not specify on a certain type of damage. They provide information on a group of DNA-lesions, their induction and repair related to structural (loop) organization of the DNA.

5. Alkaline unwinding or elution (pH > 12)

The initial induction of damage detected with the alkaline unwinding assay cannot directly be related to radiation sensitivity (168,287-294). With the alkaline unwinding assay one cannot distinguish between types of damage. However, on the basis of repair kinetics, represented by the different phases in the repair curves obtained (two phases: 286,290,291 or three phases: 295) interpretations are often made in terms of ssb-repair (fast component(s)) and dsb-repair (slowest component) (295,296). The reality of this identification, however, is questionable (297). When the slowest component in the repair kinetics is compared to the "real" dsb-repair (measured after supralethal doses, using neutral sedimentation, see also below) the half times of so-called "dsb-repair" are greater. The different repair components are -on the other hand- often interpreted as resulting from differently accessible (condensed) chromatin or repair in linker versus nucleosomal DNA (299). Although this is not totally unlikely, it seems more reasonable to explain the 2 or 3 (or more?) components as coincidental, resulting from a sum of various factors including type of damage (and its related repair process), chromatin accessibility and types of enzymes involved in the corresponding repair processes. Hyperthermia caused a "heat-dose dependent" inhibition of

all phases of repair (286,289-292). Although repair after aerobic radiation was reduced in GSH-deficient cells (> 70%) as compared to their GSH-proficient counterparts, repair after hypoxic radiation was found to be the same in both GSH pro- and deficient cells (288). The inhibition of all repair phases by hyperthermia was found to be less in thermotolerant cells although these cells showed the same hyperthermic radiosensitization as non-tolerant HeLa cells (291). Modification of survival after radiation by differences in LET (168,287) or differences in cell cycle stage or in radiosensitivity of cell lines (293) did not correspond with the initial rate of repair. Under several conditions (168,287,293), however, a relationship of radiosensitivity with an altered percentage of slowly repaired and/or unrepairable lesions was found. When a comparison was made between the repair kinetics of the xrs-5 mutant and that of its wildtype counterpart it was found that, initially, repair was somewhat retarded in the mutant, but after one hour the residual level of damage was the same in both cell lines; the kinetics of disappearance of this remaining damage was, however, significantly retarded in the mutant (294). This slow component of repair seems a good (and measurable) indicator for residual damage (difficult to measure after exposure to biologically relevant, low radiation doses).

Also for the damage detected with the alkaline elution technique, the amount of initial radiation damage does not correlate with differences in survival (270,275,299-311). Interestingly, Ward et al. (313) found that H_2O_2 could induce 10 Gy equivalents of lesions detectable with the alkaline elution technique, without any cytotoxic effect. The repair of the alkaline elution detectable lesions is equally rapid as the repair found for damage detected with the alkaline unwinding method, being almost complete in 30 minutes after a dose of 10 Gy (314). Unfortunately, no specific analysis has been done with regard to the relation of the specific phases of repair and radiosensitivity. For "overall" repair after irradiation controversial results were obtained for different LET radiation (299,315). No effect of thiol depletion on repair was reported (329). In general hyperthermia retarded repair (286,304) and a good correlation of radiosensitization with residual damage was observed (316). At lower hyperthermic temperatures, however, sometimes enhanced repair was found while cells were clearly radiosensitized (286). When comparing different cell lines and/or radiosensitive mutants, in general, no differences in repair capacities were found (306,308,311,316) although a radiosensitive CHO-mutants (EM 9) was found to be repair-deficient using this assay (317). It is important to reconcile that th efficiency of rejoining might depend on the chromatin structure of th irradiated cells; metaphase cells show a slower rate than asynchronous cell and actively transcribed DNA is repaired more efficiently (135,319).

The biological relevance of the lesions detected with the alkaline unwindin and elution methods is unclear as of yet. Often, the slow phase of repa and/or residual damage show a correlation with radiosensitivity.

Assay	Dose[1] (Gy)	$T_{1/2}$ of DNA repair (*min*)[2]	Modification of radiation sensitivity												Ref.
			LET		O.E.R.		Scavengers		Hyperthermia		Cell cycle		*Different* cell types		
			initial	repair	initial	repair	initial	repair	initial	repair	initial	repair	initial	repair	
ess	> 10L	60	nd	nd	o	*nd*	+/o	o	o	nd	nd	nd	o	+/o	A
t'type	> 50 L	10	-	o	+	nd	o	?	o	+	*nd*	*nd*	*nd*	*nd*	B
filter binding	10-200 L	a:10-120 b:120-900	nd	nd	-	nd	+	nd	+/o	+	nd	nd	nd	nd	C
alkaline unw./elution (pH > 12)	1-20L	a: 2-4 (b: 17) *b/c*: 43-200	-	+/o	o	nd	+/o	+/o	?	+/-	o	o	o	+/o	D
alkaline el.(pH 9.6)	1-40 L/LQ	a: 3-20 a/b: 40-120	+/o	+	+	nd	?	?	+	+	+/o	+/o	+/o	+/o	E
neutral el.	> 20L?	a: 3-20	nd	nd	nd	nd	nd	*nd*	nd	nd	nd	nd	o	+/o	F
neutral sed.	5-2000 L	120-240	+	+	nd	nd	nd	*nd*	nd	nd	o	o	nd	nd	G
nucleoid sed. halo assay	1-10L	a: 4-10 b: > 30	-	nd	nd	nd	nd	nd	-[3]	+[3]	nd	nd	+	o	H

Table 5: Modification of cellular radiosensitivity in relation to damage induction and repair as measured by the various assays.
+ = positively correlated to altered radiosensitivity; - = negatively, *inversely* correlated to altered radiosensitivity
o = unaltered (while radiosensitivity was altered); nd = not determined; ? = data are very contradictory and show both -,+, and o
1: induction is linear (L) or linear quadratic (LQ) (showing a "shouldered" dose-response curve)
2: half time of repair; a, b, and c indicate the various phases (*if present*) of repair
3: see Chapter 11 for further explanation of this effect.
A: references 162,270-276; B: references 139,192,277-281; C: references 134-138,270,282-285
D: references 135,168,169,286-319; see v Sonntag: 160; E: references 270,299,300,303,306-308,310,311,314,315,317,320-348
F: references 315,321 G: references 173, 349-351; H: references 178,313,314,352-357

6. Alkaline elution (pH = 9.6)

At the moment, the alkaline elution technique (often called "neutral", although the pH is 9.6) is the most widely used assay for detection of radiation-induced DNA damage. A more appropriate name for this assay might be "non-denaturing filter elution", as suggested by Kuo et al. (322). There are conflicting ideas about the types of damage that can be detected with this assay. Its (in)compatibility with the neutral (pH 7.2) elution and sedimentation (320) is still a problem (see also 1.4.2).

Radford and coworkers found a non-linear, "shouldered" curve (linear-quadratic) for the representation of damage induction at low doses (> 2 Gy). In their experiments (270,303,320,324-326,329), the curves mirror radiation survival curves. The shoulder plus slope of these curves were altered by hypoxia, the addition of cysteamine, pre-irradiation hyperthermia and were dependent on cell cycle stage, as well as by cell line dependent differences in radiosensitivity in a way that directly correlates to radiation killing (270,303,320,324-326,329). On the basis of these data, Radford et al. (320) put forward the hypothesis that the yield of "dsbs (?)" in "critical target-DNA" (a constant proportion of total "dsbs") is important for cell survival through recombinogenic misrepair. They furthermore state that "the repair of the vast majority of radiation-induced DNA dsb is irrelevant to an understanding of the lethal response in normal mammalian cells". This hypothesis was formulated in 1988 (320), in spite of several data in the literature that did not support it. Several investigators found a linear (non-shouldered) curve for the induction of damage after X-irradiation (192 plus references therein). The xrs-5 cell line does not display a shoulder in its radiation survival curve, but does show a shoulder in the DNA elution dose response curve (323). Although LET-dependent induction of DNA breaks mirror radiation cell survival curves (299,300,315), Prise et al. (331) calculated that approximately 2.5 times more lesions per lethal event were induced by X-rays than by neutrons or α-particles. Several radiosensitive cell lines showed no difference in damage induction when compared with their wildtype counterparts (306-308,311,317,320,323,335-337) or showed only a slightly higher susceptibility (310) to damage induction. Radford and colleagues suggested (320), on the basis of experiments with aphidicolin-synchronized cells in S-phase (325), that changes in "target size" or "activity in the recombinogenic misrepair" may account for these differences in induction profiles and cell survival. However, the experimental basis for this is weak, since, in contrast to the findings by Radford and Broadhurst (325), cell cycle differences in radiation sensitivity were not always found to have an effect on damage induction (332). Differences in the physicochemical properties of the DNA during the cell cycle, especially during S-phase, will affect elution profiles during alkaline elution and give rise to the observed curvilinear DNA elution dose-response curves as well as to the fluctuations in elution characteristics observed throughout the cell cycle (334). The data from Radford and Broadhurst (325) -at higher dose range- show a crossover of sensitivities for cell killing for the conditions tested, while the sensitivity for dsb- induction

continued to diverge under the same conditions. All in all, it is not likely that the survival shoulder width observed is causally related to the shoulder obtained in the DNA elution dose response curve (323; table 5).
The repair rate of lesions detected with the non-denaturing elution method is often found to correlate with radiosensitivity (335,341). L5187S cells were found to be less capable of repair than L5187R cells (306,311,346). The radiation-sensitive Jeggo mutants were found to be partly repair-deficient, although it should be noted that the relative radiation-sensitivities of the mutant lines did not correlate with the number of unrejoined lesions (307,308,317,323,336,337). In different strains of AT cells sometimes, but not always, repair deficiencies were found (335,348).

7. Neutral elution and sedimentation (pH = 7) (dsb)
Data obtained with the use of the "real" neutral elution (pH 7) assay are rather scarce, probably because of the assay's relatively low sensitivity. Less damage induction will be detected using an elution pH of 7 instead of elution at pH 9.6. Using supralethal doses (50 Gy) Evans et al. (306) showed that the repair of pH 7 labile lesions is retarded in L5187S in comparison to L5187R cells. This correlates with the higher radiosensitivity (0-4 Gy) of the L5187S cells when compared to the L5187R cells.

The neutral sedimentation technique (only dsbs are detected) has not been widely used because of its relatively low sensitivity and because it is very elaborative. The method was upgraded by Blocher (173) by means of a debatable application of a 10 Gy radiation treatment prior to cell lysis. The induction and repair was "LET-dependent" (350), but independent of the cell cycle stage (351).

Conclusions
For all types of DNA damage, detected with the assays described above, both correlations and non-correlations with survival were found. The correlations observed may just be coincidental; the "universal lethal lesion" for radiation induced cell killing might not have been detected by any of the assays available. It, however, remains unclear whether such a lesion exists. The best relation, so far, between DNA damage and radiosensitivity is found for residual damage detected with the unwinding and elution techniques (as especially indicated by the repair rates of the slow components for repair) and for dsb-induction and repair as detected with the neutral elution and sedimentation assays. The data with the alkaline assays, however, do not provide information on the types of lesions that may be important. The data with the neutral techniques do provide information on dsb only, but, unfortunately, supralethal doses are necessary for their detection. Evidence for a major role of dsb-damage and repair in radiation killing comes from studies on bacteria (314) and yeast (192). It is clear from several data using restriction endonuclease treatment of Sendai virus permeabilized mammalian cells, definitely only producing pure dsbs, that dsbs can yield chromosomal

aberrations (358-361) and leads to cell killing (360). The same data, however, also show that equal amounts of different types of dsbs (blunt ends versus cohesive ends) are differently effective in inducing chromosomal aberrations and killing. So dsbs per se are not necessarily cytotoxic and evidently can be repaired.
In the search for the pathways that lead to radiation killing, conversion of damaged DNA during the repair process into "more harmful" lesions, fidelity of the repair and/or fixation of damage (via replication or mitosis) before repair are all possibilities that play a role in the ultimate effect. Specific repair in active versus inactive genes and replicating versus non-replicating DNA has been reported (135-139,282,319). For UV-induced radiation damage, strong evidence for a major role of preferential repair of active genes in UV-induced cell death has been presented (see 1.3.5). This might also be of biological relevance for killing after ionizing radiation.

1.5 Hyperthermia

1.5.1 History

Hyperthermia, a heat treatment of cells or tissue above physiological temperature, has been recognized since the early 1900's as a possible adjuvant in cancer therapy (362). Since the 1970's new and sophisticated clinical applications of hyperthermic treatment have been started. The rationale for the use of hyperthermia in cancer therapy is provided by results obtained from in vitro studies:

- Heat (in the temperature range of 40-46^{o}C) can kill mammalian cells
- Heat (preferentially) kills cells that are relatively radioresistant such as hypoxic and S-phase cells.
- Heat killing is enhanced at low pH or when nutrient supply is poor; this situation is often found in poorly vascularized, solid tumors.
- In killing cells, heat acts synergistically with ionizing radiation and with a number of anticancer drugs.

1.5.2 Thermal dose

Unlike ionizing radiation, a "hyperthermic dose" can not be quantified as an amount of energy absorbed during heat treatment (363-365). Cell killing by hyperthermia depends on both the height of the temperature used as well on the duration of exposure to that elevated temperature (363-365). In general it seems impossible to predict the heat sensitivity of specific cell lines on basis of their tissue of origin (365). No correlation of cellular heat sensitivity with the sensitivity to UV- or X-rays is found (365).

1.5.3 Clinical application

Some remarkable results have been obtained so far in third phase studies in which a combined treatment of heat and radiation and a treatment of radiation alone have been compared (see Table 6).

Response (%)		Temperature	Time of	References
Radiation alone	Radiation plus heat	(°C)	application (min)	
14	86	42-44	40-50	U et al. 1980*
33	80	42-43	30	Kim et al. 1982*
0	62	43.5	60	Corry et al. 1982*
33-39	87	42-45	30-45	Arcangeli et al. 1983*
29	56	41-45	45	Lindholm et al. 1984*
29	68	41-44	40	Li et al. 1984*
0	24	41.6		Zee, v.d. et al. 1984**

Table 6: Tumor regression response upon radiation and radiation plus hyperthermia. Response is expressed as frequency of tumor elimination for various periods of the follow-up.

* **Data as reviewed by Perez 1984 (366)**

** **Data as reviewed by Overgaard 1984 (367)**

The results from these third phase trials now seem sufficiently promising to try a combination of heat and radiation in the treatment of primary cancers. For further improvement, the design of better applicators for hyperthermia (especially for deep-seeded tumors) as well as better insight into the biological and biochemical mechanisms underlying the heat-killing and -radiosensitization phenomena, are indispensable. Improved, better-localized application of high heat doses should lead to a higher efficiency of tumor kill, with fewer and less pronounced effects on normal tissue, and minimal pain for the patient. A better knowledge of mechanisms might lead to a better understanding of specific phenomena such as thermotolerance in heat-killing and - radiosensitization. The clinical response to heat and radiation treatments might than be optimalized by the design of better treatment schedules.

Over the last 25 years various reports on biological and biochemical reactions after treatment of cells or tissue with hyperthermia have appeared; they are reviewed in this Chapter.

1.6 Cellular and molecular thermobiology; a short introduction

1.6.1 Hyperthermia and cell survival curves

Survival curves that can be obtained after heating mammalian cells are shown in figure 9a. Although there is (still) no evidence for the existence of (a) discrete heat "target(s)" (365), the most common mathematical approach used to describe these cell survival curves after cellular heating is based on the the multi-target-single hit model (5, see 1.2.1). The linear-quadratic

method (6,7: see 1.2.1) has also been used to describe survival curves after hyperthermia (365,366). Based on the slopes (D_0) or α or β components of these survival curves, Arrhenius plots can be constructed, that will show an inflection point at around 42.5°C (when based on D_0; figure 9b: 369,370). This inflection point might be associated with the induction of thermotolerance (359,367,368). No such inflection point is found when the Arrhenius plot is based on the linear quadratic model (figure 9b: 365,368). The Arrhenius curves have often been used to identify rate-limiting steps in the mechanism of heat killing; for instance, the "activation enthalpy" for cell killing above 43°C (i.e. 600 kJ/mol) would suggest that protein denaturation is the main cause for heat-induced cell death (363,369). The absence of an inflection point when using the LQ-model (365,368,373) suggests that the inflection point found when plotting on basis of D_0 is connected with the type of analysis.
A different model to describe heat killing was proposed by Jung (374). This model assumes a two-step process. The first step is the heat-induced production of nonlethal lesions, that -in a second step- can be converted into lethal events upon further heating. After heating for a time period t, the fraction of surviving cells (S) is given by the equation:

$$S(t) = e^{\{(p/c).[1-c.t-\exp(-c.t)]\}}$$

(where p is the rate constant for production of the nonlethal lesions per cell per unit of time and c is the rate constant for the conversion of a nonlethal lesion into a lethal event per unit of time). In the Arrhenius plot both parameters p and c show a temperature dependency, and p but not c shows an inflection point at 42.5°C (Figure 9b; 374). The model implies a single event of conversion of a yet undefined nonlethal into an yet undefined lethal lesion. The model nicely describes heat killing under various conditions (single heating, thermotolerance, step-down heating) and does not need multiple targets nor repair processes.

1.6.2. Modification of thermal sensitivity

The thermosensitivity of cells can be modified by various means. First, alteration of the thermal history of cells before the test heat treatment affects their thermosensitivity. Secondly, alterations of environmental factors before, during or after the test heat treatment, can influence cellular thermosensitivity.

A. Thermal protection by affecting the thermal history.

Cells can acquire a temporary (usually < 24 hours) thermal resistance when pre-exposed to a short (3-15 minutes) high or long (2-20 hours) low hyperthermic temperature. This phenomenon is known as thermotolerance (TT) (363,370,376-378). Thermotolerance cannot only be induced in vitro (363,370,376-378) but also in vivo, in tumors and normal tissue (376,379).

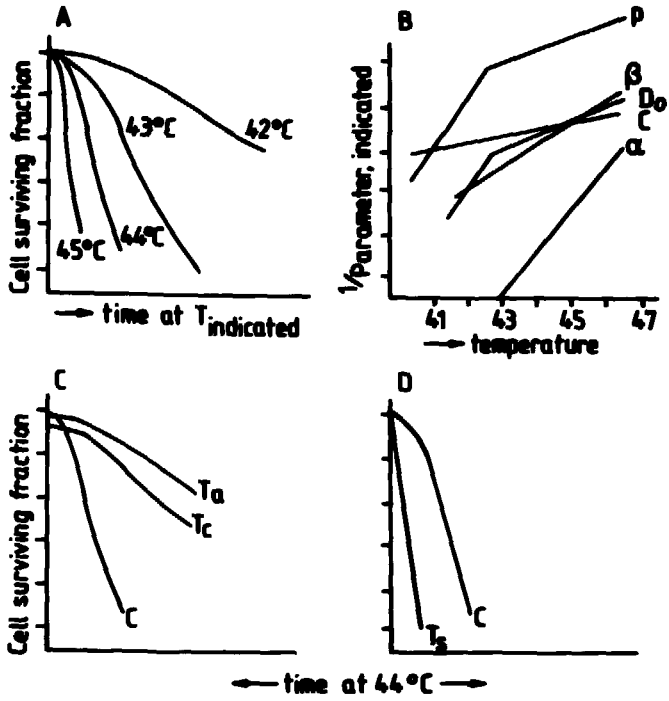

Figure 9: Graphic representation of cell survival after hyperthermia, Arrhenius analysis and effects of thermotolerance and step-down heating on survival after heating.
A: Cell survival after heating as a function of time and temperature
B: Arrhenius analysis of cell survival curves using the multitarget-single-hit model (D_0: Dewey and Westra (369)), the LQ-model ($\propto$ and β: Roti Roti and Henle (368)), or the two step model (p and c: Jung (374))
C: Effect of chronic tolerance (T_C) and acute tolerance (T_a) on hyperthermic cell killing (C relates to non preheated cells)
D: Effect of step-down heating (T_S) on hyperthermic cell survival (C relates to non preteated cells)

When cells are continuously heated at relatively low hyperthermic temperatures (usually below 42.5°C), heat killing levels off after a few hours; this is referred to as development of "chronic thermotolerance" (TT_C: figure 9a: 369,376,380-386). Chronic tolerance can be found in all cell cycle phases except S-phase cells (387) and also develops in plateau phase cells (388),

arguing against the possibility of accumulation of cells into a "thermo-resistant" cell cycle phase as the cause of the increased resistance. The resistant "tail" in the survival curve also cannot be due to a selection of a thermoresistant subpopulation of cells, since its is not observed at higher temperatures (figure 9a) and diminishes within 24 hours upon prolonged exposure to heat or return to normal growth temperature (386-389). The thermotolerant state of the cells is also evident when they are subsequently heated at temperatures above 43°C (figure 9c; 2,375,383,384,386).

The other type of thermotolerance that can be induced after a short heat pulse followed by a defined period at 37°C is often called "acute thermo-tolerance" (TT_a: Figure 9c; 291,363,375-378,383,384,386). This type of thermotolerance can be induced in all phases of the cell cycle (390-392) as well as in plateau phase cells (373,388).

Thermotolerance can be induced in many other ways than by heating and can also be inhibited in several ways. A summary of these different treatments is given in Table 7 which is compiled from data from a recent review by Henle (373).

When cells are adapted to (normal) growth at slightly elevated temperatures they can build up a transient thermal resistance (24-34 hours), referred to as "thermal adaptation" (394-396).

Stepwise repeated heating and cloning of surviving colonies will lead to the selection of heat resistant variants, of which the heat resistance is stable for over 80 generations without further selection (397-401).

Inducers of thermotolerance	Inhibitors of heat-induced thermotolerance
Alcohols	Cold temperature storage[2]
Arsenicals	Low pH[3]
Diamide	Cycloheximide[1]
CCCP	Hypertonicity
Disulfram and DDC	D_2O
Recovery from Anoxia	Glycerol
DNP	Step down heating
Calcium related drugs	Amino acid analogues
Glucocorticoids	Sugar analogues or deprivation
Prostaglandins	GSH depletion[1]
Heat	Procaine[2]
	Amiloride

Table 7: Inducers of thermotolerance and inhibitors of heat-induced thermotolerance. Data are compiled from a review by Henle (373).
1. Conflicting results have been reported. 2. Seems only to delay the development of thermotolerance 3. May not be true if data are corrected for enhanced toxicity at low pH (393)

B Thermosensitization by affecting thermal history

When a short pretreatment at a relative high temperature ($43^{o}C < T < 46^{o}C$) is immediately followed by hyperthermic treatment 1-5°C below the initial treatment temperature ("step-down-heating"), cells are thermosensitized (figure 9d; 374,375,402,403). This step-down effect might be advantageous in clinical protocols where high tumor temperatures often only can be maintained for a short time.

When cells are exposed to a short pretreatment with hypothermia (1-30°C) they will become more heat-sensitive (404-408). Also, cells that have been adapted to growth at temperatures 4-6°C below normal growth temperatures (low-temperature thermal adaptation) are more sensitive to a hyperthermic treatment (394-396).

No real, stable heat-sensitive mutants have been isolated as yet by means of selection through prior heat exposure. Recently, a heat-sensitive mutant was isolated using a replica technique following treatment of CHO cells with the mutagen ethyl methanesulphonate (EMS)(408). This mutant has been stable for more than 6 months now, and since it is also greatly reduced in its ability to develop thermotolerance, it might be a very useful cell line for future research.

C Modification of thermal sensitivity by other means

Cell survival after hyperthermia can be influenced in many ways. In Table 8, some of these ways are summarized.

There are several other means by which heat killing can be altered, but these are much less documented than those described above and are therefore not discussed here.

The action of inhibitors of RNA- and protein synthesis is addressed to some extent in 1.6.4 and 1.7. There is one other means by which heat killing can be modified that is worthwhile mentioning here. This is the modification of heat killing by post-heat incubation (potentially lethal heat damage repair) of cells under suboptimal culture conditions before plating for determination of clonogenic ability (444-446). There are not many studies concerning this phenomenon; the approach might be useful in the search for the mechanisms of heat killing.

1.6.3. Cell cycle effects

While cells in S-phase are the most resistant to radiation (19), they are the most sensitive to heat killing. Cells in G_1 appear to be the most heat-resistant cells while cells in G_2/M appear to have an intermediate sensitivity (363,369,370,447) although good data on G_2/M cells seem to be rather scarce. Hyperthermia delays cell cycle progression; especially the progression of G_1 cells is delayed (387,390,392,448,449). Several hours after hyperthermia an accumulation of cells in G_2/M occurs (449). The moment of accumulation after a heat challenge, its extent and duration appears to be dependent on the duration and temperature of that heat challenge (449). It is clearly

distinct from the radiation induced G_2-block that occurs sooner and is shorter for treatment having the same cell killing effect (450).

Heat protection	Heat sensitization
polyhydroxy compounds[1](409-411)	mono- and dialcohols (409,412)
sugars (413-415)	thiols (10,416-418)
D_2O[2](419-421)	lowering environmental pH (377,389,418-423)
mono-unsaturated fatty acids[3](424,425)	poly-unsaturated fatty acids[3](424-427)
amino acids[4](410,428)	local anesthetics[1] (426,427,429-431)
	polyamines (4432-434)
	nutrient deprivation (435-437)
	inhibitors of ADP-ribosylation[5](438-440)

Table 8: Factors affecting thermal heat sensitivity
1. mostly used with pre-incubation 2. protection only below 43°C 3. fatty acid supplementation occurs during growth in medium, using the specific fatty acids. 4. not all amino acids are protectors (414) 5. not due to a direct effect on ribosylation these inhibitors (444)

1.6.4 Molecular targets for heat killing

In order to kill a cell with hyperthermia, 10^3 - 10^5 fold more energy is expended than for radiation-induced cell killing (1,363,450). This large (and rather nonspecific) energy input by hyperthermia causes pleiotropic effects on cells, which makes it very difficult to determine (the) critical molecular target(s) and to find (a) mechanism(s) of hyperthermic cell killing. Among the effects are alterations of membranes and the cytoskeleton (see below), increases in lysosomal activity (451), disruption of the Golgi apparatus and swelling of mitochondria (452,453), decreases in ATP levels (454,455), increases (456,458) or decreases (456,459) in the degree of phosphorylation of several proteins, changes of the cell nucleus (1.6.5), and alterations in DNA, RNA and protein synthesis (1.6.6 and 1.7).

DNA and hyperthermic cell killing

Heat-induced damage to DNA seems not to be the critical event leading to hyperthermic killing (10). Whereas radiation-induced killing was enhanced by the incorporation of BrdUrd into the DNA, no such sensitization was observed for thermal killing (460) and although longer exposure to relatively high hyperthermic temperatures (generally above 43°C) may sometimes lead to a detectable amount of DNA damage (290,292,461,462), the extent of damage induction seems not to be related to the extent of heat killing (462). Furthermore, DNA repair-deficient mutants are not more heat sensitive than their wild-type counterparts (463-466). Finally, it was found that cells in S-

phase are the most heat sensitive, while radiation sensitivity is the lowest in S-phase (see 1.2.3 and 1.6.3).

Plasma membranes and hyperthermic cell killing

Since the plasma membrane is the cellular component that is in direct contact with the environment and since biomembranes are likely to be altered by small temperature changes because of the heat-induced structural alterations of membrane architecture (fluidity), it may be that the plasma membrane is the (a) primary cellular heat target (450,467). This idea was substantiated by the findings that membrane-active drugs such as local anesthetics (e.g. procaine) or amphotericin B are well-known heat sensitizers (see Table 7 and Konings 1988 (467) and references therein). Furthermore, alterations in membrane lipid composition (e.g., by incorporation of polyunsaturated fatty acids: PUFAs) were shown to result in increased membrane fluidity and increased heat sensitivity (429,431,468). Initially, reports suggested a relation between the cholesterol/protein and phospholipid/protein ratio on one hand and thermosensitivity on the other, in several cell lines (469,470). This was not found by others, in comparing other cell lines (see Konings (467)). More recent data in which thermotolerance, thermoadaptation, as well as thermoresistance were compared to the corresponding properties of normotolerant cells revealed that neither membrane lipid composition, nor cholesterol levels, or membrane fluidity should be considered principal targets in hyperthermic cell killing (see Table 9 and references 450,467).

	Relation with thermosensitivity under conditions of:			
	thermo-tolerance	thermo-adaptation	thermo resistance	different cell lines
Phospholipid composition	no	n.d.	no	no[2]
Cholesterol/protein	no	no	no[1]	yes[3]/no[2]
Cholesterol/phospholipid	no	no	no[1]	no[2]
Membrane fluidity	no	no	no[1]	no[2]

Table 9: Relation of lipid composition, cholesterol levels, and membrane fluidity to thermal sensitivity.
1: Increase as well as decrease was observed. 2: Data are derived from references 400, 450,467 and references therein, as well as from Burgman P. and Konings A.W.T., unpublished data 3: Data as from Cress and coworkers (469,470)

It is possible that membrane proteins are the primary target for heat killing. No lipid phase transitions, that could be correlated with heat killing, were shown to occur near 40-41.5°C, whereas irreversible membrane protein

denaturation was shown to occur between 40 and 47°C (467). Receptor and histocompatibility antigen binding were shown to be reduced after heating of cells (472-474). It was found that hyperthermia increases the membrane permeability for exogenous compounds such as adriamycin and polyamines ((450) plus references therein). Hyperthermia also resulted in leakage of components from the interior of the cell (450) such as potassium (475-477). It was however shown that this was not the actual cause of hyperthermic cell killing (477) and that such changes were not common to all cell lines (478,479). This suggests that impairment of Na-K-ATPase function is not related to heat killing. More specifically, the ouabain-sensitive ATP hydrolyzing activity (Na-K-ATPase) proved to be rather insensitive to hyperthermic temperatures (480,481).

Amiloride, an inhibitor of the Na-H-exchanger, was found to enhance the cellular heat sensitivity, and to partially inhibit thermotolerance (425). Also, low extracellular pH can sensitize cells to heat and inhibit thermotolerance development (see Tables 6 and 7). This points to the Na-H-membrane protein as a potential target in heat killing. However, hyperthermic cell killing of up to 99.9% did not result in changes in internal pH up to 55 hours after exposure to heat (482) and heat sensitization by low extracellular pH did not cause changes in intracellular pH that could be related to heat killing (426,427) leading the authors to conclude that thermal damage to the regulation of the internal pH is not related to thermal killing nor to thermal sensitization by low pH.

Hyperthermia might also lower the Ca-ATPase activity of the plasma membrane (and/or that of the intracellular membranes) which -in turn- could lead to an increase in cytosolic free calcium, $[Ca^{2+}]i$, by net influx of extracellular Ca^{2+} and/or liberation of Ca^{2+} from the intracellular stores, as reported by Stevenson et al. (483). Since calcium plays a central role in various cellular processes, heat-induced changes in its intracellular levels might be explicitly involved in hyperthermic cell death (363,484). There are two reports (485,486) describing a relation between extracellular Ca^{2+} levels and heat sensitivity; heat sensitivity increased when a culture medium with higher $[Ca^{2+}]$ was used. Vidair and Dewey (478) did not find a relation between influx of $^{45}Ca^{2+}$ and heat sensitivity of CHO cells. No measurements of $[Ca^{2+}]i$ were made in the above cited experiments. In EAT and HeLa S3 cells no relation between cell killing and alterations in intracellular free calcium was found, and the calcium ionophore, ionomycin (5 μM), had no effect on hyperthermic cell killing in both cell lines (487,488). The last-mentioned studies suggest, in contrast to those of Stevenson and coworkers (483), that there is no relation between changes in intracellular calcium concentrations and cellular heat sensitivity. Further research is necessary to resolve the apparent controversy.

Finally, the cytoskeleton, a filamentous protein network in contact with the plasma membrane, has been implied in playing a role in several cellular functions such as intracellular transport and communication, protein synthesis and plasma membrane stability (489,490). Heat shock was shown to bring

about several changes at the level of the cytoskeleton (452,491-493) and these changes have been suggested to be related to heat toxicity (494). However, survival and the disorganization-reorganization phenomenon of the cytoskeleton were not always affected similarly. No "heat dose" dependency was found with respect to the kind and extent of cytoskeletal alterations (493). In addition, heat-induced changes of the cytoskeleton were found not to be related to the synthesis of HSPs (see 1.7). Finally, cytoskeletal reorganization did not seem to be a prerequisite for the development of thermotolerance (490). It is, therefore, unlikely that heat-induced alterations in cytoskeletal structure play a major role in cellular heat toxicity.

1.6.5 Hyperthermic alterations of the cell nucleus

A variety of changes in the eukaryotic cell nucleus has been observed after cellular heating. This issue is the major subject of investigation in this thesis (chapters 2-7,11). The status of research up to the start of our investigations is reviewed below and (in relation to heat radiosensitization) in 1.8.4. It has been shown that an increased vesiculation within the nucleus takes place after hyperthermia (495). Flow cytometric analysis of chromatin using acridine orange staining of DNA revealed heat-induced alterations at the chromatin level, indicated by a change in the relative green (binding to double-stranded nucleic acids) over red (binding to single- stranded nucleic acids) fluorescent light emission (496). One of the most dramatic changes that was detected biochemically, immediately after cellular heating, was an increase in the protein content of isolated chromatin (305,497-502). The extent of the increase was not due to a preferential loss of DNA (498,503) and depends on both the duration of the heat treatment and the hyperthermic temperature (498-501; figure 10). Also the protein content of isolated cell nuclei after heating cells was increased (410,498,500,503-505).
Flow-cytometric measurements showed that (nearly) no cross migration of proteins took place during the isolation of nuclei (503); analysis of double-stained (FITC[1] for protein and PI[2] for DNA) of mixed (heated and unheated) populations of nuclei or of nuclei isolated from a mixed population of heated and unheated cells gave similar dual parameter histograms (figure 11). This indicates that the increase in protein mass occurs <u>in situ</u>. Little is known, however, about the identity of the increased protein mass. It is well known that under most cell fractionation conditions some proteins (e.g., DNA polymerase α) will leak from the cell nucleus (194,506). It may very well be that heating in situ increases the <u>binding</u> of certain proteins to the DNA and/or nuclear matrix. The increased binding would result in less protein loss during the isolation procedure, thereby leading to an increased protein

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[1] Fluorescein isothiocyanate, a fluorescent compound used to label proteins

[2] Propidium iodide, a fluorescent compound binding to double stranded DNA (by intercalation)

content of chromatin and nuclei when isolated. It is also possible that some cytosolic proteins enter the nucleus upon heating, as was suggested by Roti Roti (507) and by biochemical and immunological studies on HSP translocation into the nucleus upon heating cells (see 1.7). The proteins involved in the increased binding to (sub)nuclear structures appeared to be non-histone proteins (497,498).

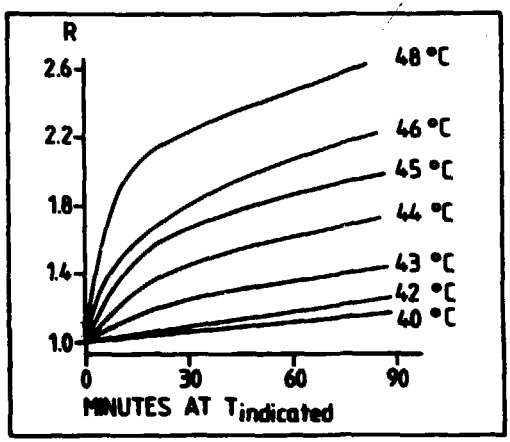

Figure 10: The kinetics of the heat-induced increase in chromatin protein content (redrawn from Roti Roti et al (500)). The relative protein content, R, is plotted as a function of time at the temperatures indicated and is defined as the protein-to-DNA ratio of chromatin isolated from heated cells, divided by the same ratio determined for chromatin from control cells.

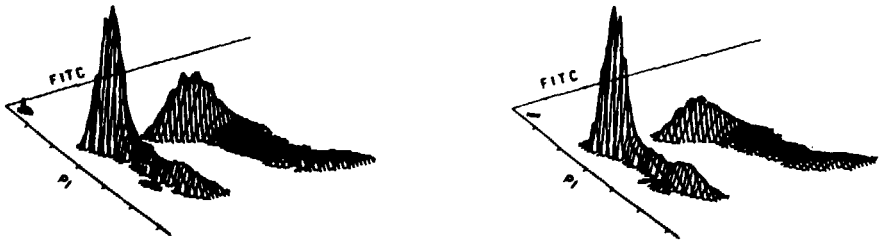

Figure 11: Dual-parameter, flow-cytometric histograms of mixed nuclei populations (redrawn from Roti Roti et al. (503)) See the text for more details. Left panel: Nuclei isolated from a mixed population of heated (30 minutes at 45°C) and unheated cells. Right panel: Mixed population of nuclei isolated separately from heated and unheated cells.

It seems unlikely that the increase is due to a heat-induced enhancement of cytoskeletal contamination to the nuclear exterior; fluorescent microscopic visualization of isolated fluorescently stained nuclei shows no such increase beyond a minor contamination with cytoplasmatic caps, equally present in nuclei from control and heated cells (504,505). It must be kept in mind that amount of protein in a nucleus isolated from a heated cell may be doubled (on a protein per nucleus basis) in comparison to a nucleus isolated from an unheated cell! Gel electrophoretic analysis of the proteins involved in the heat-induced increase in nuclear protein binding and, especially, antibody studies -in combination with electron-microscopic evaluation of nuclear morphology- will be necessary to exclude the possibility of a cytoskeletal collapse on the nucleus.

A question that needs to be resolved is: what causes the increased protein content of isolated chromatin and nuclei? Since the heating of isolated nuclei in the postnuclear supernatant of cell homogenates or in serum did not result in an increase in nuclear protein mass (501) it was concluded that some extranuclear (cellular) structure must be present to account for the heat-induced increase of nuclear protein binding. The possibility remains though that if the heat-induced increase in nuclear protein mass is caused by less loss (increased intranuclear binding) during the isolation procedure, this loss already has taken place and increased binding cannot be re-induced by in vitro heating of nuclei. Destruction of the plasma membrane with the mild detergent Tween-80 also resulted in a nuclear protein mass increase (501). This points more strongly to the possibility that the increase in nuclear protein binding results from initial, extranuclear, heat damage presumably at the level of the plasma membrane.

The nucleosomal structure seems unaffected after heating of cells; although the rate of micrococcal nuclease digestion was found to be decreased upon exposure of cells to an increasing "heat dose", the ultimate digestion level as well as the size of the monomer nucleosomal DNA remained unaltered (508,509). The increase in nuclear protein binding, however, might be responsible for the changes in DNA supercoiling observed when using the nucleoid sedimentation assay (177). On the other hand, the increased protein mass of these particles, causing altered sedimentation (177), may have effected the estimation of the degree of DNA supercoiling as judged by the titration with ethidium bromide.

From a study done by Wheeler and Warters (510) it was concluded that most of the excess nuclear proteins must be bound to the nuclear protein matrix (1.3), but less to the loop DNA, since limited nuclease digestion did not release detectable amounts of the excess nuclear protein. Since the nuclear matrix is involved in various DNA-associated processes (1.3) that are inhibited by heat (1.6.6 and 1.8.) it was hypothesized (511) that a heat-induced increase in the protein mass of (sub)nuclear structures, may responsible for the hyperthermic inhibition of DNA replication, transcription, and repair, maybe by restricting changes in supercoiling (177).

Heating of cells in the presence of alcohols and procaine (heat sensitizers) seems to enhance the heat-induced nuclear protein mass increase (505). Glycerol (a heat protector) was found to protect against such an increase (410). This suggests a relation between the extent of heat killing and the extent of nuclear protein mass increase.
Upon return to normal growth temperatures, the nuclear protein mass will return to control values with time, depending on the "heat dose" used (305,499,501,502). This process is not affected by inhibitors of DNA-, RNA- and protein synthesis nor is it affected by inhibitors of the oxidative phosphorylation or microtubulus assembly (501). Except for the inhibition of protein synthesis by cycloheximide (for which conflicting reports exist: compare Laszlo (512) and the review by Henle (373)) none of these processes seem to be a prerequisite for thermotolerance, whereas cold storage (4°C) did inhibit both tolerance development (see Table 6) and the recovery from the increased nuclear protein mass (501).
Since especially the nuclear matrix has been implicated in playing a role in DNA-associated processes it is important to provide a short overview of the effects of heat on (nuclear matrix associated) DNA and RNA synthesis. The effect of heat on protein synthesis will also be discussed (1.6.6). The synthesis of heat shock proteins after heating of cells will be covered separately (1.7). In section 1.8 the effects of heat on DNA repair will be reviewed.

1.6.6 Heat effects on DNA-, RNA- and protein synthesis

DNA synthesis

Hyperthermia inhibits the cell's ability to incorporate nucleotides into TCA-precipitable material in a "heat dose" dependent way (513-517). Initiation of DNA synthesis appears to be more heat-sensitive than DNA elongation (518), and recovers less rapidly upon reincubation at 37°C (516,519). The duration of the inhibition corresponds with the cell cycle progression delay of cells after heat treatment (515). The assembly of nascent DNA into mature chromatin is inhibited as well (509). It is unlikely that inhibition of DNA replication is caused by a depletion of precursors for DNA synthesis, since "heat doses" that inhibit replication do not affect the size of DNA precursor pools (518). The inhibition of DNA replication is also not due to a reduced rate of histone synthesis, since histone synthesis appears to have recovered when DNA synthesis is still depressed (517). There are two possibilities that might explain the heat-induced inhibition of DNA synthesis: the inactivation of enzymes involved in replication and the heat-induced increase in the nuclear matrix protein mass.

RNA synthesis

The incorporation of uridine into TCA-precipitable material also appears to be inhibited by heat (512-515). The "heat dose" dependency is reflected more in the rate of post-hyperthermic recovery than in the extent of inhibition (515). Most of the inhibition seems to occur at the level of ribosomal RNA

synthesis (450) which was suggested to be more heat-sensitive than mRNA synthesis (513). Apart from RNA synthesis, also RNA processing is affected by hyperthermia (450).
Hyperthermic inactivation of RNA polymerases and/or again changes in nuclear protein mass may be responsible for the inhibitory effect of hyperthermia on RNA synthesis.

Protein synthesis

Protein synthesis is transiently inhibited after heating cells (514,515,520). Reasons for a heat-induced inhibition of protein synthesis could be:

1. Destruction of polysomes
2. Effects on initiation factors
3. Collapse of the cytoskeleton

Roti Roti and Laszlo (450) reviewed these possibilities and concluded that the inhibition of protein synthesis most likely occurs at the initiation step via the phosphorylation of initiation factors.

All three processes are inhibited immediately after exposure of cells to heat. When the heat sensitivities of immediate inhibition of DNA, RNA and protein synthesis (measured as the incorporation of labelled precursors into TCA-insoluble material) are compared, protein synthesis turns out to be the most heat sensitive process, while DNA and RNA synthesis are less and comparably sensitive (514,515). Henle and Leeper (515) found that heating of CHO cells (17.5 minutes at 45°C) resulted in an inhibition of both DNA and RNA synthesis of about 90%, while protein synthesis was inhibited by more than 99.9%.
In comparing the recovery from hyperthermic inhibition of DNA-, RNA- and protein synthesis after "iso-heat doses" (17.5 minutes at 45°C) Henle and Leeper (515) found that RNA synthesis was suppressed for up to 12 hours, then fully recovered within 30 hours and even showed an overshoot for up to 50 hours after the treatment, while DNA synthesis remained suppressed for 6 hours, and then recovered to maximally 30% of the pre-treatment level (20-50 hours post heating). In these experiments protein synthesis started to recover 2 hours after the heat treatment, and returned to normal within 20 hours.
The relation of the inhibition of the biosynthesis of nucleic acids and proteins to thermal killing remains to be elucidated. The level of inhibition of RNA synthesis was found to be independent of the state of thermotolerance, but tolerant cells showed an enhanced recovery from this inhibition (521). Laszlo's recent data on the heat-induced inhibition of protein synthesis seem to indicate that also here only the post-heat recovery and not the extent of inhibition by heat is related to survival from hyperthermia (521). Some data with thermosensitive mutants recently showed a non-correlation between the extent of protein synthesis inhibition and recovery on one hand, and the extent of heat killing on the other (522).

1.7 Heat shock proteins (HSPs)

One of the general reactions occurring in cells upon exposure to heat is the inhibition of protein synthesis (523). After a heat shock a small specific subset of proteins, called heat shock proteins (HSPs) is, however, still (even at enhanced rates) synthesized. In 1962, Ritossa (524) was the first to observe a specific puffing pattern in heated Drosophila chromosomes, which later (525) was found to be a reflection of the activity of HSP genes.
HSPs are characterized (526) by the following features:

- they are induced upon exposure of cells to environmental stress (such as heat)
- their genes have the so-called "Pelham-box" (or Heat Shock Element-(HSE)) : see transcriptional control (below).

There are 3 major HSPs (70, 84, 110 kD) as well as some small HSPs (8, 20-30 kD), and some glucose-regulated HSPs (or GRPs: 78-80, 95-100 kD) (526,527). The HSP 70 class has been most extensively studied. Within this class of proteins a distinction can be made between constitutive HSPs (induced by stress but synthesized at significant levels in unstressed cells) and inducible HSPs (synthesized after stress only: see reference 528). These proteins share extensive sequence homology (492). In addition, a third class of HSP 70s exists that is constitutively synthesized. Their synthesis is not elevated upon stress (529); they are called "Heat Shock Cognate Proteins (HSCs)" (527). Most HSPs can undergo extensive covalent modifications, such as phosphorylation, methylation, glycosylation or ADP-ribosylation, which might affect their function and/or stability (527). HSPs can be induced by other stresses than heat, such as sodium-arsenite (Na-Ar: 492,530), ethanol (530), chronic hypoxia (530), amino acid analogues (492,530) or $CdCl_2$ (531).

1.7.1.Transcriptional control

It has been shown several years ago that exposure of cells to heat results in the specific induction of HSP gene transcription, while other genes are (transiently) not transcribed (524,525,532). Craine et al (533) combined cytoplasm of heated cells with DNA of non-heated cells and found specific transcription (in vitro) of HSP genes, while histone genes were not transcribed. This proved the need for a specific cytoplasmic factor in promoting HSP transcription. The heat-induced promotion of HSP transcription was found to be independent of the sequence -per se- of the transcribed region of the HSP 70 gene (534). HSP genes have a normal TATA promoter sequence (a short AT-rich DNA sequence about 30 bp upstream of the initiation site of the gene: 535), that is protected from DNase I attack before, during and after heat shock (534,536-538). Upon heat shock, however, yet another sequence, upstream from the TATA-box, is protected from DNase I attack. This region, with the consensus sequence: CTGGAATnTTCTAGA (an inverted repeat), was shown to be a secondary promoter. It was called "Heat Shock Element (HSE)" or "Pelham-box" (534,536-538). This element has

been found in Drosophila as well as in human cells (534,539,540) and is evolutionary highly conserved (541). Introduction of the HSP70-promoter region in a plasmid containing non-HSP-genes enabled transcription of these genes in cells transfected with the plasmid after exposure to heat (542,543). It is thought that a heat-inducible, cytoplasmic factor (called "Heat Shock Transcription Factor": HSTF) interacts with the HSE, causing local unwinding of the DNA helix. The change in chromatin arrangement leads to a better exposure of the TATA-box. This, in turn, results in enhanced transcription (527,534). The level of induction of HSP genes seems to be related to the number of HSEs (527,5454). Activation by HSTF is independent of protein synthesis and the HSTF must thus be present in some form in unstressed cells (545,546).

1.7.2 Translational control

HSP synthesis is probably both transcriptionally and translationally controlled. HSPmRNAs are -in vivo- preferentially translated over non-HSP-mRNAs (547). Although HSPmRNAs often have unusually long 5'non-translated sequences, little secondary structure, and an abundance of conserved sequences (548), these structural features seem not to be responsible for their preferential translation, since there is no competition with non-HSPmRNAs during translation in vitro (547). The overall decay of polysomes after a heat shock (548) does not, in general, result in dissociation of mRNAs from ribosomes (549); mRNAs, other than HSPmRNAs are not translated though. Their translation recovers during the post-heating period (547,550). HSPmRNAs <u>are</u> translated, however, and Sanders et al. (551) suggest that other factors than the polysomal structure itself must be responsible for that preferential translation. A dephosphorylation of the S6 ribosomal protein (552,553) was suggested to be one of the regulatory mechanisms involved. Dephosporylation of the initiation factor eIF-4b and phosphorylation of the initiation factor eIF-2a (554) may also be play a role in the regulation.
The relative importance of translational control versus transcriptional control remains unclear. Non-heat-shocked cells seem to contain sufficient HSP-mRNAs to account for the elevated HSP synthesis upon heat shock (555) and enucleated oocytes show a clear HSP-response upon heat shock (556) indicating that HSP genes need not to be actively transcribed. Another interesting feature of most HSPs (but not HSCs) is that the genes by which they are coded for do not contain introns (557,558). This avoids the need for (heat-sensitive) splicing (559) and allows the proteins to be made as rapidly as possible.

1.7.3. Functions of HSPs

<u>a) under normal physiological conditions</u>
Since most HSPs are synthesized constitutively, a search was started to elucidate their function(s) under physiological conditions. Insight into the

physiological function also might give information on their function in situations of stress. A function of HSPs in development was suggested, for instance, during early rat or mouse embryo development (560,561; see for a review Bond and Schlesinger (562)). Serum stimulation caused an elevation in HSP synthesis, suggesting a role of HSPs in cell proliferation (563-566). The increase in HSP70 synthesis in S-phase (565) and after partial hepatectomy in rats (567) also point to a role of HSPs in proliferation.
The high degree of homology between HSP70 and the dnaK protein of E. coli, a protein involved in the disassembly of protein-protein interactions during DNA-replication and in the modulation of the heat shock response (568), suggests a role for HSP70s in the modulation of protein-protein interactions in relation to dynamic DNA structure (568,570). The (ATP driven) uncoating reaction of clathrin by HSP70 also suggests an involvement of HSP70s in the assembly or disassembly of protein-protein interactions (550,569). Very recently, it was suggested that HSP70 might be involved in the translocation of proteins across intracellular membranes, acting as a protein "unfoldase" in a reaction that is ATP-dependent (571,572).

b) under stress conditions

Based on the properties and functions of HSPs as described above, hypotheses have been made about the functions of HSPs under stress conditions. HSPs are thought to stabilize proteins and to prevent aggregation of proteins; they are also thought to be involved in the disaggregation or solubilization of abnormal, degraded protein complexes in heated cells (527,573,574). An interesting finding was the ATP-binding capacity of HSP70 (550). This observation led to the following model for the role of HSPs in the "repair" of protein aggregates (figure 12).

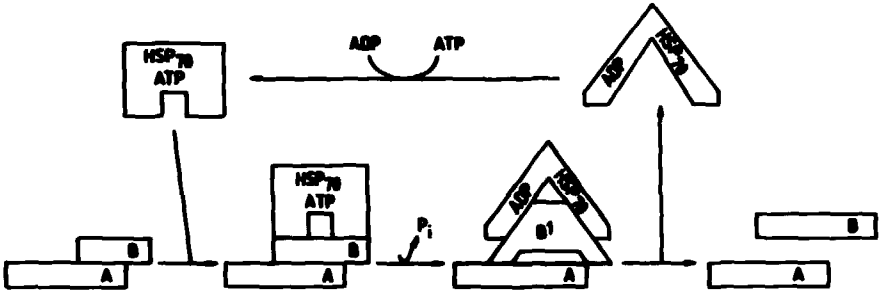

Figure 12: HSP-mediated disaggregation of abnormal protein-protein aggregates, driven by ATP hydrolysis (redrawn after Pelham (601)).

In Table 10, the role of various HSPs in protection and/or recovery of several cellular elements is given.

HSP (MW)	Protection/Recovery of	Ref.
110	nucleus/nucleolus	575
90	cytoskeleton	576
78	endoplasmic reticulum	569
72	first nucleoli, than ribosomes	550
70	nucleus	577
70	nucleolus	578-580
70	nucleolus/nuclear matrix	569
68	cytoskeleton	581-583

Table 10: Suggested protection and repair functions of HSPs during or after heat-induced changes in the structures listed.

Based on these findings, Schlesinger speculated in his review (526) that HSP70s participate in the "scaffolding" of protein complexes as well in their dissociation, the latter driven by ATP hydrolysis.

1.7.4. Relation between HSPs and thermosensitivity

In view of their functions (as discussed above), HSPs might play a role in modulating the thermosensitivity of cells.

Treatment	Thermotolerance	HSP synthesis	Ref.
heat	+	+	363
sodium-arsenite	+	+	363,550
ethanol	+	+	363
chronic hypoxia	+	+	363
amino acid analogues	D	I(*)	363
D2O	D	D	363
step-down heating	D	I	363
cycloheximide	+	-	363
Low heating rate	I	D	590,591
heat resistant var.	+	D	583,585
dinitrophenol	+	-	592,593

Table 11: Relation between HSP synthesis and thermotolerance
+= occurring; -= not occurring; I= increased; D= decreased
***= these HSPs, whose primary structure is altered because of the incorporation of these analogues, are non-functional.**

Although the absolute levels of HSPs (especially HSP70) have been found elevated in some heat-resistant CHO variants (583,584) and the kinetics of increasing and decreasing levels of HSPs often correlate with the appearance and disappearance of thermotolerance (583,585), HSP-levels are no general indicators of a cell's thermosensitivity (586-588). In Table 11 the effects of various treatments on the development of thermotolerance and HSP synthesis are compared. Although heat causes an enhanced rate of HSP synthesis (various reports; see e.g. Hahn (363)) other studies do not detect a direct relation with thermosensitivity (589-594).

HSP (MW)	Cellular distribution				relat. to TT	Cell type	Ref.
	normal	1thHT	recovery	2ndHT			
22-30	cytopl	n.matr	nd	nd	nd	various	527
24	cytopl	cytopl	nd	nucl.	nd	CEF	595
21-27	-	n.matr.	nd	nd	nd	Drosophila	596
46-48	-	n.matr.	nd	nd	nd	CHO	597
70	-	n.matr.	nd	nd	nd	Drosophila	596
	-	n.matr.	nd	nd	nd	CHO	597
	-	n.matr.	nd	nd	nd	various	569
70	cytopl	nucleus	nd	nd	nd	Drosophila	598
	cytopl	nucleus	cytopl	nd	specul.	various	526
	cytopl	nucleus	nd	nucl.	nd	CEF	595
	-	nucleus	nd	nd	nd	Drosophila	577
	cytopl	nucleus	nd	nd	nd	HeLa	599
	c.skel.	nucleus	nd	nd	nd	mammals	600
	-	nucleus	nd	nd	nd	plants	579
70	c.skel	nucl.	nd	nucl.	no	CHO	601
	cytopl	nucleol.	ribos	nd	yes	Drosoph./CEF	550,602
	random	nucleol.	nd	nd	nd	various	578
	cytopl	nucleol.	cytopl	nd	no	HeLa	531
78	cytopl	E.R.	nd	nd	nd	various	569
84	cytopl	cytopl	cytopl	nd	nd	various	527
84	cytopl	cytopl	cytopl	nd	nd	Drosophila	598
84	cytosk.	nucleus	cytopl	nd	nd	Neurobl.	603,604
85	-	n.matrix	nd	nd	nd	CHO	597
87	cytopl	cytopl	nd	cytopl	no	CHO	601
89	cytopl	cytopl	nd	nucl?	nd	CEF	595
95-100	golgi	golgi	golgi	nd	nd	various	569
110	nucleo	nucleo	nucleo	nd	nd	mammals	569
	-	nucleo	nd	nd	nd	10T1/2	575
110	cytopl	cytopl	nd	cytosk	no	CHO	601

Table 12: Intracellular translocation of HSPs -= unclear; nd= not done; TT= thermotolerance (see text for further details).

Another way in which HSPs might be involved in modulating thermosensitivity is via their translocation between different compartments of the cell during and after the heat treatment. Various translocations of the different HSPs have been reported, by both immunological and biochemical approaches (see Table 12). Using arsenite (as an inducer of thermotolerance) Welch (550,602) found only nuclear and not nucleolar HSP localization. In an excellent immunological study, Ohtsuka et al. (531) showed that nucleolar translocation kinetics did not correlate with the usual kinetics of thermotolerance development and decay. This translocation of HSP to the nucleolus was thought to be involved in the restoration of hyperthermic inhibition of RNA processing. They also did not observe any nucleolar staining when cells were treated with arsenite, cadmium-chloride, 8-hydroxyquinoline or ethanol (all inducers of thermotolerance). Survival was, however, not determined in these studies and the quantification of the data is somewhat questionable. Murnane et al. (601) suggested that HSP-translocation played no role in thermotolerance. Because of poorly defined cell-fractionation procedures as well as insufficient quantification of the data, their conclusion seems not justified. Clear-cut relations between HSP-translocations and survival after hyperthermia and thermotolerance can not be deduced from the data obtained so far.

1.7.5. Possible triggers for HSP synthesis and HSP translocations

a) cytoskeletal alterations:
Since part of the translational machinery seems to be associated with the cytoskeleton (605) and heat causes a collapse of the cytoskeleton (493,518, 606), heat-induced cytoskeletal rearrangements might serve as a trigger for HSP-synthesis. However, it was shown that cytoskeletal alterations do not result in changes in the synthesis of proteins in general, and that cells with a disrupted cytoskeleton are still able to respond normally to heat shock (493,518). So, changes in cytoskeletal organization are unlikely as a trigger for HSP synthesis.

b) ionic modifications of the intracellular environment:
After heat shock the plasma membrane of cells seems to be affected, leading to the alteration of the ionic composition of the intracellular environment (see also 1.6). Since intracellular ionic strength and gene expression may be interrelated (607) such changes after cellular heating could very well be involved in the HSP gene activation. Although in some cells the monovalent cation concentration seemed to be altered immediately (608) or 2-16 hrs after hyperthermia (468,476), this was not observed under all conditions or in all cell lines investigated (477,478,609). Boonstra et al. (609) showed that under conditions that led to the induction of HSPs, no changes in the concentration of monovalent cations occur. Therefore, the concentration of monovalent ions most likely does not play a key role in triggering HSP synthesis.

Another ion that might trigger HSP-synthesis is calcium. After heat shock, changes in calcium-metabolism were observed (478,479,610-613). Lamarche et al. (613) showed that when cells were incubated and heated in the absence of extracellular calcium, synthesis of HSPs was inhibited (and heat-sensitization was observed). There data are in contrast with the findings of Kim and Lee (614) who observed normal HSP synthesis after cells had been heated in the absence of extracellular calcium. In addition, it was shown that calcium-depleted Drosophila cells were still able to synthesize HSPs upon exposure to heat (613) and that inhibitors of the calcium-binding protein calmodulin (CAM) did not affect HSP synthesis in heat-shocked cells, although heat sensitization was observed (615). Since also heat-induced changes in intracellular calcium were found not to be related to heat killing under several conditions (1.6.4), it is not very likely that calcium acts as a signal for HSP synthesis.
Witzel et al (616) suggested that a changed intracellular pH could act as trigger for HSP synthesis, which was contradicted by Drummond et al. (612).

c) abnormal proteins:
It has been suggested (574,617) that intracellular accumulation of damaged or abnormal proteins might act as trigger for HSP synthesis. Heat shock as well as other inducers of a HSP response can be expected to cause (partial) denaturation of intracellular proteins (618). Normally, aberrant proteins are degraded in the cell via an ubiquitin-dependent reaction (see 619). If mammalian cells are transfected with plasmids carrying genes coding for truncated proteins, the degradation of such proteins is completely inhibited upon exposure of the cells to heat (620). The ubiquitin-degradation system itself is not particularly heat-sensitive; it rather seems overloaded after heat-shock (617). Temperature-sensitive mutant mouse cells with a thermolabile ubiquitin- activation enzyme synthesize high levels of HSPs at the non-permissive temperature (574). The level of ubiquinated histones H2A and H2B drops dramatically upon heat shock, and abnormal proteins appear to be non-ubiquinated (617). This indicates a shortage of ubiquitin that might be responsible for the non-ubiquination of the heat shock transcription factor (HSTF). This HSP-gene activator (normally ubiquinated) can thus become active (527,617,621). Its binding to the HSE (see transcriptional regulation and figure 13), will result in enhanced HSP synthesis as well as in increased ubiquitin synthesis (526); the ubiquitin gene itself was found to be heat-inducible (622). The concerted action of the synthesized HSPs and the ubiquitin system can take care of aberrant, damaged proteins and protein-protein-interactions, thereby restoring a normal level of demand for ubiquitin. This mechanism would be inherently self-regulating and thereby explain the transient nature of HSP transcription even when cells are maintained at high temperature for longer times (523). It would also explain the fact that HSP synthesis continues when amino acid analogues are used (577). The resulting HSPs are aberrant and non-functional.

Interestingly, microinjection of non-functional proteins led to the induction of HSP synthesis (623). Also, induction of HSP was found when cells were treated with agents that induce high molecular weight (abnormal) protein complexes; this leads to thermotolerance as well (624).

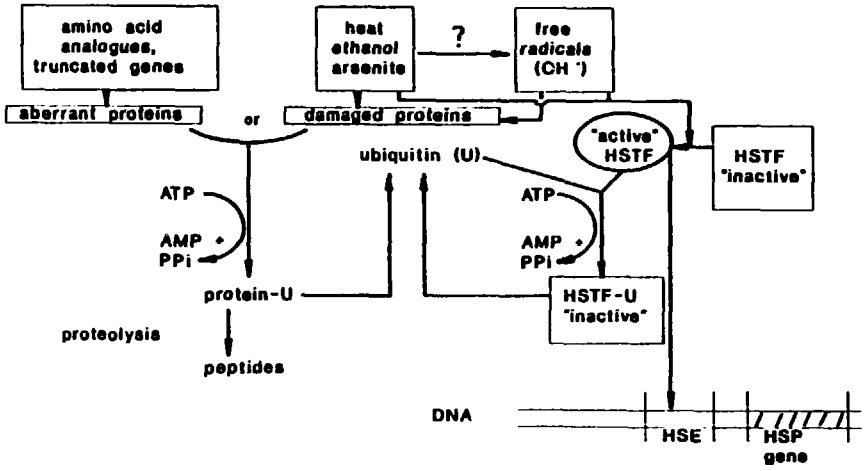

Figure 13: Schematic representation of the mechanism by which aberrant or damaged proteins may act as triggers for HSP-synthesis (527).

1.7.6. Increased nuclear (matrix) protein mass and HSPs

The observed increase of intranuclear protein binding (see 1.6.5) might be the trigger for HSP synthesis. The heat-altered protein-protein interactions in the nucleus might than -in turn- be restored to normal by HSP-action. The appearance of HSPs in the nucleus, nuclear matrix or nucleoids upon the exposure of cells to heat (see table 12) may be instrumental in this respect. Since the nuclear matrix has been implicated to play a dynamic role in DNA processing (1.3), alteration of the nuclear structure and its restoration (by HSPs) might be (one of) the means for a cell to survive an exposure to heat.

1.8 Heat and radiation

1.8.1 The synergism of heat and radiation

The synergism between heat and radiation is interpreted as a heat-induced increase in the cell's sensitivity to radiation. Optimal synergism is obtained when heat and radiation are applied simultaneously; when exposure to heat and radiation are separated in time, the synergism decreases with time, the fastest when radiation precedes hyperthermia (625,626; figure 14).

Radiosensitization can be quantified by the calculation of the thermal enhancement ratio (TER) which is defined as:

$$TER = \frac{\text{effect of radiation plus heat}}{\text{effect of radiation alone + effect of heat alone}}$$

The effect can be calculated for a fixed dose of radiation, on the basis of iso-survival levels or on the basis of the parameters that describe radiation survival curves (D_0, D_q, and n, or α and β: 1.2.1).

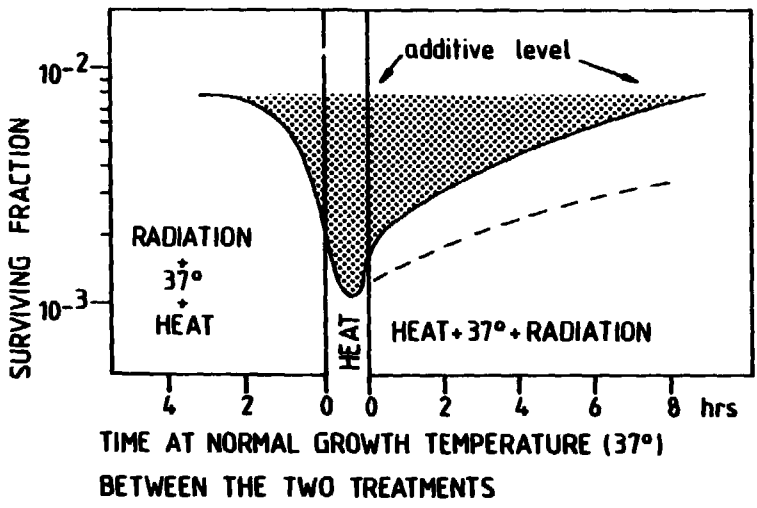

Figure 14: Effect of separation of heat and radiation on cell survival. Asynchronous cells received radiation before, during or after hyperthermia. The synergism is indicated by the shaded area (taken from Jorritsma: 297, deduced form reference 316).

1.8.2 The relation between heat killing and -radiosensitization

Usually, hyperthermic radiosensitization increases with increasing "heat dose", but it may become saturated at high heat doses (627). The extent of heat

radiosensitization in the various phases of the cell cycle is correlated to heat and to not radiation sensitivity (447,628). Modifications of heat sensitivity often but not always affect the extent of radiosensitization similarly (see Table 13).

	Effect on killing by		Reference
	heat alone	heat plus radiation	
Synchronization (S-phase)	+	+	447,628
Procaine	+	+	629,630
Glycerol	-	o	627
Low medium pH	-	o	289,424,631
	-	-	632,633
Hypothermia	+	o	404
Membrane fluidization	+	o	430,634
Step-down heating	+	+/o	see text
Thermotolerance	-	-/o	see text

Table 13: Effect of modifying conditions on hyperthermic cell killing and hyperthermic radiosensitization. + = sensitizing; - = protecting; o = no effect

The impact of thermotolerance (both chronic and acute) on heat radiosensitization has been a topic of extensive investigation.
In studying continuous heating at relatively low, hyperthermic temperatures several investigators noted the development of chronic thermotolerance, also expressed at the level of radio- sensitization; prolonged heating at these relatively low (41 or 42°C) temperatures did not result in any further radiosensitization in some cell lines (375,633,635-637). None or very little tolerance was observed for other cell lines (636) or after step-up (42-44°C) heating (291,375).
Also with regard to acute thermotolerance controversial results have been obtained. At first, different data were found for the (residual) radiosensitizing effect of a heat dose used to induce thermotolerance; although in most cases tolerant cells had radiosensitivities identical to those of nontolerant cells (632,638-641). Others found that D_q (637,642,643) or D_0 (291) had not fully recovered. When (if necessary) corrected for these residual radiosensitization effects, tolerant cells that were heated again had acquired also an effect of tolerance at the level of heat radiosensitization, i.e., the second heat dose could not sensitize for radiation as much as it did in nontolerant cells (632,637,638,641,642). This turned out to be not a general phenomenon, however; in other cell lines little or no effect of thermotolerance on heat radiosensitization was found (291,375,643). Even within one single cell line (CHO) contradictory results were obtained (compare 636,642:

tolerance for TER, to 639,640: no tolerance at the level of TER) for which no good explanation can be given.
Step-down heating was found to have a potentiating effect on heat radiosensitization of V-79 hamster cells (644) but not in Hela, EAT (375) or CHO (645) cells.
When normal fibroblasts were compared to AT cells, it was noticed that these cell lines displayed similar heat sensitivities but that the extent of radiosensitization was less in the AT cells (466). Raaphorst and colleagues (463,464) obtained similar data for heat sensitivity, but they observed that the AT cells could be radiosensitized by heat to the same extent as normal fibroblasts. In the AT cells, however, there was no loss of synergism between heat and radiation when the modalities were separated in time. This was taken as to suggest that the AT cells were unable to recover from heat and radiation treatments, while normal fibroblasts could. XP- and FA-fibroblasts also showed no altered heat sensitivity as compared to normal fibroblasts, but the TER was significantly lower in these cells (465).
The data discussed above suggest that the mechanisms underlying heat-induced cell death and radiosensitization, must, at least in part, be different. Especially the controversial effects of thermotolerance still need further elucidation.

1.8.3 Molecular targets for heat radiosensitization

Since the synergistic action of heat and radiation is due to a radiosensitization by heat and not to a heat sensitization by radiation (646) it is reasonable to assume that heat enhances the effect of radiation on the DNA, the target for reproductive cell death after radiation (1.2.2). Hyperthermia increases the amount of radiation induced chromosomal aberrations correlating with an increased radiation sensitivity (18).
Hyperthermia could enhance the radiation effect on DNA in two main ways:

1. Heat synergistically increases the initial amount of DNA damage
2. Heat lowers the ability of the cell to deal with the radiation damage

1. Initial damage by heat and radiation

As has been discussed in 1.6.4, heat (in the higher "dose range") can cause DNA lesions, that are not involved in heat-induced cell death. With regard to the type and extent of DNA damage after a combined treatment of cells with heat (usually lower "doses" than those that produce DNA damage) and radiation, a variety of results has been obtained. Some of these results have already been mentioned in 1.4; they are summarized in Table 14. It is unclear at the moment why there is such great variance between the data on the effects of heat on radiation-induced initial damage to the DNA. The differences are not cell-line or method-specific (see Table 14), nor are they correlated with the temperature range used in the various studies. The detectability of DNA damage after the combined treatment might have been affected by heat-altered chromatin structure (1.6.5) but this is hard to

evaluate. The data in Table 14 suggest that there might be only a minor effect (if any) of heat on the initial extent of radiation-induced damage.

Type of assay	Amount of initial damage: Heat plus radiation compared to radiation alone	Cell line	Ref.
Ess	same	Mouse L and V79	303
T'type damage	same	HeLa S3	279
Filter binding	same[1]	V79 and L1210	283,303,647
	more	Mouse L	283
Alkaline unwinding	same	HeLa S3 and CHO	289,292
	somewhat more	EAT	286,290
	somewhat less	HeLa S3	291
Alkaline elution	same	V79 and CHO	303
	same	L1210	648
	more	CHO	304,305
	less	HMV-1	302
Alk. sucr. gradient	same	CHO	304,502
	more	CHO	649
Neutral sucr. grad.	same	CHO	649
Non-denaturing filter elution	more	V79 and Mouse L	270,324,341

Table 14: The effect of hyperthermia on the initial extent of radiation-induced damage, as measured by the assays indicated. 1: sometimes apparently more DPCs are found, but this appeared to be due to qualitative, rather than quantitative, differences in the type of crosslinks measured (647).

2. Repair of radiation-induced DNA lesions and hyperthermia

Hyperthermia was shown to inhibit the repair of radiation-induced DNA lesions in nearly all cell lines investigated, as determined using all the various assays described in 1.4 and above (279,283,286,289-292,304,316,341, 502,648,649). The inhibition of repair was "heat dose" dependent, as shown in figure 15A, and the number of residual DNA lesions upon radiation given before, during or after heat (figure 15B), was found to be inversely proportional to cell survival (figure 14). With respect to the data reported by Mills and Meyn (316) it must be considered that for determination of residual DNA damage (figure 15B) much higher radiation doses were used than for survival (figure 14). Heat (< 42°C) sometimes accelerates repair (292,304) while radiosensitization is evident. Also, the finding that thermotolerance in HeLa S3 cells is expressed at the level of DNA repair but not at the level of heat radiosensitization (291) shows that hyperthermic effects on DNA repair rates alone are not sufficient to explain radiosensitization and

that more data are needed to fully understand the mechanism of heat radiosensitization (see chapter 12). Still, however, heat effects on DNA repair (rates as well as fidelity of repair, fixation-repair balance and preferential repair of specific sequences) seem -at least presently- the most likely cause for thermal radiosensitization.

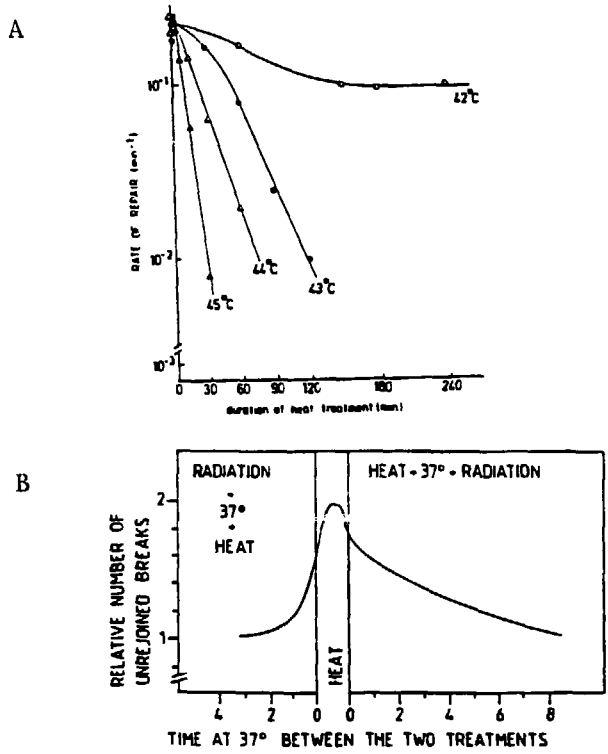

Figure 15:
A: Kinetics of DNA damage repair (alkaline unwinding assay) after different heat treatments (redrawn after Jorritsma and Konings (286))
B: Separation (in time) of heat and radiation treatments: effect on residual damage (alkaline elution; adapted from Mills and Meyn (316)).

1.8.4 Possible mechanisms involved in hyperthermic inhibition of DNA repair rates

Heat might inhibit DNA repair in at least two ways:

1. By hyperthermic reduction of the cellular activities of DNA repair enzymes
2. By hyperthermic alteration of chromatin structure, causing a decrease in the accessibility of the damaged sites to the repair machinery.

Both possibilities are investigated in this thesis and only a short introduction on these issues is given below.

1. Hyperthermic reduction of cellular repair enzyme activities.

For the excision of t'type damage, involving either DNA glycosylase, exinuclease or 5'-endonuclease activity (figure 7B), it was shown that a sonicate (presumably with these enzymes available to perform a repair task) of heated (30 minutes at 45°C) HeLa S3 cells was as proficient in removing this t'type damage from chromatin of unheated cells as was a sonicate from control cells (650: figure 16). So, these enzymes apparently are not very heat-sensitive.

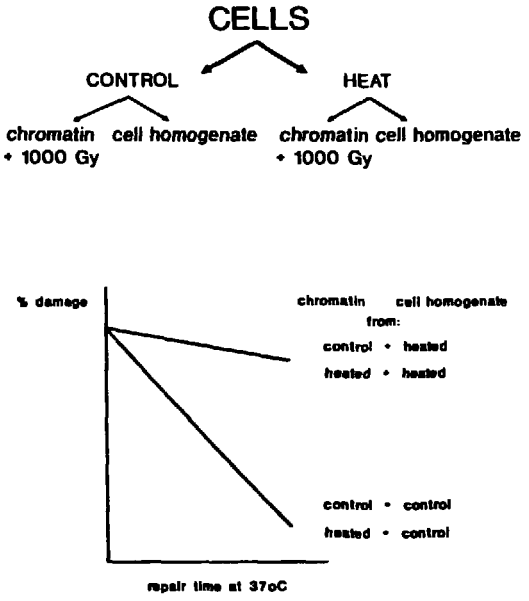

Figure 16: Schematic view of an experiment on the excision rate of t'type damage from irradiated chromatin isolated from heated and unheated cells by cell homogenates from heated and unheated cells. (adapted from Warters and Roti Roti (650))
HT = heat treatment of the cells (30 minutes 45°C)
C = unheated control cells

The activities of DNA polymerase α and β were greatly affected upon heating cells. These two enzymes are among the most extensively investigated with respect to their possible role in hyperthermic inhibition of repair. The activities are usually determined in cellular homogenates of heated and unheated cells by measuring the rate of incorporation of labeled nucleotides in exogenously added, "gapped" (DNase I treated) DNA. The individual activities of DNA polymerase α and β are distinguished by adding a specific

inhibitor of polymerase ∝ during the assay (N-ethylmaleimide (NEM) or aphidicolin); the resulting activity is defined as β-activity. The activity measured without inhibitors minus the β-activity is defined as ∝-activity (γ and δ activity are neglected). A schematic outline of the assay is given below (figure 17).

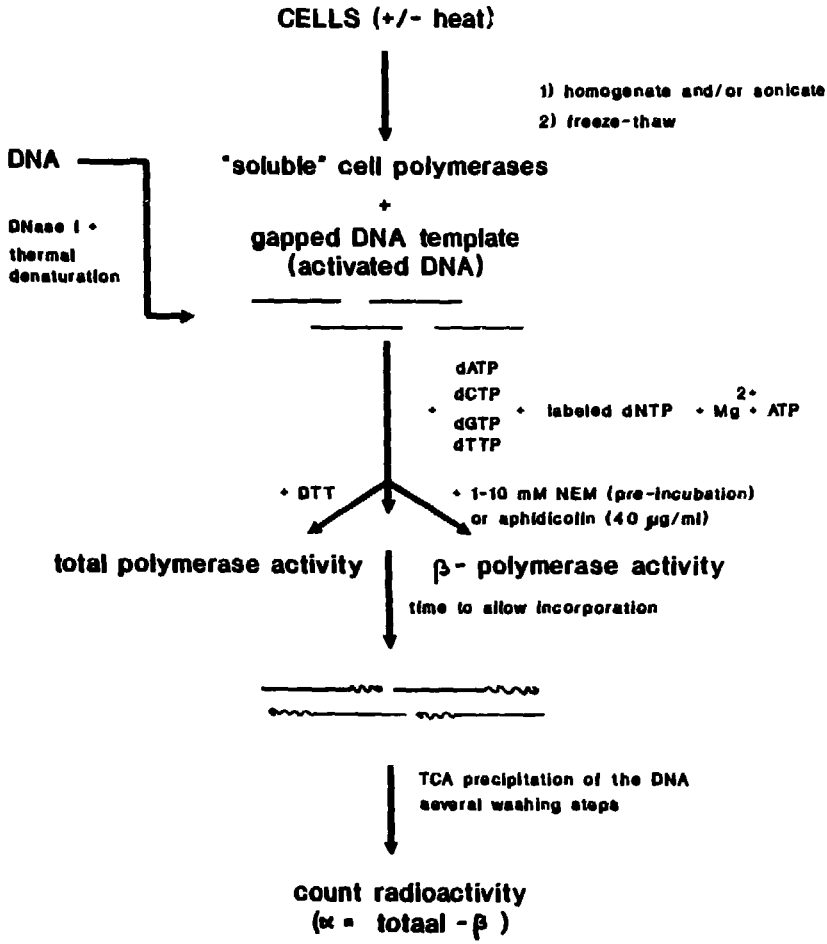

Figure 17: Schematic representation of the procedure used in determining the effects of hyperthermia on cellular DNA polymerase activities.

A "heat dose dependent" loss of activity was found for both enzymes. Polymerase -α, in general, is less heat-sensitive than polymerase -β (389,651-653: figure 18).

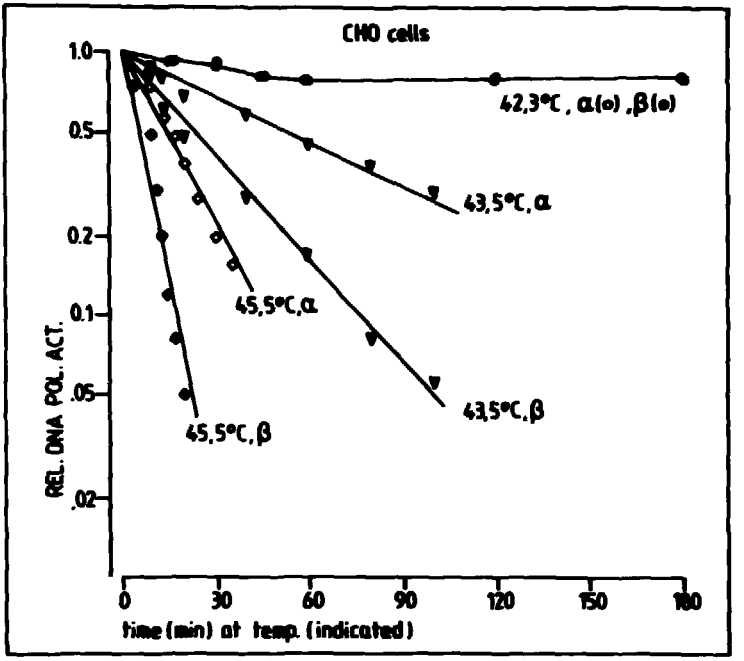

Figure 18: The effect of hyperthermia on the activities of DNA polymerase α (open symbols) and β (closed symbols) in CHO cells. (redrawn after Spiro et al. (651))

It was shown by Dewey and Esch (389) that continuous heating of cells at 42°C resulted in thermal tolerance for loss of polymerase activity. It is known (but not measured here (389)) for CHO cells that chronic thermotolerance is also expressed at the level of heat radiosensitization (633,635). So, it was suggested (but not quantitated) by these and other investigators (389,651-653) that the inactivation of the DNA polymerases could be responsible for heat radiosensitization.
Up to the start of the investigations presented in this thesis no information was available on the heat sensitivity of other DNA repair enzymes such as DNA topoisomerase II, exonucleases, AP-endonucleases or DNA ligase.

2. Hyperthermic reduction of DNA accessibility

As mentioned in 1.6.5, heat induces an increase in the protein mass of isolated chromatin and nuclei. This increase has been held responsible for a reduced accessibility of the DNA as detected by a decreased rate of

digestibility of heated chromatin by micrococcus nuclease (508-510). For the excision of t'type damage it was shown (650; figure 16) that the increased protein mass of the chromatin mainly determines the rate of repair. Reduced accessibility to the DNA in chromatin may very well be the rate-limiting step in the hyperthermic inhibition of repair of this type of damage. Whether this is just true for t'type damage or also for any other type of DNA damage has yet to be established. Mills and Meyn (305) also reported a correlation between hyperthermic inhibition of repair (as measured by the alkaline elution assay) and increased protein mass of the chromatin. Clark et al. (502) reported that post-heat recovery from hyperthermic inhibition of repair (as judged by the alkaline sucrose gradient assay) was still incomplete while the protein-to-DNA ratio of the isolated chromatin was already restored completely. DNA repair rates and chromatin alterations were measured in asynchronous cells while radiosensitization was examined for G_1- and S-phase cells only, so that a direct comparison cannot be made. An important question to be answered is whether or not heat-altered chromatin accessibility is a (the) main factor in radiosensitization. How this altered chromatin accessibility is related to the increased intranuclear protein binding has yet to be elucidated.

1.9 Aim of the investigations, scope of the thesis

The application of heat in the treatment of cancer, especially in combination with radiation, has been shown, in third phase clinical trials, to be promising. Better insight in the underlying mechanisms of both heat killing and -radiosensitization is helpful in achieving a more effective use of heat in cancer therapy. The use of hyperthermia may also lead to a better understanding of the processes that can lead to the loss of clonogenic ability of a cell. It may, furthermore, help to elucidate the role of DNA damage induction and repair in radiation-induced cell death.
In Chapters 2 and 3 experiments are described concerning the role of heat-induced increase in intranuclear protein binding in hyperthermic cell killing. If the increase which is observed immediately after heating of cells is related to cell death, the extent of this increase should be reduced for cells that are made thermotolerant or for cells heated in the presence of glycerol; the presence of procaine or ethanol would, in contrast, be expected to enhance this heat-induced increase. Different types of isolation procedures (detergent versus nondetergent) are used. In Chapter 7 indications are given with respect to the identity of some of the proteins involved in the increased binding to nuclear structures (DNA polymerases from the nucleoplasm). In Chapters 4-6 it is described how heat affects the protein composition of subnuclear structures (nuclear matrix and nucleoids), where in these structures the excess proteins might be bound and how this affects the structure and degree of supercoiling (using a new, so-called halo-assay) of the DNA loops. One-dimensional SDS-polyacrylamide gel electrophoresis was

performed to further identify the proteins involved in the increased heat-induced intranuclear protein binding, and using immunoblotting, the intracellular distribution of HSP70 immediately after heat and as a function of post-heating time is investigated in more detail (Chapters 5 and 11).
In Chapters 7-10 the putative role of heat-induced loss of DNA polymerases α and -β activity in heat-induced radiosensitization is tested. Three cell lines with different heat sensitivities were compared with respect to the extent of heat-induced loss of polymerase activities in relation to the extent of radiosensitization (Chapter 8). Using the polymerase α inhibitor aphidicolin, the specific role of DNA polymerase α in hyperthermic inhibition of repair was examined. In Chapters 7 and 10 the effect of thermotolerance on DNA polymerase inactivation was investigated and related to heat radiosensitization and its disappearance as a function of post-heating time. In parallel, these studies allowed for further evaluation of the relationship between heat-induced cell death and heat radiosensitization.
In Chapters 10 and 11 the possible relation between increased, intranuclear protein binding and heat radiosensitization, and the repair inhibition by hyperthermia was investigated. In Chapter 11, using the halo-assay, evidence is presented for a heat-induced reduction in the accessibility of radiation-damaged DNA in relation to the heat-induced, increased intranuclear protein binding.

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CHAPTER 2

HEAT-INDUCED NUCLEAR PROTEIN BINDING AND ITS RELATION TO THERMAL CYTOTOXICITY

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SUMMARY

When nuclei were isolated from exponentially growing HeLa S3 cells immediately after a treatment with hyperthermia and/or procaine-HCl, an increase in nuclear protein binding was observed. The extent of this increase however did not correlate with cell survival all under the conditions of the various treatments. For example, increase up to 40% in nuclear protein binding as result of a procaine treatment did not lead to a decrease of survival, while a 40% increase of nuclear protein binding as a result of hyperthermia corresponded with over 90% cell killing. In addition the extent of heat-induced enhancement of nuclear protein content was approximately equal for thermotolerant and heated control cells, or for cells heated in the presence of procaine. The rate of decay in nuclear protein binding upon post-heat incubations at 37°C of the cells however was enhanced in tolerant cells and retarded in cells heated in the presence of procaine as compared to heated control cells. These results show that in spite of suggestions in other reports neither the initial rate of enhanced protein binding nor the extent of the protein bound to the nucleus seems a reliable measure for heat toxicity. The capacity of the cell to reverse this heat-induced protein binding must be considered.

2.1 Introduction

The increase of the protein to DNA ratio in isolated nuclei (Kampinga et al. 1985), chromatin (Tomasovic et al. 1978, Roti and Winward, 1978, 1980) and nuclear matrices (Warters et al. 1986) as measured immediately after a hyperthermic treatment of cells has been suggested to be related to hyperthermic cell killing (Warters and Roti Roti 1982). Enhanced nuclear protein binding is thought to be caused via heat-induced membrane damage (Roti Roti and Winward 1980, Kampinga et al. 1985). Disruption of important nuclear processes by this nuclear protein binding may then be the reason for cell killing (Warters and Roti Roti 1982). Incubation of heated cells at 37°C may result in restoration to normal protein to DNA ratios depending on the hyperthermic dose given (Roti Roti and Winward 1978, Warters et al. 1986). Preliminary investigations in our laboratory indicated that the extent of enhanced nuclear protein binding does not always correlate with cell killing by heat (Kampinga and Konings 1985). We have extended our studies on this issue and initiated research on the process of decay of the enhanced protein binding after the heat treatment. Procaine treatment was used to sensitize cells for heat killing and pretreatment of cells with heat was used to obtain thermotolerance.

2.2 Materials and methods

2.2.1 Cell culturing

HeLa S3 cells were grown at 37°C in a shaking incubator as suspension culture in Jokliks modification of minimal essential medium (Flow, Irvine, Scotland) supplemented with 10 per cent fetal calf serum (Gibco, Paisley, Scotland). Exponentially growing cells were used in all experiments.

2.2.2 Treatment conditions

2.2.2.1 Procaine toxicity

The HeLa S3 cells growing in suspension were harvested by centrifugation at 800 g during 5 min. and resuspended in Jokliks medium supplemented with 10 per cent fetal calf serum to a cell density of 10^6 cells/ml. To test procaine toxicity procaine HCL (10 mM: final concentration) was added to the medium and the cells were incubated for various time periods at 37°C. The pH was maintained at 7.4 throughout the experiment. Procaine was washed out prior to the plating of the cells.

2.2.2.2 Hyperthermia alone

The cells were harvested by centrifugation and resuspended in prewarmed medium to a final concentration of 5.10^6 cells/ml. To achieve thermotolerance the cells were heated at 44°C during 15 min. (survival level > 90 per cent) and incubated at 37°C for 5 hours (1.10^6 cells/ml). Control and thermotolerant cells were harvested and heated at 45°C in fresh prewarmed medium.

2.2.2.3 Procaine and hyperthermia

After harvesting, the cells were resuspended in medium containing 10 mM procaine to a final concentration of 10^7 cells per ml. Before giving a heat treatment, the cells were

± 0.05 and 1.04 ± 0.03 respectively. These nuclei were isolated by the detergent method and the observations are consistent with earlier data (4). The procaine-induced increase in nuclear protein mass as observed after isolation of nuclei with the non-detergent method (2) seems to be based on a rather weak Triton-sensitive binding.

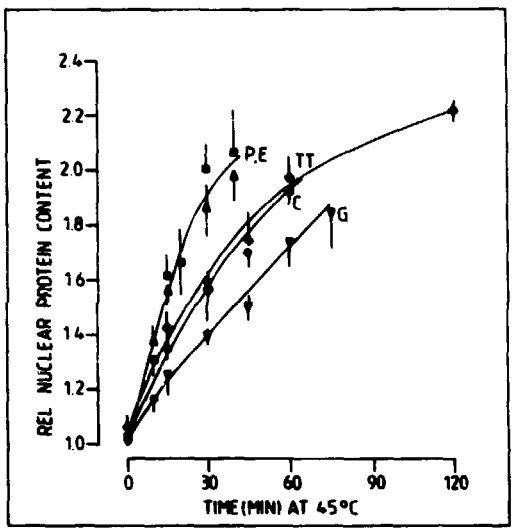

Figure 2: Effect of thermotolerance (TT), glycerol (G), ethanol (E), and procaine-HCl (P) on hyperthermia (45°C) induced changes in nuclear protein content. Glycerol was present 1 hour before and during the heat treatment. Nuclei were isolated directly after the hyperthermic treatment. The relative nuclear protein content (unheated control = 1.0) as revealed by the mean FITC fluorescence, is plotted versus heating time at 45°C. (mean ± s.e.m.; n > 3). The curves were fitted by eye. Symbols as in figure 1

It is known that after hyperthermia, cell-cycle redistribution occurs (10,13) with cells accumulating in G_2 with time after heat treatment. Since the mean FITC distribution of G_2 nuclei is about 1.78 times higher than for G_1 nuclei (9,12), cell- cycle redistribution might have influenced our results, especially those involving thermotolerance. Therefore we measured the effects of heat on nuclear protein mass-increase in nuclei from mid-S cells only to correct for possible cell-cycle rearrangements (figure 4).We found separate correlation curves for tolerant and nontolerant cells, in agreement with the finding for the total population of nuclei (figure 3). Therefore cell-cycle redistribution could not account for the observed deviation between survival and nuclear protein mass for tolerant and nontolerant cells.

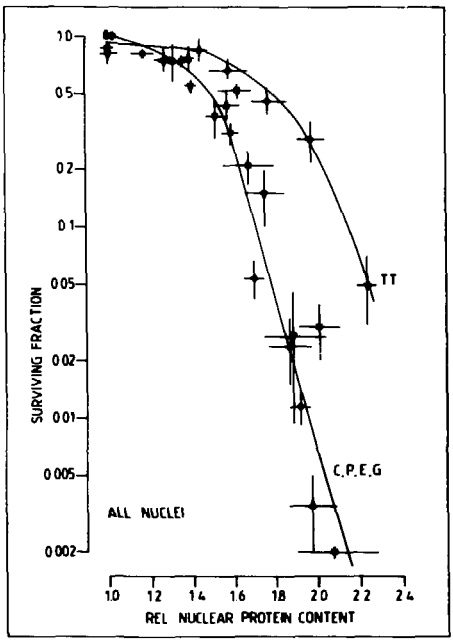

Figure 3: Hyperthermic cell survival as a function of heat-induced changes in nuclear protein content. Hyperthermic cell survival (data from figure 1) is plotted as a function of nuclear protein content (data from figure 2). At the 10% survival level tolerant cells contain 1.27 times more nuclear proteins as compared to nontolerant cells. Glycerol was present 1 hour before and during the heat treatment. Nuclei were isolated directly after the hyperthermic treatment. The two curves were fitted by eye, one for control, 5 mM procaine, 0.41 M ethanol, and 0.5 glycerol, and one for thermotolerant cells (15 min 45°C + 5h 37°C). Log-linear regression analysis revealed a "threshold" of 1.2. Symbols as in figure 1.

Hereafter the rate of removal of the increased nuclear protein mass during post-heating incubations was studied. As shown in figure 5A, single heat treatments causing near isosurvival (see figure 1: 30'45°C control cells; 15'45°C in the presence of 5 mM procaine or 0.41 M ethanol or 45'45°C in the presence of 0.5 M glycerol) showed similar initial increase in nuclear protein mass (~1.6-1.7 x) as well as the same rate of recovery upon post-heating time at 37°C. Thermotolerant cells showing a 1.6 fold increase in nuclear protein mass after cellular heating (30'45°C) however, showed a faster rate of recovery which might be related to their higher survival (figure 1) after this heat dose. In figure 5A the dotted line represents the curve for tolerant cells heated 60 minutes at 45°C (near isosurvival as compared to the nontolerant cells). Except for the zero hour point (initial increase) this curve closely resembles the curves for non-tolerant cells. The same was true for treatments causing a ~2 fold increase in nuclear protein mass (figure 5B).Therefore, no direct relation between the initial nuclear protein mass increase and the rate of its reduction after hyperthermia was found. The reduction of nuclear protein mass during post-heat incubations was also analyzed for mid-S phase nuclei. The removal kinetics were not significantly different when comparing these mid-S nuclei with the total population of isolated nuclei (data not shown).

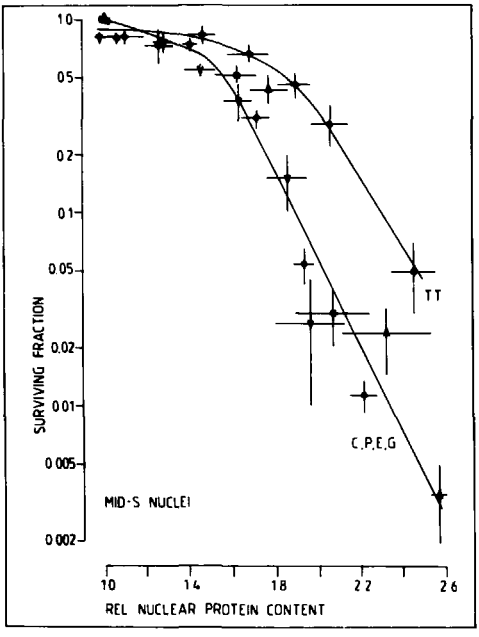

Figure 4: Hyperthermic cell survival as a function of heat-induced changes in nuclear protein content. Only mid-S nuclei were analyzed. Hyperthermic cell survival (data from figure 1) is plotted as a function of nuclear protein content (data not shown elsewhere). Glycerol was present 1 hour before and during the heat treatment. Nuclei were isolated directly after the hyperthermic treatment. The two curves were fitted by eye one for (control cells, 5 mM procaine, 0.41 M ethanol, and 0.5 M glycerol, and one for thermotolerant cells (15 min 45°C + 5h 37°C).
Symbols as in figure 1

3.4 Discussion

In a joint effort of our two laboratories, we have resolved our apparent controversies on the possible relation of heat-induced increase in the nuclear protein mass with heat killing. The analysis of the effect of various heat modifying conditions on the increase and post-hyperthermic restoration of nuclear protein mass, as measured by a detergent-based nuclear extraction, leads to the conclusion that both increase as well as recovery have to be measured in order to get a good correlation with hyperthermic cell killing.

3.4.1 Non-toxic increase of nuclear protein mass

The data in this report and earlier results (2,4) on procaine-induced nuclear protein binding are consistent with the suggestion (2) of the existence of loosely and firmly bound proteins to nuclear structures as induced by non-toxic procaine treatments or toxic heat treatments respectively. When nuclei are isolated with the TX-100 method these loosely bound proteins will detach from nuclear structures and will no longer be detected ((4), data not shown).

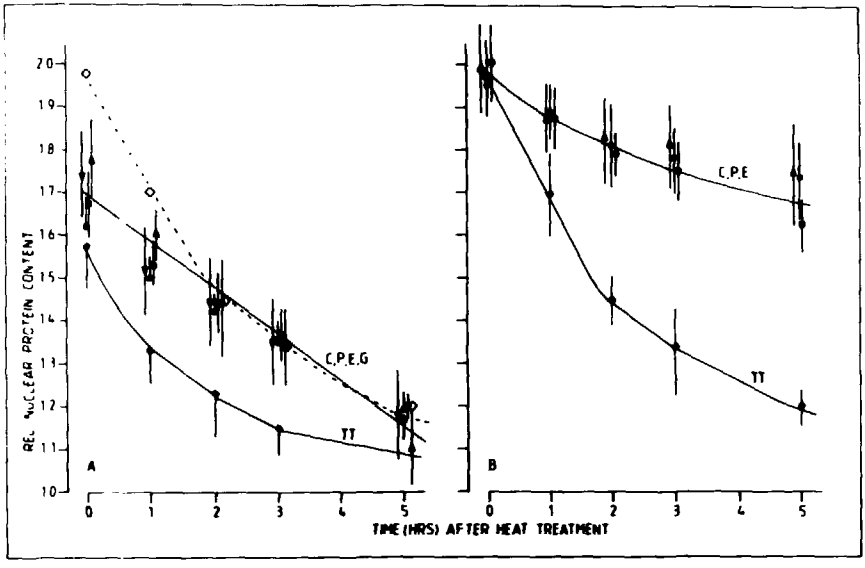

Figure 5: Effect of thermotolerance (TT), glycerol (G), ethanol (E), and procaine-HCl (P) on the decay of hyperthermia (45°C) induced changes in nuclear protein content. Panel A: decay after an initial heat-induced increase in nuclear protein mass of 1.58-1.77x. The dotted line represents decay after an initial increase in nuclear protein mass of 2.0x for tolerant cells heated 60' at 45°C, which is near isosurvival to the single heat treatments shown in this panel (see also figure 1). Panel B: decay after an initial heat-induced increase in nuclear protein mass of 1.95-2.01x. For both panels, glycerol was present 1 hour before and during the heat treatment. Cells were washed twice after the heat treatment with complete fresh (37°C) medium and reincubated at 37°C for the indicated periods of time before nuclear isolation. (mean ± s.e.m.; n > 3). Two curves were fitted by eye, one for control, 5 mM procaine, 0.41 ethanol, and o.5 glycerol, and one for thermotolerant cells (15 min. 45°C + 5h 37°C).

The loss of the nuclear enzymes DNA polymerase α and β from nuclei when isolated and the fact that a decrease of that loss is observed after hyperthermia (5) already indicates that various nuclear proteins are loosely bound (and bound more tightly after cellular heating). The higher protein content in nuclei isolated with the non-detergent sucrose gradient method (60 pg per nucleus, unpublished data) in comparison to nuclei isolated with the Triton X-100 method used here (47 pg per nucleus; (11)) indicates that different types of binding do play a role when comparing the two isolation methods.

3.4.2 Increased nuclear protein binding and cell killing

The results of the experiments with single heat doses as given in the current report reveal a correlation between the amount of increased nuclear protein mass and cell killing by hyperthermia as described earlier (1,4,6,10, 14). With the data available so far, it seems that the initial change in nuclear protein mass is a good predictor for heat killing when heat sensitivity is modified externally by ethanol, procaine or glycerol. The simple measurement of immediate increase in nuclear protein content will however not always be sufficient. After modification of heat sensitivity by alteration of the physiological state of cells (e.g. nutritional manipulations (16), heat-induced thermotolerance development[2] (2,7, this report)) both initial increase and recovery have to be taken into account. This result is consistent with the previous observation that a single correlation curve is not always sufficient (2,7). The status of thermotolerance seems to act like a dose modifier; a 1.27 fold greater increase in nuclear protein mass (figure 3) can be sustained to reach a 10% isosurvival level. With both methods for the isolation of nuclei (detergent as well as non-detergent) it has now been demonstrated (2,7, this report) that the amount of heat-induced nuclear protein mass increase in control and thermotolerant cells is the same, while the rate of recovery is faster in thermotolerant cells.

As the current data show, these phenomena cannot be explained by heat effects on the cell cycle distribution. Although heat causes an G_2 accumulation (10,13), and G_2 nuclei have more nuclear protein than nuclei from G_1 cells, based on FITC-fluorescence (9,12), corrections for possible cell cycle rearrangements, by only measuring S-phase nuclei, did not change our findings; heat-induced nuclear protein mass increase was similar for tolerant and nontolerant cells. Two separate correlation curves were found (figure 4) for mid-S nuclei from tolerant and nontolerant heated cells. Major alterations in cell cycle distribution do not start until 10-14 hours after cellular heating of HeLa cells (10), which suggests that cell cycle redistribution could also not have had any major effects on our recovery data (measured up to 5 hours after hyperthermia), which was found to be true (data not shown).

It appears that heat killing may be related to the amount of increased nuclear protein mass and the duration of its association with the nucleus. Both these parameters can be combined to include both the amount of increased nuclear protein mass as well as the extent of its removal within 5 hours post-heat time at 37°C. For experiments not involving tolerance Warters et al (15) suggested that a 15% increase in nuclear matrix protein mass might be sustained prior to the onset of cytotoxicity. Our data (figure 3) on nuclear protein mass increase suggest a comparable threshold (about

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2 C.A. Wallen and M. Landis. Removal of excess nuclear protein from cells heated in different physiological conditions. 8th Ann. Meeting of the NAHG. Philadelphia. abstract Ae-9 (1988).

20%) and are similar to data obtained before (1). Therefore we tested the "excess nuclear protein hours (ENPH)" above a 20% threshold (time of its presence in hours) as an independent variable for heat-induced cytotoxicity. As an example, figure 6 shows the ENPH for the heat plus removal curves of tolerant and nontolerant cells for a 30 min 45°C heat treatment. The ENPH is the area under the heat plus removal curve, representing the integral of the relative nuclear protein mass increase above 1.2x control during the heat plus 5 hours (arbitrary) post-heating period. When ENPH was plotted versus the extent of heat killing a good correlation was found (figure 7; correlation coefficient: r= 0.965). For certain heat treatments, nuclear protein mass has not returned to control values in the time span of 5 hours post-heating. However, we have arbitrarily chosen a 5 hour cut-off point to avoid secondary increases in nuclear protein content due to cell cycle rearrangements (10). Although the parameter ENPH does has its limits, it is adequate for our first approximation and appears to give a good correlation with heat killing. This issue will need a more detailed analysis. We are currently working at refined methods quantifying the relation between increased nuclear protein mass and probability of cell killing by hyperthermia. The present analysis reveals that both immediate increase as well as recovery of nuclear protein mass increase have to be determined in order to test its relation with heat killing. Similar observations were done recently by Wallen and Landis[2].

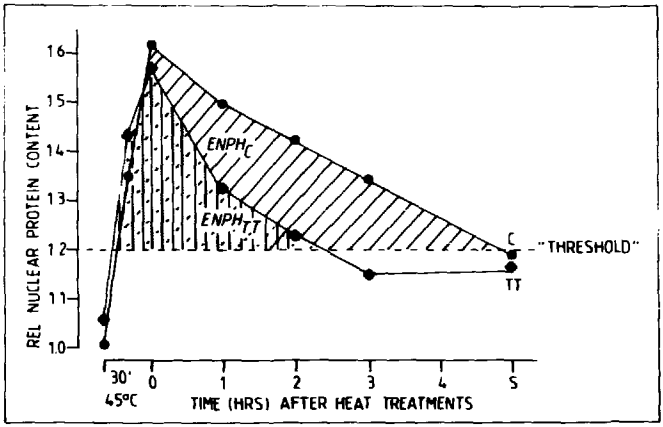

Figure 6: Schematic presentation of the calculation of the "excess nuclear protein hours (ENPH)" parameter. The parameter is the integral of the area under curves above a 1.2 fold threshold. The curves presented are the curves after 30'45°C heat treatments of control (C) and thermotolerant (TT) cells as shown in figure 5. The shaded areas represent the ENPH-values.

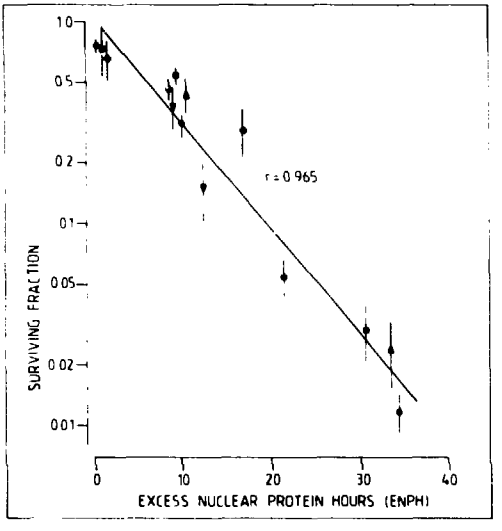

Figure 7: Hyperthermic (45°C) cell survival as a function of the "excess nuclear protein hour (ENPH)" parameter under the various heating conditions. (circles) control cells; (diamonds) thermotolerant cells (15'45°C + 5 h 37°C); (inverted triangles) 0.5 M glycerol; (triangles) 0.41 M ethanol; (squares) 5 mM procaine. Glycerol was present 1 hour before and during the heat treatment. Cells were washed twice after the heat treatment with complete fresh (37°C) medium and reincubated at 37°C for the up to 5 hours after cellular heating before nuclear isolation. The ENPH values (as calculated from figure 4 and from data not shown) were plotted versus hyperthermic cell survival (data from figure 1). The curve was fitted by linear regression.

3.4.3 Possible mechanism involved in the decay of increased nuclear protein mass

The presence of heat shock proteins (HSPs) in the nucleus (nucleolus) has been suggested to play a role in recovery processes after hyperthermic treatments of cells[3] (16-21). These HSPs were suggested to be involved in

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recovery from heat-induced changes in nuclear (nucleolar) structures (16, 18, 20,) and hyperthermic inhibition of RNA-processing (20). The recovery was inhibited when cells were heated in the presence of actinomycin-D, due to inhibition of HSP-synthesis (20). Here as well as in an previous study (2), we report on a faster recovery from heat-induced increase in nuclear protein mass in tolerant cells. It is tempting to speculate that the increase of (the inducible) HSP 72/73 in nuclear structures 5-6 hours after the initial heat dose (16, 19, 21), leading to the status of thermotolerance, is responsible for this enhanced recovery from nuclear protein mass increase and resumption of nuclear functions. The HSPs may function in releasing the heat induced abnormal protein binding to the (sub)nuclear structures by inducing conformational changes in the attached proteins.

Changes in the nuclear structure appear to be one of the most likely causes of heat-induced cell death. It is possible that this change in nuclear protein content is a consequence of primary actions of heat at the plasma membrane level as was suggested before (22). The change in chromatin structure then leads to the impairment of a variety of nuclear functions. Not only the extent, but also the duration of this infliction determines whether a cell will survive the hyperthermic treatment or not. Synthesis and reallocation of HSPs (from the cytoplasm to the nucleus) may be of major importance in the ability of the cell to recover from potentially lethal disturbances at the level of the nucleus as caused by hyperthermia.

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CHAPTER 4

EFFECT OF HYPERTHERMIA ON DNA LOOP-SIZE IN HeLa S3 CELLS

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SUMMARY

Nuclear matrices of heated and non-heated HeLa S3 cells were isolated and average DNA loop-sizes were compared. The heat treatment (30 minutes at 45°C) resulted in an ultimate survival level of the cells of about 10 per cent. The loop-size determinations were done on nuclear material isolated from the cells directly after the heat treatment. In the nuclear matrices isolated from the heated cells about 1.8 times more protein was bound as compared to the matrices from control cells. Enzymatic analysis using DNase I digestion followed by centrifugation on neutral sucrose gradients was performed. Also, halo visualization was combined with autoradiography. Both methods revealed no gross alterations in DNA loop-sizes. The possible function of DNA loop organization in the effect of hyperthermic interference with DNA related processes is discussed.

4.1 Introduction

Nuclear DNA is attached to a structural protein entity, termed the nuclear matrix (Dijkwel et al. 1979; Mullenders et al. 1982, 1983a; Berezney 1984). This protein structure can be obtained by extraction of purified nuclei with high salt solutions (Dijkwel et al. 1979; Mullenders et al. 1982, 1983a). The organization of eukaryotic DNA into repeating DNA loops, anchored to this nuclear matrix is assumed to play an important role in the regulation of nuclear processes in addition to its function in DNA packaging (see for review Berezney 1984). Both DNA replication (Wanka et al. 1977; Dijkwel et al. 1979; Vogelstein et al. 1980; Mullenders et al. 1983b; Berezney 1985) and transcription (Pardoll and Vogelstein 1980) seem to be associated with the nuclear matrix. As far as repair processes are concerned, no close association was found with the nuclear matrix in human fibroblasts after 30 J/m^2 UV-irradiation (Mullenders et al. 1983b), although some investigators do suggest a role for the DNA matrix attachment sites in DNA repair (McCready and Cook 1984, Harless and Hewitt 1987). Hyperthermic treatment of mammalian cells leads to cell killing and to sensitization of cell killing by UV- and X-irradiation (Dewey et al. 1980; Dewey 1984). The synthesis of DNA, RNA and protein is inhibited by lethal heat doses (Dewey et al. 1980; Dewey 1984; Jonsson et al. 1984). Radiation sensitization by heat is probably due to the observed hyperthermic inhibition of repair of damaged DNA (Corry et al. 1977; Jorritsma and Konings 1983; Bodell et al. 1984). Besides inactivating DNA repair enzymes (Spiro et al. 1982; Kampinga et al. 1985) heat has been shown to alter the protein to DNA ratio in nuclei (Kampinga et al. 1985) chromatin (Roti Roti and Winward 1978; Tomasovic et al. 1978) and DNA protein matrices (Warters et al. 1986) isolated from heated cells. A distinct alteration in DNA to protein ratio may have important implications for the organization of chromatin within the nucleus. Thus far no information is available on possible heat- induced alterations of DNA loop organization. Since this organization might be important for the regulation of various nuclear processes, alterations at this level might be of great relevance. We therefore determined whether a hyperthermic treatment (30 min. 45°C), leading to a cell survival of about 10 per cent, affects protein binding in DNA matrices and alters DNA loop organization of HeLa S3 cells, using high salt extraction (2M NaCl) of nuclei, matrix DNA degradation kinetics and fluorescence halo technique.

4.2 Materials and methods

4.2.1 Cell culturing and cell labelling conditions

HeLa S3 cells (ATCC no. CCL 2.2.) were grown in suspension cultures in Jokliks modification of minimal essential medium (Flow, Irvine, Scotland) supplemented with 10 per cent Fetal Calf Serum (Gibco, Paisley, Scotland). Doubling time was about 26 hours.For determinations of protein binding, asynchronous, exponentially growing cells were uniformally labelled in their DNA with 2.8 x 10^2 Bq/ml [methyl-^{3}H]thymidine (s.a. 7.4 x 10^{10}

Bq/mmol) for about 48 hours and uniformally protein labelled with 1.39 x 10^5 Bq/ml [^{14}C]-leucine (s.a. 1.29 x 10^{10} Bq/mmol) (both NEN, Dreieich FRG) for the same time period. For loop-size determinations the cells were uniformally labelled for about 36 hours with either [methyl-^{3}H]thymidine, 1.85 x 10^3 Bq/ml (1.85 x 10^{11} Bq/mmol) or [methyl-^{14}C]-thymidine, 3.7 x 10^3 Bq/ml (1.85 x 10^9 Bq/mmol) (both Amersham GB). After labelling, the cells were chased for at least 2 hours at 37°C in complete, label-free medium.

4.2.2 Measurement of DNA loop-size using DNAse I digestion of DNA-matrix complexes

After cell labelling and chase, the [^{3}H]-labelled cells were spun down (5 min at 250 x g), resuspended in complete medium(1 x 10^6 cells/ml), preheated at the desired temperature and heated 30 minutes at 45°C in a precision waterbath,while the [^{14}C]-labelled cells resuspended and held at 37°C.The heated and nonheated cells were mixed, pelleted (5 min 260 x g) and washed once with phosphate buffered saline (50mM Na-phosphate pH 7.4/0.9 M NaCl). Nuclei were isolated by homogenizing the cells in 0.1 % Triton X-100/5 mM Tris-HCl pH 8.0 through a hypodermic needle (Dijkwel et al. 1979). After 10 min centrifugation, at 1000 x g the nuclear pellet, free of cytoplasmic contamination as judged by phase- contrast microscopy, was dispersed in 6 ml of 10 mM NaCl/25 mM Tris-HCl pH 8.0, and mixed with 6 ml 4 M NaCl/25 mM Tris- HCl pH 8.0. All these steps were carried out at 0-4°C. After gently lysing the nuclei in the 2M NaCl (final conc.) for protein extraction, 2 ml samples were taken for DNAse I digestion (Sigma, electrophoretically pure). DNAse I (Sigma E.C. 3.1.21.1; 0-200 μg/ml) digestion was carried out for 15 minutes at 37°C in the presence of 5 mM MgCl . The digestion was stopped by putting the samples on ice and adding 100 μl 0.5 M EDTA. The digested nuclear lysates were layered on neutral 15-30 per cent sucrose gradients containing 2 M NaCl/25 mM Tris-HCl pH 8.0, prepared on a 65 per cent sucrose cushion containing 0.4 g/ml CsCl. Centrifugation was performed in a Beckman SW41 Ti rotor for 45 minutes at 30 000 rpm and 20°C. After centrifugation, the DNA-nuclear protein matrix (the nuclear protein skeleton with its associated DNA; the amount depending on the DNase I digestion) was recovered from the sucrose cushion (fractions 1 and 2) as the fast-sedimenting complex (Mullenders et al 1982). The gradients were fractionated from the bottom and the radioactivity was determined as described previously (Mullenders et al. 1983a). The fraction of DNA attached to this nuclear matrix protein skeleton was plotted versus the DNase I digestion as percentage of total cpm [^{3}H] or [^{14}C] per gradient respectively.

4.2.3 Measurement of DNA loop-size using the halo fluorescence technique and autoradiography

Exponentially growing HeLa S3 suspension cells were cultured in monolayer in petri dishes for 2 days, split and grown on sterilized coverslips. (non-pretreated glass). For autoradiography cells were incubated for 2 hours in the presence of 5.55 x 10^{10}Bq/ml [^{3}H]thymidine (1.85 x 10^{10} Bq/mmol) to label S-phase cells. The cells were heated for 30 minutes at 45°C by submerging the coverslips in sealed petridishes in a precision waterbath and DNA halo structures of heated and nonheated cells were prepared according to the method of Buorgiorno-Nardelli (1982) as described previously (Mullenders et al. 1984). Briefly, coverslips were subsequently dipped in ice cold detergent buffer (NP), high salt (2 M NaCl) buffer and high salt buffer plus 100 μg/ml Ethidium bromide. After exposure to 254 mm UV (4 W/cm2) at 1 cm distance to obtain maximal halo expansion, photographs of halos were made under a fluorescence microscope (Zeiss: green light excitation). The coverslips with the labelled cells were washed with low salt buffer, without salt and distilled water, air

dried and prepared for autoradiography using Kodak NB2T emulsion. After exposure and photographic processing, the halos were stained with 5 per cent aqueous Giemsa, and analyzed with a Leitz microscope connected to an Artek Model 880 automatic grain counter (Lonati-Kalligani et al. 1983).

4.2.4 Measurement of protein binding to DNA-nuclear matrix complexes

The DNA- and protein labelled cells were, after a 2 hours chase, heated at 45°C for various periods of time. DNA-nuclear matrix complexes were isolated as described in 4.2.2 (without DNase digestion). The nuclear matrix fraction was precipitated (10 min at 1000 x g) in 10% TCA/ 2% sodium-pyrophosphate (NaPy). After washing (10 min at 1000 x g) the samples twice with 5% TCA/ 2% NaPy and once with 100% ethanol, the remaining pellets were dried and dissolved in solueen-350 (Packard). Radioactivity was counted using liquid scintillation.Protein binding was measured using a $[^{14}C]$ Leu/$[^{3}H]$ TdR ratios and expressed relative to the ratio observed for unheated controls (=1.0)

4.2.5 Determination of cell survival

Cell survival was determined using the cloning assay on soft agar as described before (Kampinga et al. 1985). Plating efficiency of unheated control cells was about 70%.

4.3 Results and discussion

Heat treatment of the HeLa S3 cells resulted in progressive cell killing upon increasing heating time at 45°C and concomitantly the protein binding to DNA-nuclear matrix complexes increased (figure 1). During the matrix isolation there was a small loss of DNA ($[^{3}H]$thy), that was the same for heated and unheated cells (not shown). So, the increase in $[^{14}C]$Leu/$[^{3}H]$thy ratio was indeed due to an increased protein binding to the DNA-nuclear matrix complexes of heated cells. Our data however do not reveal whether the enhanced protein binding is predominantly to the DNA or to the nuclear matrix protein skeleton. Data from Wheeler and Warters (1982) and Warters et al (1986) show that most (if not all) of the protein binding is to the protein skeleton. The large increase of normal protein to DNA ratios in DNA- nuclear matrix complexes isolated from heated cells, also found by Warters et al. (1986), might possibly affect the structural organization of DNA at the nuclear matrix and result in alteration of the DNA loop-size. Alterations in DNA loop-size can be detected by digestion of DNA matrix complexes with DNAse I (Linskens et al. 1987). Relative loop-size increment or decrement by heat will be reflected by an increased or decreased rate of release of DNA from DNA matrix complexes. For reasons of accuracy with respect to DNAse digestion studies, we have employed a double labelling procedure using $[^{3}H]$- and $[^{14}C]$-thymidine. Relative alterations in average loop-size will be indicated by a release of both labels to a different extent during the course of the DNAse I digestion.

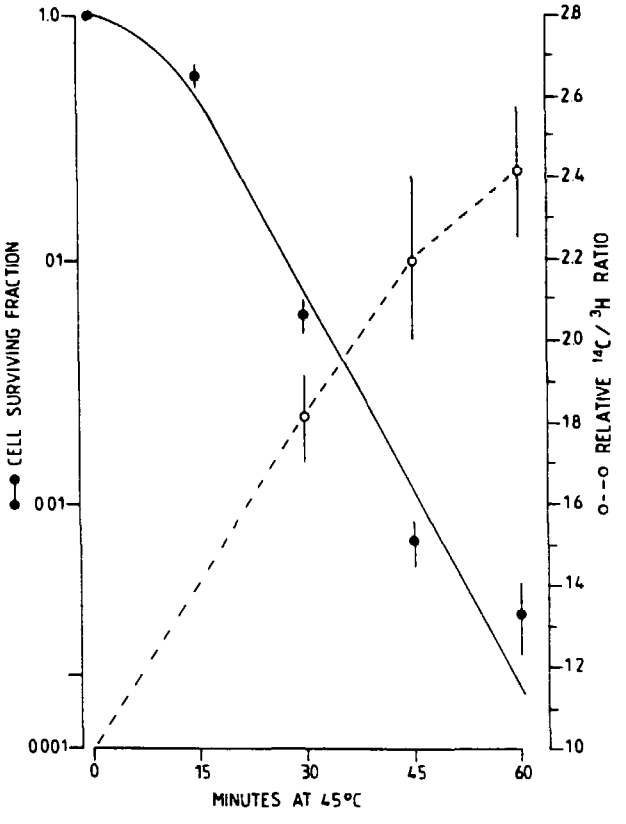

Figure 1: Effect of hyperthermia (45°C) on cell survival (closed symbols) and relative protein binding to DNA-nuclear matrix complexes (open symbols). Cell survival was determined using the soft agar cloning assay and plotted relative to untreated control cells (= 1.0). Relative protein binding to DNA-nuclear matrix complexes (no DNAseI used) was determined immediately after the heat treatment of [^{14}C] leu/[^{3}H]thy-labelled cells. The [^{14}C]leu-[^{3}H]thy ratio was plotted versus the ratio in nuclear matrices from unheated cells (= 1.0). Mean values (± SEM) of 3 independent measurements are given.

As shown in figure 2 no alterations in digestion rate between heated and nonheated cells were observed. Occasionally some small differences were observed, indicating somewhat smaller loop-sizes after hyperthermia. However, this effect was not significant. The same results were found when we heated the [$1^{4}C$]-labelled cells instead of the [^{3}H]-labelled cells (data not shown).

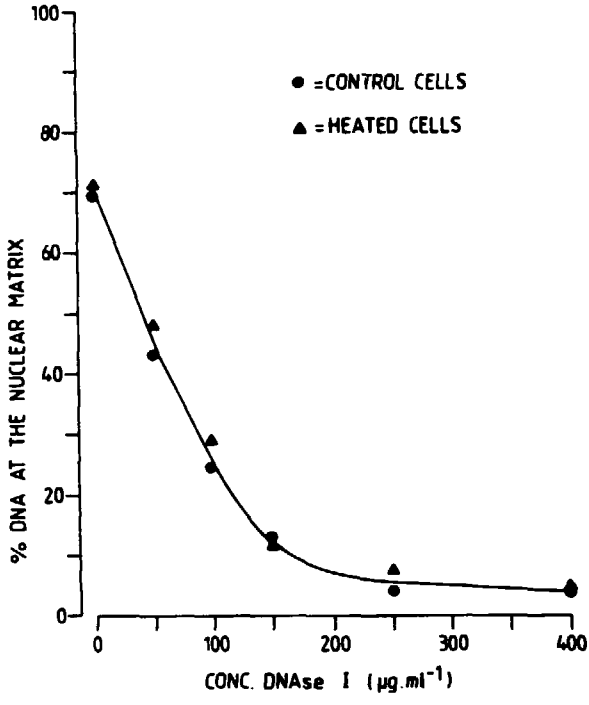

Figure 2: Effect of hyperthermia (30 min. 45°C) on the DNAse I digestion of nuclear matrices. C-labelled control (circles) and H-labelled heated (triangles) cells were mixed and nuclear matrices were isolated and digested for 15 minutes with increasing concentrations of DNAse I at 37°C. After centrifugation over a sucrose (15-30%) gradient, the fractions were collected. The fraction of labelled-DNA coisolated with the rapidly sedimenting nuclear protein skeleton (=% DNA at the nuclear matrix) was plotted versus increasing concentration of DNAse I. Mean values of 3 independent measurements are given.

The most obvious explanation thus is that the loop-sizes in heated and unheated cells are the same. However, two other possibilities might explain a similar rate of digestion. It is possible that in the heated group, the loops are smaller but more rapidly digested. This is not very likely though, since nuclei from heated cells are slightly less accessible for DNase I digestion (Roti Roti et al. 1985). The loops in the heated cells could also be larger but less accessible. This is not very likely too since the enhanced protein binding seems to be localized at the nuclear protein skeleton and not to its associated DNA (Wheeler and Warters 1982; Warters et al. 1986). This makes

it unlikely that the "naked and histone-less" DNA of our high salt treated nuclei (DNA-nuclear matrix complexes) is protected from DNase I attack. Additional evidence is necessary to draw final conclusions. Therefore, DNA halo matrices structures were prepared from heated and nonheated cells and DNA-loops were measured. No significant differences (figure 3) in DNA loop-sizes were observed either. Fluorescence micrographs (figure 3A,B) and computer aided measurements of loop-sizes of S-phase labelled cells (figure 3C,D), revealed a loop size of about 30 μm for both the heated and unheated cells. The autoradiographic analysis of the matrix structures prepared from cells labelled for 2 hrs, showed that the distribution of grains in heated and unheated matrices was very similar: on average 19.6% and 20.2% of the total grains within a structure, were overlaying the matrix region in unheated and heated cells, respectively. So, in spite of the fact that DNA nuclear matrix complexes, isolated from cells heated 30 minutes at 45^{o}C have protein to DNA ratios about 1.8 times higher than control cells (figure 1) and although hyperthermia interferes with nuclear processes (Dewey et al. 1980; Dewey 1984; Jonsson et al. 1984), gross alterations in DNA loop organization as examined by the current techniques are not found. Hyperthermia has been shown to cause structural alterations in nuclei, nuclear matrices and chromatin, which especially are reflected in an increased protein mass (Roti Roti and Winward 1978; Tomasovic et al. 1978; Kampinga et al. 1985; Warters et al. 1986) and in a decrease in the sedimentation velocity of isolated nuclei (Kampinga et al. 1985). Although heat did not alter the basic nucleosomal structure (Warters and Roti Roti 1981) nor the linking number of the DNA (Roti Roti and Painter 1982), it did increase the ability of high concentrations ethidium to generate rapidly sedimenting nucleoid DNA (Roti Roti and Painter 1982) which is consistent with the concept of tightening the DNA-protein interactions. More compact nucleoids after heating cells (Roti Roti and Painter 1982) maybe due to additional DNA attachment sites at the nuclear matrix, resulting in reduction of the average loop-size. Such alterations could be responsible for the inhibition of replication and transcription upon heat treatment (Dewey et al. 1980; Dewey 1984; Jonsson et al. 1984). The results of the present investigation show that hyperthermia has no or only a minor effect on the average loop-size. Consistent with these results, Small et al. (1985) reported that the organization of genes (coding for heat shock proteins) at the DNA-nuclear matrix complex was not altered by heat treatment of cells. It has to be borne in mind that under normal conditions less than 1000 bp are involved in DNA-matrix attachment (Mirkovitch et al. 1984; Cockerill and Garrard 1986), while the average loop-size is about 30-50 kb (Mullenders et al. 1983a). Doubling the number of nucleotides involved in matrix attachment (e.g. by the protein accumulation) would alter the average DNA loop-size only 2-3 per cent, which may not be detected in the assays we performed. Nevertheless it is clear that more detailed structural analyses of the organization of genes at the level of the nuclear matrix is necessary to draw further conclusions with respect to heat effects on nuclear DNA organization.

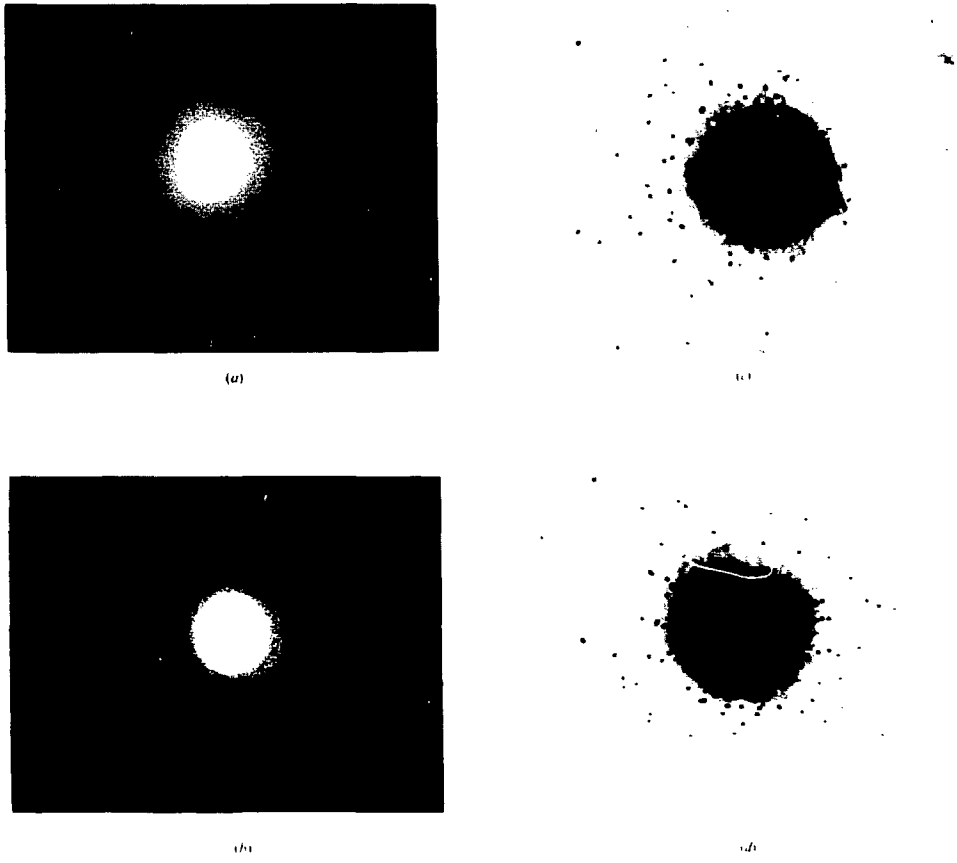

Figure 3: DNA halo preparations from control (panel A,C) and 30 min. at 45°C heated (panel B,D) cells. After staining with EtBr, the halos were analyzed using fluorescent microscopy. Typical halos are shown in panel A,B. Halos from labelled cells were used for autoradiography to visualize S-phase labelled cells after Giemsa staining (panel C,D).

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CHAPTER 5

CHANGES IN THE STRUCTURE OF NUCLEOIDS ISOLATED FROM HEAT-SHOCKED HeLa CELLS

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SUMMARY

Using a technique to detect changes in DNA supercoiling which allows one to visualize both DNA unwinding and rewinding in presence of the intercalating dye, propidium iodide (PI), we show that hyperthermic treatment (30 min at 45°C) of HeLa S_3 cells alters the response to the intercalating dye. Depending on the treatment, conditions we observed a reduction in the maximum size of the DNA loop that can be measured at the relaxation point (PI concentration 5-7.5 μg/ml). Cellular heating also affected all degrees of DNA rewinding (measured as a function of PI concentrations between 10 and 50 μg/ml). By 6 hours after cellular heating these heat effects had disappeared. This time interval correlated with the time necessary for recovery from a heat-induced increase to normal nuclear and nucleoid protein content. Using gel electrophoresis we show that the nucleoids (DNA plus nuclear matrix proteins) after heat exposure are enriched in several polypeptides and that there is a specific increase in HSP 72/73. We hypothesize that the altered response to the intercalating dye after cellular heat-shock is due to an increase in polypeptides associated with the nuclear matrix thereby altering the DNA-nuclear protein matrix anchor points.

5.1 Introduction

Several reports have shown that hyperthermia alters the protein content of chromatin (Roti Roti and Winward, 1978), nuclei (Kampinga et al., 1985, 1987, Roti Roti et al., 1986) and nuclear matrices (Warters et al., 1986, Kampinga et al., 1987a, Wright et al. 1988). Correlations between the extent and/or removal of these excess nuclear proteins with the extent of heat-induced cell killing were reported (Roti Roti & Wilson, 1984, Kampinga et al., 1987). In addition, it was shown that the increase in nuclear protein might be related to the heat induced inhibition of repair of radiation-induced DNA damage (Warters and Roti Roti, 1978). The biochemical basis for these correlations however, remains unelucidated. Heat exposure did not alter the nucleosome structure (Warters et al., 1980) nor did it cause gross changes in maximal DNA loop size (Kampinga et al., 1988a). However, the major part of the heat-induced excess nuclear proteins seems to be associated with the nuclear matrix obtained after 2 or 3 M NaCl treatment of nuclei (Warters et al., 1986, Wright et al. 1988). The attachment sites of DNA at the nuclear matrix define domains for supercoiling (Nelkin et al., 1982) and changes in supercoiling are believed to be important in replication (Mattern and Painter 1979a, b) and transcription (Axel et al., 1973). It has been reported, using conventional nucleoid sedimentation techniques, that heat might alter DNA supercoiling ability (Roti Roti and Painter, 1982). However, this conclusion could not be made firmly because of the fact that heat increased the mass of the nucleoid due to the heat-induced increase in protein content. Since these proteins are associated with the nuclear matrix (Warters et al., 1986) the change in mass of the nucleoid as well as DNA supercoiling changes could account for the observed sedimentation changes (Roti Roti and Painter, 1982). Therefore, we have studied the effects of heat on DNA supercoiling using an image analysis based technique (Roti Roti and Wright, 1987) to directly measure DNA supercoiling changes independent of sedimentation, by titration with the intercalating, fluorescent dye propidium iodide (PI).

5.2 Materials and methods

5.2.1 Cell Culturing

HeLa S_3 cells were maintained in exponential growth by daily subculturing in Joklik MEM (Gibco, Grand Island, NY) supplemented with 3.5% each of calf and fetal bovine serum (GIBCO). The cells were grown in suspension in spinner flasks (Bellco) and doubling time was between 17 and 22 hours.

5.2.2 Heating Conditions

Exponentially growing cells were harvested by sedimentation for 5 min at 150 x g and resuspended in complete medium, prewarmed at the desired temperature (50 x 10^5 cells/ml). For heat-recovery studies, heated cells were 5-fold diluted in complete medium at 37 °C and reincubated at 37 °C for the desired period of time.

5.2.3 Fluorescent Halo Assay

The halo assay was performed as described by Roti Roti and Wright (1987). Cells were resuspended to a concentration of 1 x 10^5 cells/ml in Eagle's spinners salt solution. Cells were then lysed for various periods of time at room temperature in the dark on poly-L-lysine-pretreated, 4 well, Lab-Tek slides (Miles Scientific) by dilution into the dye-lysis solution (1:1 in 2M NaCl, 10 mM EDTA, 2 mM Tris pH 8.0, 0.5% Triton X-100 plus 2 times the desired PI concentration). Besides 1.0 M, we also used (the standard) 2.0 M final NaCl concentration for nucleoid isolation. Since our halo data were essentially the same for both salt concentrations and the lysis solution with 3 M NaCl (to obtain 2 M final concentration after 1:2 dilution of the cells) tends to show some precipitation, we used 1.0 M NaCl (final concentration) for all our analysis unless indicated otherwise. After lysis the halos were visualized (610 nm long pass filter) with an inverted fluorescent microscope (using 520-570 nm excitation light).

Automated measurements of halo size and density were performed using a Model 3000 Image Analyzer (Image Techn. Corp., NY). The images were visualized via a SIT TV camera and monitor and analyzed by an IBM PC based image analysis system. For each specific PI concentration exciting light levels were set to obtain a constant background lumination. The same setting was used for each experimental condition. Background light emission was measured and automatically subtracted during measurements. The threshold (grey level) was set either high for overall halo or low for core measurement. Each field measured was selected for uniformity of focus. The system was programmed to select all of the image pixels above the grey level setting and measure the diameters of the ensuing shapes. Size calibration within the system was done using a stage micrometer.

5.2.4 Isolation of Nuclei and Nucleoids

Nuclei were isolated as described before (Roti Roti et al., 1982). Briefly, cells were washed 3 times in Eagle's spinner salt solution and three times in TX-100 solution (1.0% Triton X-100, 0.08 M NaCl, 0.01 EDTA pH 7.2) and the resulting nuclei were washed once with TMNP (10 mM Tris-HCL, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulphonylfluoride (PMSF)). Nuclear protein content was determined flow cytometrically as described before (Blair et al., 1979). Nucleoids were prepared as described above by 1:1 dilution of cells (in Eagle's spinners salt solution) with lysis buffer (2M NaCl, 10 mM EDTA, 2 mM Tris pH 8.0, 0.5% Triton X-100 plus PI). They were spun down (15 min, 15000 x g) and the resulting pellet was resuspended in TNMP and used for gel electrophoresis after DNase I (Worthington Biochemical, checked for electrophoretic purity) digestion (overnight at 4°C). The lysis was performed at room temperature as well as at ice temperatures in the presence of protease inhibitor PMSF (0.1 mM). No differences in the polypeptide patterns were observed using SDS-PAGE (data not shown) when these different methods were used. So, under the conditions of lysis at room temperature no proteolysis of importance seems to occur. The presence of PMSF (0.1 mM) was sufficient to inhibit protease activity both during lysis and DNase I digestion; further addition of 0.5 μg/ml leupeptin and 0.5 μg/ml Aprotinin revealed no differences in the polypeptide patterns.

5.2.5 Protein gel Electrophoresis

Nuclei and/or nucleoids were dissolved in TMNP (10 mM Tris pH 7.4, 5 mM $MgCl_2$, 10 mM NaCl, 0.1 mM PMSF) and DNase I digested (0.25 mg/ml). Thereafter they were mixed with

equal volumes of 2x sample buffer (125 mM Tris, pH 6.8, 2.0 M Glycerol, 100 mM DTT, 7 mM SDS) and boiled for 5 minutes prior to electrophoresis. Bromphenol blue was added and the samples (10-60 μl), with protein from equal numbers of nucleoids were loaded and electrophoresed through a 3.75 percent stacking and into a 12.5 percent polyacrylamide gel. Gels were stained in 0.2% Commassie Brilliant Blue (in 45% methanol, 7% acetic acid) for 2.5 - 3 hours and destained in 5% methanol, 7% acetic acid. Gel scanning was performed using an LKB 2202 ultroscan laser densitometer.

5.3 Results

Figure 1 shows schematically how a PI dependent, DNA unwinding-rewinding curve was obtained.

The computerized system allows one to make more measurements per treatment condition within less time than the conventional method involving photography and hand measurement of the projected image of the nucleoid (Roti Roti & Wright, 1987; Vogelstein et al., 1980). For control cells, the results from the image analysis system were calibrated against the results from the photographic measurements reported previously (Roti Roti and Wright, 1987) which had been internally calibrated against ocular micrometer measurements. One hundred nucleoids were measured and averaged per experimental point. All experiments were repeated 3 or 4 times as indicated in the figure legends. Experimental variation was expressed as the standard error of the mean and is plotted on each figure when one SEM is larger than the symbol. Prior to image analysis each sample was coded and measured without knowledge of the experimental treatment of a given sample.

To determine the effects of heat-shock on the ability of DNA to undergo supercoiling changes HeLa cells were exposed to 45°C for 30 min and then subjected to the fluorescent halo assay. The procedure involves cell lysis at room temperature in 1.0 M NaCl (see Materials and Methods). We, therefore, varied the lysis time prior to assaying for halo diameter to control for the effect of this factor. Following 15 min lysis we observed higher rewinding efficiency as reflected by a more rapid decrease in halo diameter in nucleoids from heated cells as compared to those obtained from control cells (figure 2). DNA rewinding was complete at 20 μg/ml PI in nucleoids from heat-shocked cells while 50 μg/ml was required for nucleoids from control cells. Little effect was observed on DNA unwinding, the PI concentration required for supercoil relaxation, and DNA loop size at the relaxation point. However, with increasing lysis time we observed (figure 3), that at the relaxation point (PI = 7.5 μg/ml), the DNA loops increased in diameter while the core diameter remained constant indicating a time dependent loosening of the DNA anchor points. Loosening was not evident (as seen by the constant halo diameter) in nucleoids form heat-shocked cells until after 45 min of lysis. Beyond 60 minutes of lysis time the nucleoids from control cells began to fall apart (loss of distinct morphological features and discernible 3-

dimensional structure) and no meaningful measurements could be obtained. In contrast, the halos from heated cells, showed a slower increase in DNA loops extended more rapidly from nucleoids from heat shocked cells than those from control. Further, the nucleoids from heated cells appeared to be more stable to longer lysis times with a slight increase in size up to 48.0 ± 1.0 μm at 120 min.

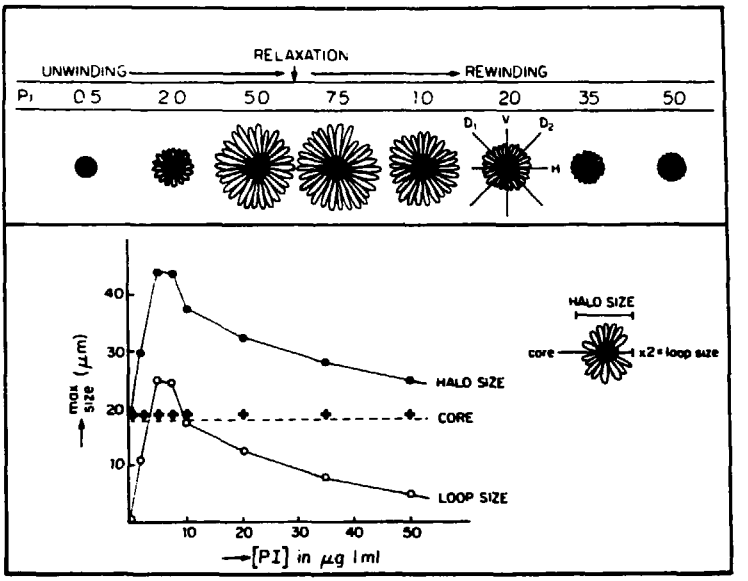

Figure 1: Illustration of the Fluorescent Halo Method for Measuring DNA supercoiling Changes. The upper panel illustrates our interpretation of the fluorescent DNA halo in terms of loops extending and contracting as a function of PI concentration. The left-handed superhelical domains start to unwind at low PI concentrations. With increasing PI concentrations these domains become fully extended at the relaxation point and then rewind into right-handed supercoiled domains. The lower panel illustrates the typical data that results from the technique. Four diameters (2 Diagonal, 1 Vertical, 1 Horizontal, illustrated under 20μg/ml PI, upper panel) were measured and arbitrarily the maximum value per nucleoid was used for relative measures while the average was used for absolute measures. The parameters of halo size, core diameter and loop size, illustrated in the lower panel, were obtained and plotted as a function of PI concentration.

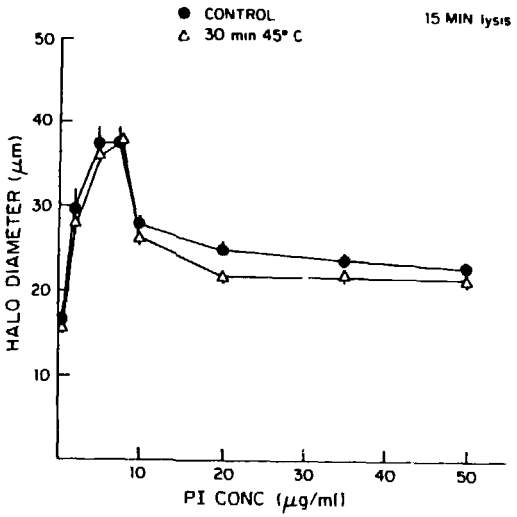

Figure 2: Effects of Hyperthermia and Short Lysis Time Upon DNA Unwinding and Rewinding in the Presence of PI. HeLa cells were exposed to 45°C for 30 min and then analyzed by the fluorescent halo assay as described in the Material and Methods section and illustrated in figure 1. The plotted points represent the mean of 3 repeated experiments and the bars represent one standard error of the mean.

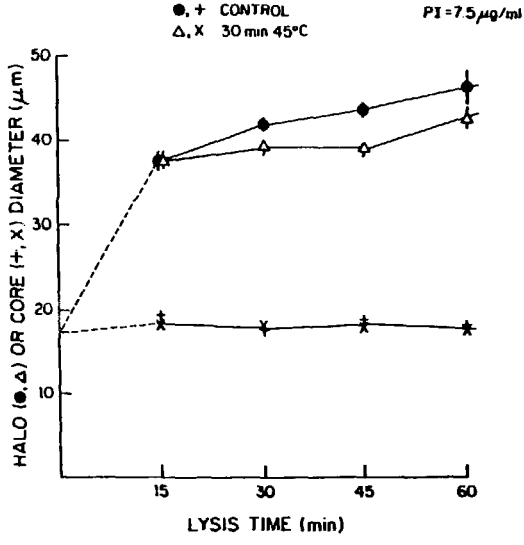

Figure 3: The effect of Lysis Time on Nucleoid Diameter. After exposure to 45°C for 30 min, HeLa cells were subjected to the fluorescent halo assay. In this experiment the PI concentration was held constant at 7.5 μg/ml while the lysis time was varied. Both core and halo diameters (see figure 1) are plotted as a function of lysis time. The plotted points are the mean of 4 experiments and the bars represent one standard error of the mean.

Since the difference between control and heated was maximal, we measured the total PI curve after 45 minutes of lysis (figure 4). Both unwinding, DNA loop size at the relaxation point and rewinding were altered by heat. Except at 0.5 μg/ml PI the difference between two curves at all PI concentrations was statistically significant to the 95% confidence level for each pair of points. The PI concentration needed to get full relaxation was not changed by heat, neither was the core size.

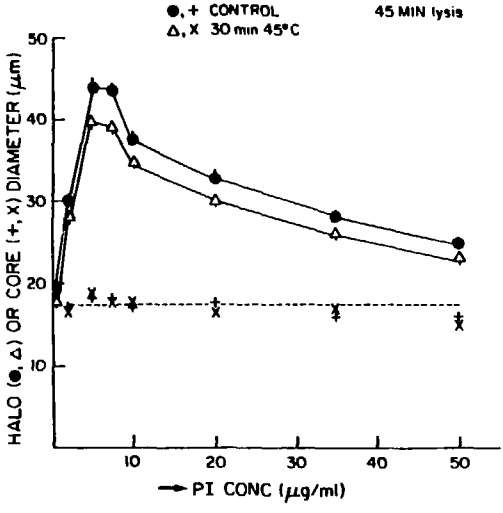

Figure 4: The effects of Hyperthermia and Long Lysis Time on DNA Unwinding and Rewinding in the Presence of Varying PI Concentration. The experiment is the same as illustrated in figure 2 except that the lysis time was 45 min and both the halo and core diameters are plotted as functions of PI concentration. The plotted points represent the mean of 3 experiments and the bars represent one standard error of the mean.

As we know from earlier observations (Roti Roti and Winward, 1978; Roti Roti et al., 1986; Warters et al., 1986, Kampinga et al 1987,1988a), heat-shock causes an increased protein content of chromatin, nuclei and nuclear matrices. As shown in figure 5 (for nuclei) this increased protein content decreases within 6 hours incubation at 37°C, after a 30 minute 45°C heat treatment of cells. Our hypothesis was that the additional protein mass tightens the DNA interaction points at the nuclear matrix thereby causing the heat-induced changes observed using the halo assay (figures 1-3). If this notion were correct, then one would expect that these effects would be gone when the protein was removed. Therefore, we measured halos immediately after the 30 minute 45°C heat treatment and after a post heat incubation of 6 hrs at 37°C. In figure 6, it can be seen that the PI curve obtained 6 hours after the heat shock closely resembles that of unheated controls. This result is consistent with the hypothesis that the heat-induced excess nuclear proteins remain with the nucleoid under the isolation conditions and suggests that this increase might be related to the heat-induced changes observed in the halo assay.

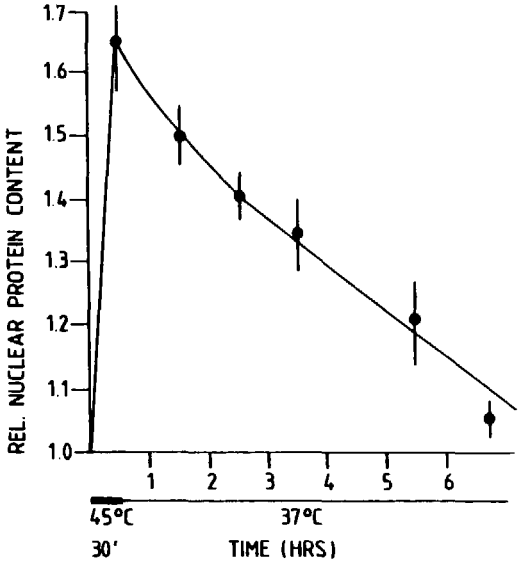

Figure 5: Relative Changes in Nuclear Protein Content Following Hyperthermia. At various time intervals after exposing HeLa cells to hyperthermia the relative nuclear protein content was measured using the flow cytometric assay of Blair et al. (1979). The protein content of the nuclei is plotted relative to that for nuclei from control cells. The points represent the means of 10 experiments and the bars represent one standard error the mean.

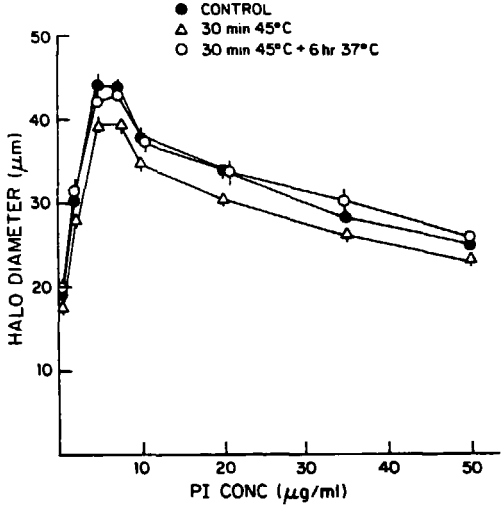

Figure 6: Effects of Hyperthermia and Recovery Time on DNA Unwinding and Rewinding in the Presence of Varying Concentrations of PI. After exposure to 45°C for 30 min HeLa cells were either allowed to recover at 37°C for 6 hours or immediately subjected to the fluorescent halo assay as described previously. The plotted points represent the mean of 3 experiments and the bars represent one standard error of the mean.

The polypeptides associated with the nucleoids were characterized by one dimensional SDS-polyacrylamide gel electrophoresis. The gel in figure 7A shows the polypeptides associated with nucleoids from control and heat-shocked cells at various PI concentrations. Five major bands, (see Table 1) were found associated with nucleoids from control cells at PI concentrations 7.5 μg/ml and above. Some residual histones were found at PI concentrations of 0.5-5 μg/ml. The presence of histones was dependent upon lysis time (figure 7 B) and independent of whether or not the cells had been heated (figure 7 A and B). The nucleoids for heat-shocked cells consistently contained additional polypeptides (see Table I) showing that the heat-induced excess proteins were present in the nucleoids under the experimental conditions used to study DNA supercoiling changes. As can be seen in figure 8, the protein mass of nucleoids was reduced to near control amounts with time after heat treatment of the cells. Although still somewhat elevated, most of the non-HSP 72/73 excess nuclear proteins appeared to be removed 6 hours after hyperthermia. The increase in nucleoid protein mass seems not to be due to a collapse of the cytoskeleton on the nucleoids after hyperthermia, since no heat-induced differences were found in the amount of the cytoskeleton proteins actin (MW = 42.5 kD) or vimentine (MW = 53 kD). In addition, Laszlo et al. (1987) showed that heat-induced excess nuclear proteins did not include increase in cytoskeletal proteins.

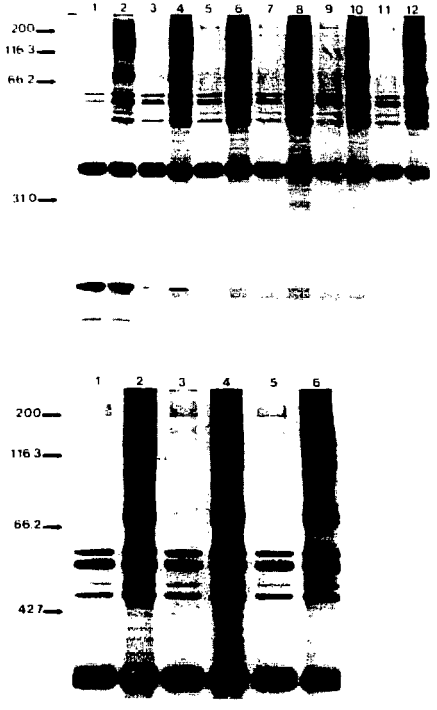

Figure 7: One dimensional Polyacrylamide Gel Electrophoretograms of the Polypeptides Associated With Nucleoids Under Various Conditions. The upper panel shows the polypeptides associated with nucleoids from control and hyperthermia treated cells as a function of PI concentration. Even number lanes are from heated cells while odd number lanes are from control. From left to right the pairs of lanes are from nucleoids in 0, 0.5, 2, 5, 7.5 and 50 μg PI, respectively after 45 min of lysis. The lower panel shows the effect of lysis time on the polypeptides associated with nucleoids at 7.5 μg/ml PI. The lanes are paired according to control and heated with increasing lysis time (15, 30 and 45 min) from left to right.

Major bands[1](MW in kD)			Minor bands[1](MW in kD)		
control	45°C 30 min.	relative change[2]	control	45°C 30 min.	relative change[2]
56	56	+/-	200	200	+/-
52	52	+/-	160	160	+
47	47	+/-	140	140	+
44	44	+/-		125	++
42.5	42.5	+/-	95	95	+
			87	87	+
				78	++
				72	+++
			71	71	+
			69	69	+
			64	64	+

TABLE 1 Polypeptides Coisolating with Nucleoids. Histones and DNase I are excluded from the analysis 1. Distinction between major and minor bands is specified for control nucleoids 2. Relative change due to heat shock is indicated +/- = no or slight change; + = increased significantly; ++ = new polypeptide in nucleoids from heated cells, that is absent in nucleoids from unheated cells; +++ = new major band in nucleoids from heated cells, that is absent in nucleoids from unheated cells

Figure 8: One Dimensional Polyacrylamide Gel Electrophoretograms of the Polypeptides Associated with Nucleoids After Hyperthermia. After incubation for 0, 3, 6 or 9 hours post-heating at 45°C for 30 min nucleoids were prepared from the HeLa cells and polypeptides recovered for electrophoretric analysis as described (see Materials and Methods). The lanes show the polypeptides associated with nucleoids from control cells : lane 1; cells immediately after hyperthermia: lane 2; and cells at 3, 6 and 9 hours after hyperthermia (lanes 3-5 respectively). Lanes are loaded with protein from equal numbers of nucleoids (isolated from 5 x 10^5 cells). Migration positions of molecular weight standards are shown in the left most lane.

5.4 Discussion

The results obtained in this study are consistent with and expand upon the observation that heat shock (45°C 30 min) alters nucleoid sedimentation (Roti Roti and Painter, 1981). Both techniques demonstrated that the ability of a DNA intercalating dye to cause DNA loop rewinding appeared to be more efficient following heat shock. However, by using the "fluorescent halo" technique it was possible to demonstrate that when lysis time was between 30 and 45 minutes the halo size at maximum unwinding was reduced in the nucleoids from heat shocked cells relative to those from control. Since halo diameter depends directly upon DNA loop extension as opposed to sedimentation which depends upon extension and the mass of the particle, this result is consistent with the previous assertion (Roti Roti and Painter, 1981) that heat shock alters the ability of DNA to undergo supercoiling changes. Also, it was shown that heat increases nucleoid stability (in lysis buffer)

The reduced rate of DNA extension with lysis time as observed after cellular heating (figure 3) might be explained by differential release of histones from the DNA. However, halo size continues to increase and the differential between control and heated is maximized after the removal of all histones (at 45 min of lysis time, figure 7A). In addition, limited digestion of chromatin with micrococcal nuclease was the same for chromatin from heated and unheated cells revealing no heat-induced differences in the basic nucleosome structure (Wheeler and Warters, 1982). Furthermore, we found similar lysis time effects when we repeated these experiments using 2 M NaCl which released all histones immediately (data not shown). Our results seem to be inconsistent with the observation that heat shock (45°C 30 min) did not alter DNA loop size (Kampinga et al., 1988a). However the latter method involved UV nicking of the DNA followed by staining in 100 μg/ml Ethidium Bromide, which should maximally extend the DNA loops but also could remove subtle changes e.g. at the DNA-nuclear matrix anchor points. The data in figure 3 indicate that heat-shock does indeed induce subtle changes which can be detected as a reduction in the rate of DNA extension (i.e. increasing halo diameter with lysis time) in our relatively gentle method of nucleoid preparation. Also, these changes are reflected in figures 4 and 6 where nucleoids from heat-shocked cells had fluorescent DNA halos consistently smaller than those from control for all PI concentrations between 2 μg/ml and 50 μg/ml. The DNA loops ultimately reached the same length in the nucleoids from heated as that for control nucleoids. At 120 min of lysis time, the halo diameter for nucleoids from heat-shocked cells was 48.0 ± 1.0, approximately equal to diameter of halos from control nucleoids after 60 min of lysis time. This result is consistent with that of Kampinga et al. (1988a) suggesting that heat shock does not alter the number of DNA nuclear matrix anchor points, but probably alters the nature of the anchor point (see below).

Heat-induced excess nuclear proteins coisolate with the nucleoid under all lysis conditions studied. Therefore, these polypeptides could play a role in

the hyperthermia induced changes as detected with the halo assay described here. Indirect evidence that the heat-induced increase in protein content of nuclear structures is responsible for the observed effect, detected using the halo assay, comes from the recovery data (figures 5, 6 and 8). After recovery of nuclear/nucleoid protein content to near control levels (approximately 6 hours) we observe that the heat effects on DNA halo assay have disappeared. Electrophoretic analysis of these polypeptides reveals that several are enhanced in abundance while others are newly associated with the nucleoids from heat-shocked cells (Table I). Considering the known translocation of HSP 70-73 from the cytoplasm into the nucleus (Welch and Feramisco, 1984; Ohtsuka et al., 1986b), it is not surprising that HSP 72/73 becomes a major component of the nucleoids from heated cells. This protein appears to be the heat-inducible form of the HSP 70-73 polypeptides as revealed by gel blotting (Kampinga et al., 1988b). However, it is surprising that a large number of polypeptides are present in increased amounts or appear de novo in the nucleoids from heat-shocked cells. A reasonable assertion would be that one or more of these polypeptides plays a role in the heat-induced increase in nuclear matrix stability and the restriction of DNA supercoiling changes. Since HSP 72/73 remains elevated up to 6-9 hours post hyperthermia, it is not very likely that this protein is responsible for the supercoiling changes induced by heat.

Since it was shown by Wheeler and Warters (1982) that most (if not all) heat-induced excess nuclear (matrix) protein mass is bound to the nuclear skeleton and not to its associated loop DNA, it is suggested from our data that excess nuclear proteins causes stabilization of the DNA-matrix anchor-points, thereby affecting the reactions to the intercalating dye. Besides acting at the DNA-nuclear matrix attachment points delaying DNA unwinding, the increase in nucleoid protein mass might also act at the nuclear lamina restricting the DNA from being extruded out of the nuclear core during lysis. The latter might have been caused by the intermediate filament collapsing onto the nuclear periphery (Welch and Suzan 1985), that also could have caused the observed increase in nucleoid stability. However, cytoskeletal proteins do not seem to be included in the heat-induced increase in nuclear (Laszlo et al. 1988) or nucleoid (no increase in actin, MW 42.5 kD or vimentin MW 52 kD: figure 7,8) protein mass. Thus, it seems unlikely that cytoskeletal collapse could have caused the observed effects on DNA supercoiling and nucleoid stability as shown in this report. Recent data by McConnell et al. (1987) and ourselves (Kampinga et al 1989) suggest that cellular heating causes accessibility changes at the level of topoisomerase II. No heat-induced changes for nuclear pore or lamina antibody staining were found (McConnell et al, 1987). We, therefore, conclude that the delayed release of DNA loops (figure 3) and the altered un- and rewinding patterns (figures 2,4,6) are most likely caused by the heat-induced excess proteins by stabilization of the DNA nuclear matrix anchorpoints which could affect DNA supercoiling and/or matrix stability.

Because DNA supercoiling is important in replication, transcription and DNA repair (Mattern and Painter, 1979a, b; Axel et al, 1973; Cook et al, 1978) the restriction of DNA supercoiling might be part of the mechanism by which heat inhibits these processes and thereby leads to cell killing. It must be noted however that all the measurements are done with histone-less DNA and the relevance of our findings for the biological activity at the level of the DNA remains to be elucidated. Complementary to restrictions in DNA supercoiling ability, it may be that increased stability of the nuclear matrix is also part of the mechanism by which heat shock alters cellular function. Assuming that the nuclear matrix must have certain dynamic characteristics in order to function, it is not difficult to imagine that increased stability would accompany loss of dynamic properties thereby restricting nuclear matrix function. Recent reports (Evans and Hancock, 1985 and McConnell et al, 1987) show that a heat-shock will cause a consistent set of changes resulting in nuclear matrix stabilization. These investigators also show that in vitro thermal stressing of nuclei can cause a stabilization of the nuclear matrix, albeit to some extent distinct from the stabilization induced by cellular heating. This indicates a rigidification of previously soluble nuclear components (rather than a protein migration into the nucleus) as a biochemical response to thermal stress. The finding of McConnell et al. (1987) that matrix stabilization is also dependent on the duration of the isolation procedure might be related to the observed differences with lysis time (figure 2,3,4). Whether their data can be directly related to our findings is unclear at the moment. Although the existence of the nuclear matrix in situ may remain controversial, our results (the present study and Wright et al., 1988) show that subnuclear structures can be recovered from non heat-shocked cells and that such structures are altered and more stable from cells which have been heat-shocked.

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CHAPTER 6

REDUCED DNA BREAK FORMATION AND CYTOTOXICITY OF THE TOPOISOMERASE II DRUG 4'-(9'-ACRIDINYLAMINO)METHANE SULFON-m-ANISIDIDE WHEN COMBINED WITH HYPERTHERMIA IN HUMAN AND RODENT CELL LINES

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SUMMARY

*The interaction between hyperthermia and the anti-cancer drug 4'-(9'acridinylamino)methanesulfon-m-aniside (mAMSA) was studied in both the human HeLa S3 and the rodent Ehrlich Ascites Tumor cell line. For both cell lines it was found that hyperthermia preceding the drug treatment reduced the extent of mAMSA induced DNA breakage as well as mAMSA cytotoxicity. Formation and resealing of mAMSA induced DNA break formation was found to be related to cytotoxicity. Hyperthermic protection for the action of mAMSA was found not to be a result of changed permeability for the drug. The data also do not support the possibility that heat has caused inactivation of the putative target enzyme of mAMSA, topoisomerase II. It is suggested that the hyperthermic protection for the mAMSA drug action is due to a hyperthermic alteration of the chromatin organization, especially at topoisomerase II target sequences that are found to be enriched in the nuclear matrix (Cockerill and Garrard, Cell **44**, (1986, 273). We show here that heat has caused an alteration of protein binding to the nucleus that seems related to the hyperthermic inhibition of mAMSA induced DNA break induction. It is concluded that preheating cells before treatment with mAMSA should not be used, at least not in this sequence, in cancer therapy.*

6.1 Introduction

Hyperthermia (1) and anti-neoplastic agents such as 4'-(9'-acridinylamino)-methanesulfon-m-aniside (mAMSA: (2)) are potential modalities that can be used in the treatment of cancer. Besides the direct action of hyperthermia on the proliferative capacity of cells, hyperthermia also potentiates radiation induced killing (1) and the action of several drugs (3). Evidence indicates that the synergistic action between heat and radiation is due to hyperthermic inhibition of radiation induced DNA damage. Hyperthermia inhibits the repair of radiation induced DNA strand breaks (4-6) and base damage (7,8). As a cause for this inhibition of DNA repair, it was suggested that heat causes alteration in the structural organization of eukaryotic DNA, rendering the damaged DNA less accessible for the repair process (7, 9-11). Changes in chromatin organization comprise an increase in protein content of nuclear (sub)structures especially the nuclear protein skeleton, often called nuclear matrix (9-16). Concomitantly with these changes, the supercoiling properties of the DNA, that is bound to this nuclear matrix structure in superhelical loops (17,18) was found to be altered after cellular heating (13,16)[2]. These supercoiling changes were consistent with the idea of enhanced constraint of the DNA loops at the anchorpoints of the DNA at the nuclear matrix. The DNA sequences that are anchored at this nuclear matrix, called matrix associated regions (MARs), were found to be enriched (19-23) in consensus sequences (GTnA/TAY ATTnATnnG: 24) for topoisomerase II cleavage. Moreover topoisomerase II was found to be an important constituent of the nuclear matrix (25). The enzyme topoisomerase II is involved in the superhelical control of eucaryotic DNA (26), which seemed affected by hyperthermia as noticed above. mAMSA is an antineoplastic agent that uncouples the breakage rejoining cycle of topoisomerase II, stabilizing an intermediate, often referred to as "cleavable complex" (26-31). Dissociation of this intermediate with SDS, proteinase- K and/or alkali (28) will result in the detection of DNA breaks (one break (topo II site) per 2.10^5 nucleotides (30)). The ratio of DNA-protein crosslinks (DPC)-to-DNA breaks is 1:1 (32). The break formation was often found to be related to the cytotoxicity of the drug (32-35) but not always (36,37).
We have studied the effect of the interaction of heat and mAMSA for two reasons. At first, hyperthermia has shown to influence radiation induced DNA damage and repair, resulting in enhanced radiation toxicity, probably through interference with the structural DNA organization. It is of interest for our knowledge of thermochemotherapy to reveal whether heat could also affect DNA damage induction (and repair) as induced by mAMSA, in relation to cytotoxicity. Heat has been shown to increase the cytotoxicity of several chemotherapeutic agents such as bleomycin, 1,3-bis(2-chloroethyl)-1-nitro-

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2. Kampinga.H.H., Wright.W.D., Konings.A.W.T., and Roti Roti, J.L. Changes in the structure of nucleoids isolated from heat-shocked HeLa cells. 1988, submitted.

sourea, and cisplatin, while it was found to be less effective with other drug such as daunorubicin or 1-β--D-arabinofuranosylcytosine (3,38,39). It is of interest to investigate whether heat can also influence the action of mAMSA. Secondly, the use of mAMSA, since it interacts with topoisomerase II, might give us more detailed information about the suggested action of heat-induced changes in nuclear protein binding on the topoisomerase II sites. The preferential action of topoisomerase II in the nontranscribed spacers in the 87A7 locus of Drosophila (22), that were identical to the MARs (19,20) implies that topoisomerase II inhibitors such as mAMSA will specifically cleave in these MARs (22,40,41). This specific action of mAMSA with the MARs is consistent with the antiproliferative capacity of mAMSA and the role of the nuclear matrix in replication (42-44). If, as suggested, these MARs are indeed affected by hyperthermia, this might be reflected in a different cleavage pattern of mAMSA given after the heat treatment, and then give insight in the possible mechanism by which this heat-induced change in chromatin organization at the nuclear matrix, affects DNA repair.

6.2 Materials and methods

6.2.1 Materials

Two cell lines were used in this study. Ehrlich Ascites Tumor (EAT) cells were grown in suspension culture in RPMI 1640 (Flow, Irvine, Scotland) and 10% foetal bovine serum (Hyclone, Logan, Utah). HeLa S3 cells were also grown in suspension in Joklik-MEM (Flow) with 10% foetal bovine serum (Hyclone). The cultures were in exponential growth (doubling times for EAT about 13 hours and for HeLa S3 about 26 hours) and more than 95% of the cells excluded trypan blue. Uniform DNA labelling was obtained by adding ^{3}H-Thymidine (NEN, Dreicich, West Germany; specific activity 20 Ci/mol) to the medium at a final concentration of 2uM for 36-48 hours. After labelling, the cells were washed with complete medium and chased for at least 1.5 hours in labelfree medium plus 2uM unlabeled thymidine. After the chase, the cells were washed again and resuspended in fresh complete medium at a concentration of 10^{6} cells/ml. mAMSA (4'-(9'-acridinylamino)-methanesulfon-m-aniside; NCI-USA), a generous gift of Dr J.L. Roti Roti (Washington University, St Louis, USA), was dissolved in 100% DMSO at a concentration of 10 mM and frozen at -20^{o}C until use. All other standard laboratory chemicals were purchased from Sigma (St Louis, USA) or Merck (Darmstadt, West Germany).

6.2.2. Condition for hyperthermia and mAMSA treatment

Hyperthermia was performed in precision water baths (+0.05^{o}C) under conditions of gentle agitation. Suspensions of 10 ml cells (1.10^{6}/ml) were heated for various periods of time at the desired temperature. Directly after the heat treatments, 0.54 ml of the cells were added to 0.06 ml mAMSA of the desired concentration (appropriately diluted in complete medium) and treated with the drug at 37^{o}C up to 1 hour. Samples were taken for determination of cell survival or for determination of DNA break formation. Also in analogous experiments 10^{7} cells were taken directly after the cellular heating for nuclear isolation to determine nuclear protein changes. For studying repair of mAMSA-induced DNA damage, cells were washed twice with drug-free, complete medium at 4^{o}C to remove the drug and incubated

at 37°C for various repair times. The solvent concentration (DMSO) at the highest mAMSA concentration used (2 μM) was 0.02%, which had no effect on any of the endpoints measured (data not shown).

6.2.3 Determination of mAMSA induced break induction

DNA break formation by mAMSA was determined using the, slightly modified (5), alkaline unwinding method of Ahnstrom and Edvardsson (45). Triplicate 0.1 ml samples containing 1.10^5 cells were cooled to 4°C. An icecold solution of 0.03 M NaOH, 0.9 M KCl and 0.008 mM Na_2HPO_4 was rapidly added. Alkali unwinding was allowed for 30 minutes at 20°C in a water bath. After rapid neutralization with 3 ml of 0.015 M HCl, 1.8ml SDS (2.5% stock solution) was added and the samples were stored at -20°C. After thawing the samples, they were sonicated with a Branson sonifier (50 watts) for 20 seconds and 2 ml from each tube, raised to a temperature of 60°C, was applied to a hydroxylapatite column (150 mg DNA-grade hydroxylapatite, BDH chemicals) maintained at 60°C in an aluminum block. Single stranded DNA (ssDNA) and double stranded DNA (dsDNA) were separated by elution with 0.15 M Na-phosphate buffer (pH 6.8) and 0.4 M Na-phosphate buffer (pH 6.8) respectively. All buffers were pre-heated at 60°C before applying to the columns. Eluates from the 0.15 M phosphate (4 ml: ssDNA) and from the 0.4 M phosphate (3 ml: dsDNA) were acidified with 1 ml acetic acid (0.5N) and made up to 8 ml with water. To obtain gels 8 ml of hydrocount (Lumac, Landgraaf, The Netherlands) was added. The gels were allowed to cool and counted in a liquid scintillation counter. Corrections for quenching were made by the external standard/channel ratio method. As a calibration curve X-irradiated cells (0-9 Gy of X-irradiation using a Phillips-Muller MG 300 X-ray machine operating at 200 kV and 15 mA at a dose rate of 6 Gy/minute) were used (5). The amount of mAMSA induced damage was expressed in Gy-equivalents producing a similar ratio of ssDNA:dsDNA. The mean ± SE of 3 or more independent experiments are given for all experiments.

6.2.4 Determination of cell survival

Colony forming ability of the both cell lines was tested by applying 0.1 ml of an appropriately diluted sample to 0.5 % soft agar plates as described before (5). The mean ± SE of 4 independent experiments are given.

6.2.5 Nuclear isolation and flow cytometrical analysis of nuclear protein content

Nuclei of both HeLa and EAT cells were isolated using the, slightly modified, method of Blair et al (46). After washing the cells three times in phosphate (50 mM) buffered NaCl (0.25 M), they were resuspended in a TX-100 solution (1% Triton X-100; 0.08 M NaCl; 0.01 M EDTA; pH 7.2). The cells were centrifuged (5 min at 260 g), resuspended in the TX-100 solution and resuspended through a hypodermic needle two times. After pelleting and washing once more in the TX-100 buffer, the nuclei were washed once in TMNP (10 mM Tris-base; 10 mM NaCl; 5 mM $MgCl_2$; 0.1 mM PMSF; pH 7.4). Recovery of nuclei was usually about 80% of the initial numbers of cells used. Isolated nuclei were stained overnight with fluorescein isothiocyanate (FITC: 30 μg/ml) and propidium iodide (PI: 35ug/ml). All procedures were carried out at 4°C. After staining, the nuclei were spun down, resuspended in TNMP plus PI (3. 10^7 nuclei/ml) and analyzed on a FACS flowcy-

meter/sorter[3]. Nuclear protein content was measured by computing the mean of the FITC fluorescent distribution. The mean FITC fluorescence (±SE) of the treated samples was plotted relative to that of nuclei from control cells for 3 independent experiments (46,47).

6.3 Results

With several methods it has been shown possible to detect mAMSA induced DNA strand breaks (28). Here we show that also the alkaline unwinding method, followed by hydroxylapatite chromatography (5,45) is able to detect an mAMSA induced break induction in HeLa S3 (figure 1A) and EAT (figure 1B) cells in a dose dependent manner. Addition of SDS (5%) or proteinase K (0.25 mg/ml, 15 minutes at 37°C) prior to the alkali treatment in the assay, did not result in a further increase in drug-induced DNA strand breaks (data not shown). The induction of mAMSA induced breaks saturates rapidly in time (< 15 minutes) in both cell lines (figure 1). A 30 minute treatment of mAMSA was used in all further experiments.

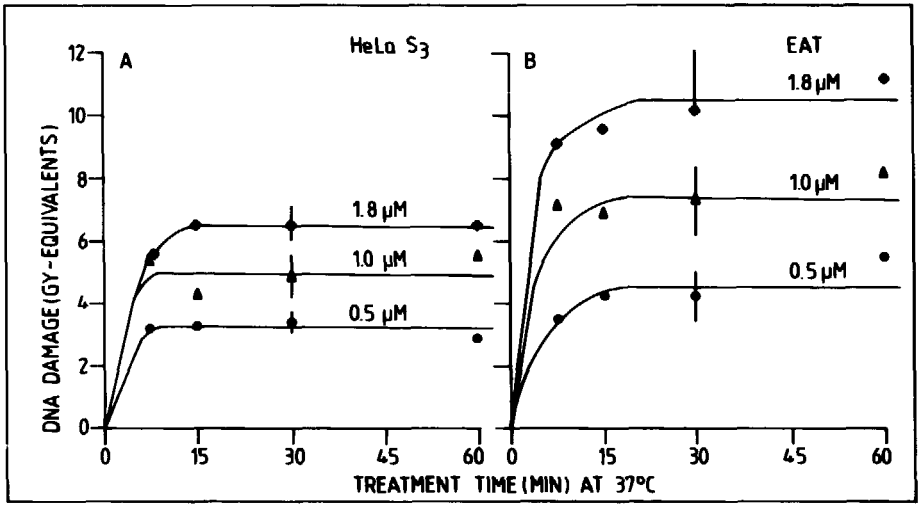

Figure 1: Induction of DNA breaks by various concentrations mAMSA for various periods of incubation time in Hela S3 (A) and EAT (B) cells. Both cell lines were treated with 0.5, 1.0 or 1.8 μM mAMSA for various periods of time at 37°C. DNA break formation was determined as described in materials and methods. DNA breaks are expressed as amount of Gy-equivalent damage.

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[3]. Kampinga,H.H., Kruk van der G., and Konings.A.W.T. Interaction between hyperthermia and radiation. Role of DNA polymerase inactivation. 1988, submitted.

In figure 2, it can be seen that preheating HeLa cells for 30 minutes at 45°C or the more heat sensitive EAT cells for 30 minutes at 44°C, resulted in an inhibition of mAMSA (0.1-2 μM) induced break formation. The rate of break formation by mAMSA was not affected (data not shown). In figure 3, the heat dose dependent inhibition of mAMSA (1.0 μM) induced break formation is given. The amount of Gy-equivalents induced in unheated cells is plotted as 100 percent and the hyperthermic inhibition is given as the fraction of Gy-equivalent damage after preheating in comparison to mAMSA break induction of the unheated samples. Corrections were made for the induction of alkali labile sites by heat alone (up to 2 Gy-equivalents for the highest heat doses given) (48). Higher temperatures and/or longer exposure time to hyperthermia before the mAMSA treatment caused progressive inhibition of break formation by the following mAMSA treatment in both Hela (figure 3A) and EAT (figure 3B) cells.

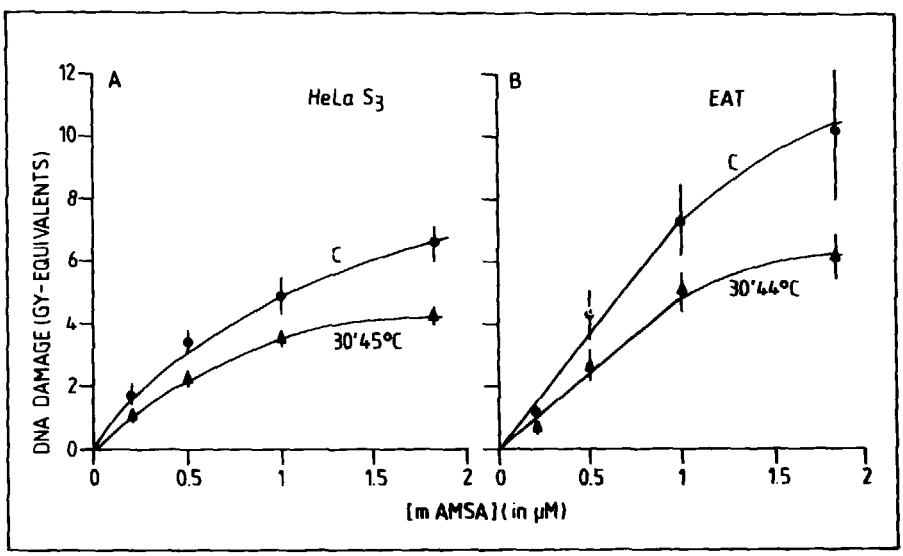

Figure 2: The effect of hyperthermia on mAMSA induced DNA break formation in HeLa S3 (A) and EAT (B) cells. After a hyperthermic treatment of HeLa S3 (30' 45°C) and EAT (30'44°C), heated (triangles) and unheated (circles) cells were exposed to various concentrations of mAMSA (30'37°C). Hereafter DNA break formation (in Gy equivalents) by mAMSA was measured as described in materials and methods. Corrections for hyperthermia induced DNA breakage (48) were made.

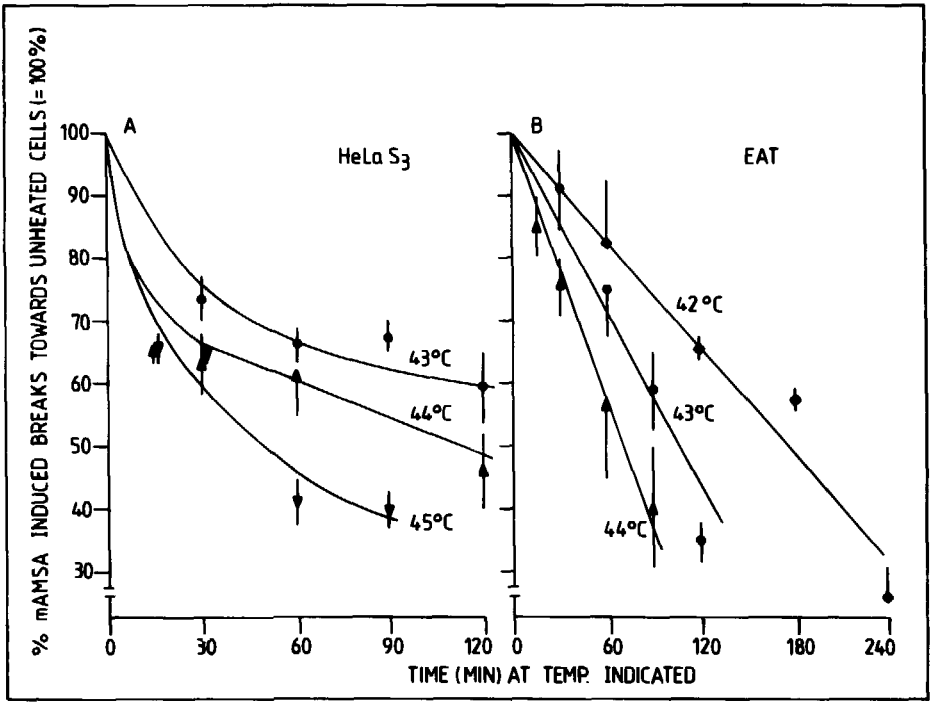

Figure 3: The effect of various "doses" of hyperthermia on mAMSA induced DNA break formation in HeLa S3 (A) and EAT (B) cells. After exposure of HeLa S3 and EAT cells to various doses of hyperthermia (42°C-45°C), heated and unheated cells were exposed to 1.0 μM mAMSA. The inhibition of mAMSA induced breaks by hyperthermia was calculated by dividing the mAMSA induced Gy-equivalents after heating by the Gy equivalents in unheated cells, and multiplying by 100 to convert the fraction to a percentage. The percent values are plotted. Corrections for heat-induced DNA breakage (48) were made.

When analyzing the heat induced changes in nuclear protein content as revealed by FITC fluorescence, we found response curves as depicted in figure 4. As expected (15,46,47), it was found that cellular heating increased the amount of protein (higher FITC signal) in nuclei isolated from Hela (figure 4A) and also EAT (figure 4B) cells in a "heat dose" dependent manner. This might be related to the "heat dose" dependent inhibition of the ability of mAMSA to cause topoisomerase II associated breaks (figure 3). Besides the possibility of heat causing a structural modification of the matrix associated DNA regions (MARs) two alternative explanations can be given for the hyperthermic inhibition of mAMSA induced break formation. The first is that the topoisomerase II enzyme itself is heat sensitive and as a conse-

quence may be inactivated by the cellular heating. Therefore total enzyme activity may be diminished, resulting in producing less breaks. This was tested by looking at the rejoining capacity of the cells after treatment with mAMSA.

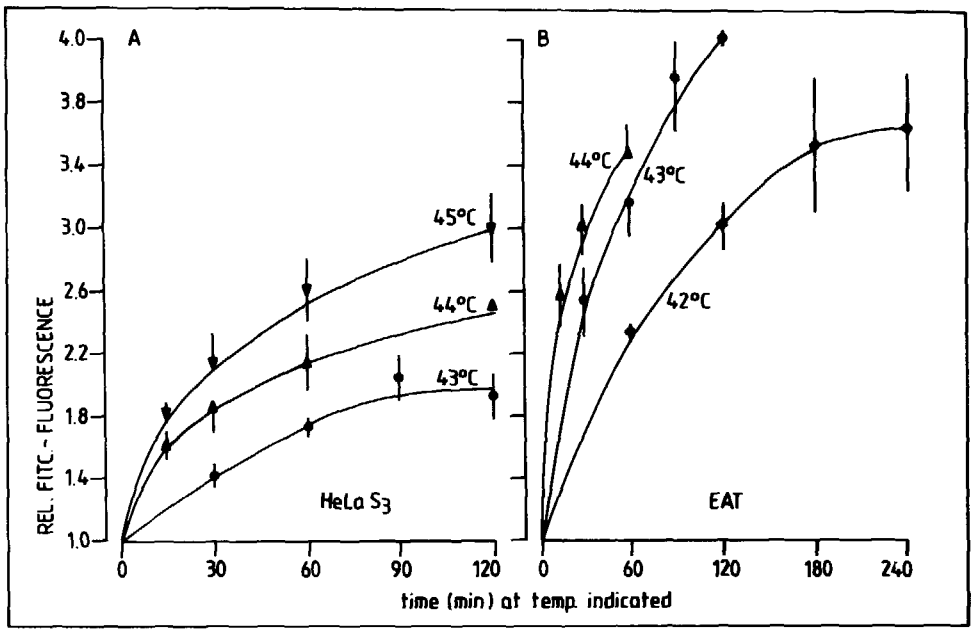

Figure 4: The effect of various "doses" of hyperthermia on the relative FITC fluorescence of nuclei isolated from HeLa S3 (A) and EAT (B) cells. Nuclei from heated and unheated cells (42°C; 43°C; 44°C; 45°C) were isolated directly after the cellular heating according to the method described in materials and methods. After staining the nuclei with PI and FITC overnight, they were analyzed flowcytometrically. The relative increase in FITC-fluorescence (towards nuclei isolated from unheated cells = 1.0) is plotted versus cellular heating.

The resealing of such breaks seems to occur solely by the action of topoisomerase II (49). Measuring resealing rates will thus provide information on the total cellular enzymatic activity of topoisomerase II in the intact cell after or during hyperthermia. Resealing activity was measured after various treatment schedules and at 37°C as well as at 44°C (EAT) or at 45°C (HeLa); residual damage was plotted as a percentage of the initial damage (figure 5). Resealing rates were always slightly enhanced when measured at

elevated temperatures (figure 5: open symbols). Also it can be seen that preheating before mAMSA (figure 5: triangles) or heating during the mAMSA treatment (figure 5: inverted triangles) never retarded the resealing rates at normal nor hyperthermic temperatures. In contrast, often a slight enhancement of repair was observed.

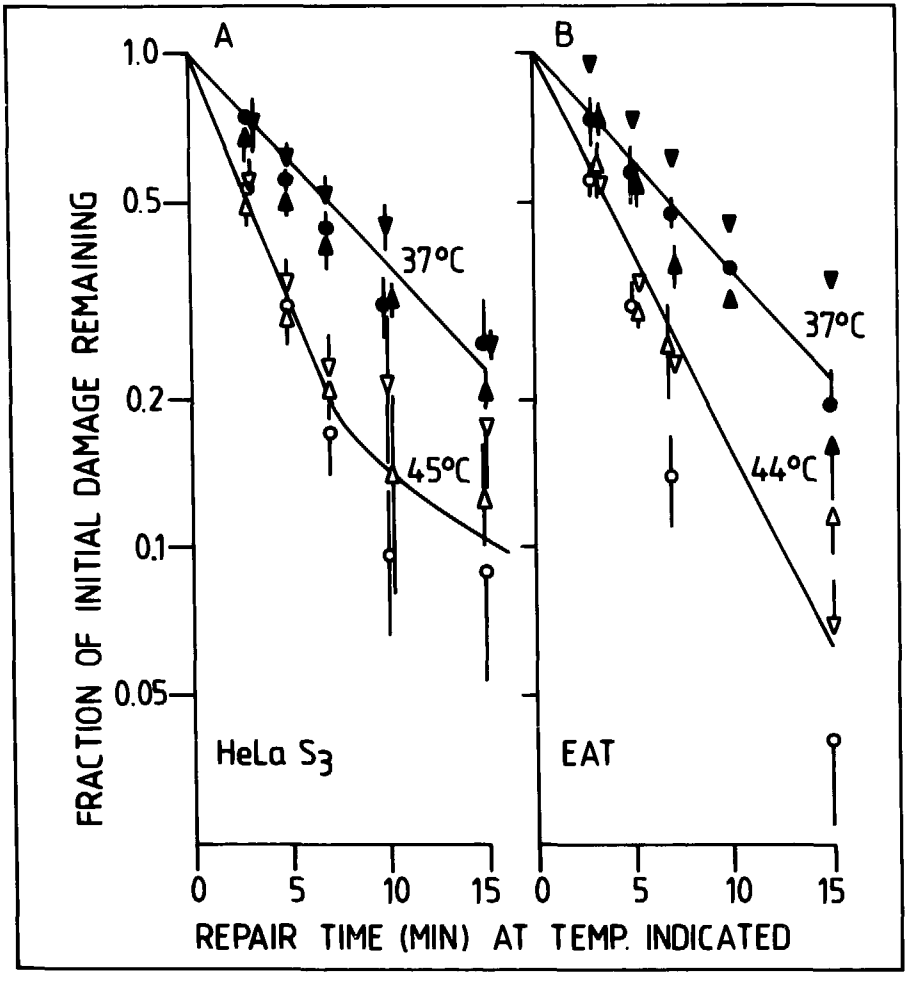

Figure 5: The effect of hyperthermia on the resealing rate of mAMSA induced DNA breaks in HeLa S3 (A) and EAT (B) cells. After treatment with 1.0 μM mAMSA (30 minutes), cells were washed and reincubated in fresh, drug-free, medium for various periods of time at 37°C (closed symbols) or 45°C (HeLa S3; open symbols) or 44°C (EAT; open symbols). The amount of Gy-equivalent DNA damage was determined as described in materials and methods. The amount of residual damage is plotted as a percentage of the initial damage at t = 0 min. repair (=100%). (circles: 37°C - mAMSA at 37°C - repair; triangles: 44/45°C - mAMSA at 37°C - repair; inverted triangles: 37°C - mAMSA at 44/45°C - repair).

Thus the resealing capacity of topoisomerase II seems not heat sensitive in these two cell lines. A second possibility for the hyperthermic protection for mAMSA induced break formation is that the preceding heat dose has decreased the membrane permeability for mAMSA, thus decreasing the intracellular concentration of mAMSA. This possibility was tested by permeabilizing the cells after cellular heating, but before mAMSA treatment, with 5 (EAT) or 10 (HeLa) μM digitonin (15 minutes on ice). Permeabilization was checked by measuring trypan-blue exclusion. The treatment with digitonin made over 75% of all cells (HeLa and EAT, heated and unheated) permeable to this dye. It was found (figure 6) that a preceding heat treatment was still protective for break induction by mAMSA in these permeable cells, the effect even being somewhat more pronounced than for the intact cell. Also it can be seen that the permeabilization itself caused a slight protection for break induction. Thus, the protecting effect of heat for mAMSA induced break induction cannot be attributed to a decreased uptake of mAMSA due to the heat treatment.

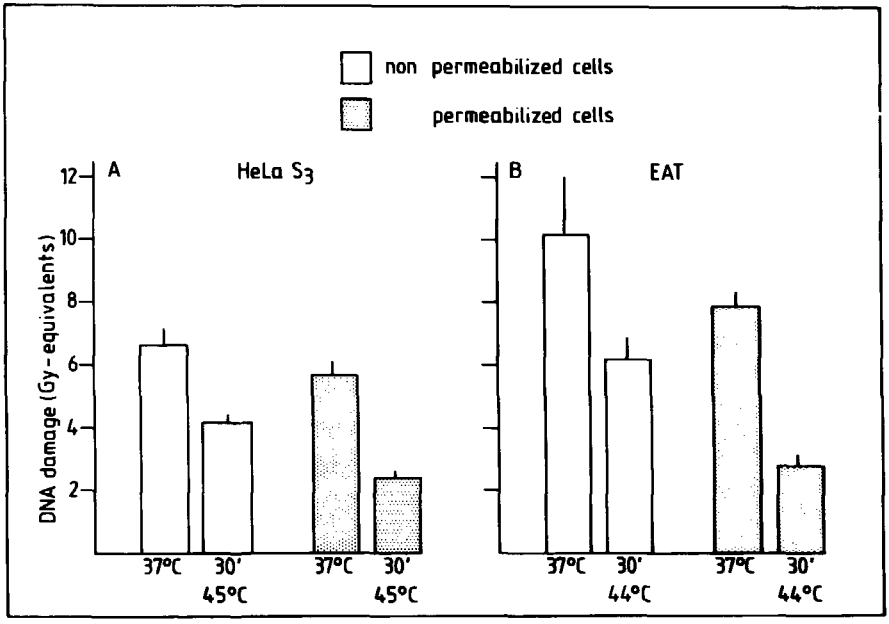

Figure 6: The effect of cell-permeabilization after hyperthermia on mAMSA induced DNA break formation in HeLA S3 (A) and EAT (B) cells. After hyperthermia (HeLa S3: 30'45°C: EAT: 30'44°C), heated and unheated cells were permeabilized with digitonin (see text in results) and both permeabilized and non-permeabilized cells were then exposed to mAMSA (2.0 μM, 30'37°C). Thereafter the amount of Gy-equivalent damage was determined as described in the materials and methods.

Hyperthermia is used to enhance the cytotoxicity of various drugs (3,38,39). From the data in figure 7, it may be concluded that a preceding heat treatment resulted in an protection for mAMSA toxicity in both cell lines. This protection was found for heat treatments that caused no more than 10% killing by itself; so we are not looking at a specific heat and mAMSA resistent subpopulation of cells.

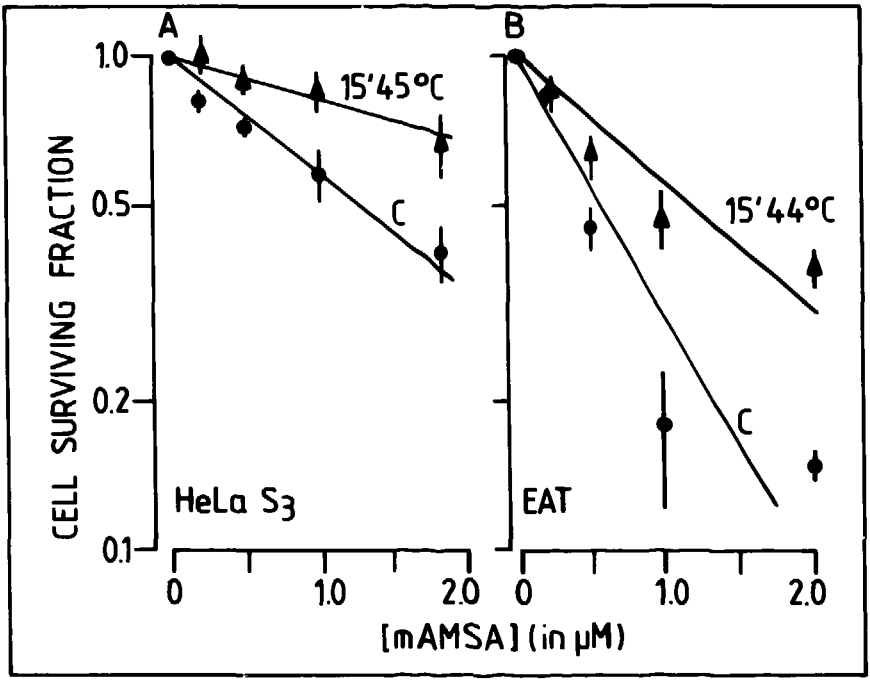

Figure 7: The effect of hyperthermia on the cytotoxicity of mAMSA in HeLa S3 (A) and EAT (B) cells. After a hyperthermic treatment of HeLa S3 (15' 45°C) and EAT (15'44°C), heated and unheated cells were exposed to various concentrations of mAMSA (30'37°C). Hereafter the clonogenic ability of the cells was tested using the soft agar cloning technique (5). Corrections were made for cell killing by heat alone (less than 10% killing for both cell lines).

6.4 Discussion

6.4.1 Heat and drug interaction

The potentiating effect of heat has been observed for many drugs (3,38,39), but was not found for mAMSA, when heat preceded the mAMSA treatment. A decrease of cytotoxicity by the hyperthermic pretreatment was observed. The effect seems not to be caused by inactivation of the total cellular activity of topoisomerase II, the putative target for mAMSA, because the resealing

activity of the cells was found not to be inhibited by the heat treatment (figure 5). This is a rather indirect measurement that gives no direct information on the topoisomerase II incision activity. However, it was shown by Warters et al. (50) that the in vitro ability of cell homogenates from heated cells to perform plasmid decatenation (a topoisomerase II incision dependent process) also was not heat sensitive. Thus although it cannot be fully excluded, it seems not very likely that our results can be explained in terms loss of cellular topoisomerase II activity due to hyperthermia.
The inhibiting effect could also not be attributed to a hyperthermic decrease in drug uptake, since the same protective effect of heat was found after cell permeabilization. Cell killing of mAMSA when given simultaneously with elevated temperatures was also inhibited, as shown in a study of Herman (51). It is interesting to notice that preheating cells can potentiate the toxicity of several, also DNA intercalating (e.g. cis-DDP), drugs(39), but often not of drugs that act on topoisomerase II (27) such as daunorubicin (39,52), adriamycin (53-55) and VP-16 (56). Although for adriamycin, this was suggested to be an effect of decreased uptake (54,55), Rice and Hahn (53) already showed that altered uptake alone could not explain the hyperthermic protection for adriamycin toxicity and suggested that also structural changes in chromatin structure had to be taken into account to fully explain the heat protection found. We would like to propose that hyperthermic inhibition of the action of topoisomerase II directed drugs, is not related to uptake, but rather might be due to a change in the putative target site for topoisomerase II, being the MARs (19-24). The adriamycin uptake data (54,55) are hampered by the fact that a different drug-topoisomerase-II-DNA-interaction will result in an increased passive loss of adriamycin (after preheating the cells) even when washing at $4^{o}C$ (57), resulting in a lower fluorescent signal, when measuring intracellular adriamycin concentrations flow cytometrically. The action of mAMSA on topoisomerase II is at least not totally dependent of the intercalating character of the drug, but seems to be more specific directly acting on the topoisomerase II enzyme (31). Moreover heat also seems to protect for the action of the non-intercalative drug VP-16 (56). Thus the hyperthermic protection against mAMSA (and other topoisomerase II drugs) toxicity must be mediated via an alteration of the conformation of the topoisomerase II site, either by decreasing the accessibility of the topoisomerase II site for the topoisomerase-II-mAMSA-complex or of the topoisomerase-II-DNA-complex for mAMSA.

6.4.2 mAMSA toxicity; DNA break formation and repair

In this study a relation between mAMSA induced break formation and toxicity was found; hyperthermia decreased the number of breaks induced by mAMSA (figures 2 and 3) as well as mAMSA cytotoxicity (figure 7). Furthermore it can be seen that mAMSA induced more breaks in EAT cells (figure 1B,2B) than in HeLa S3 cells (figure 1A,2A) which is in accordance with the higher toxicity of isodoses of mAMSA in EAT cells as compared to HeLa cells (figure 7A,B). Break formation and cytotoxicity of mAMSA were

often found to be related (32-35). Whether the formation of breaks is sufficient to kill however is doubtful, since initial breaks can already be detected without the occurrence of cell death (36). The capacity to repair these breaks has also to be taken into account. In heat treated cells an enhanced rate of repair was found (figure 5), which also may contribute to the reduced toxicity of mAMSA. Also the fidelity of repair of these breaks may play a role in drug toxicity; cells in S-phase show relatively more kill per break (37,58) and were shown to develop the highest number of sister chromatid exchanges after drug treatment (59).

6.4.3 Heat induced chromatin alterations as revealed by mAMSA; importance for heat killing and radiosensitization

As indicated above, the hyperthermic inhibition of the action of mAMSA might be related to alterations in chromatin structure. Heat somehow has decreased the accessibility of topoisomerase II sites in the DNA for cleavage or of the topoisomerase-II-DNA complex for mAMSA. These interaction sites are known to be located near/at the control regions of active genes (22,60); these sites were found to be identical to the DNA sites (MARs) that are located at the nuclear matrix (20,21,23,41). The MARs are thought to be important for the regulation of several DNA processes (61) including replication, transcription and maybe also repair. All these processes are inhibited by hyperthermia and this as such might be responsible for heat killing and hyperthermic radiosensitization. In this study we have shown data that suggest that these sites are indeed altered, in a way that correlated with the increase in nuclear protein binding (figure 4). The duration of this infliction might then determine the extent of heat killing (62)[4].

For heat radiosensitization the actual status of the DNA at the nuclear matrix at the moment of irradiation was related to repairability of DNA damage and heat radiosensitization (7-9,10,11). Changes of the regulative sites of DNA at the matrix might have affected the preferential repair that seems to occur at the nuclear matrix (63-65) and which might involve topoisomerase II activity (66,67).

In conclusion, it was shown that prehcating cells before mAMSA application (and maybe, in general drugs acting on topoisomerase II) protects against DNA break formation and cytotoxicity and therefore should not be used together (at least not in this sequence) in cancer therapy. Furthermore, the data are supportive for the idea that hyperthermia changes the nuclear organization of DNA by altering the attachment sites of the DNA at the nuclear matrix, probably by changing the nuclear (matrix) protein binding. Consequentially cells might die from this infliction and/or become more sensitivity to X-irradiation.

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CHAPTER 7

HEAT-INDUCED ALTERATIONS IN DNA POLYMERASE ACTIVITY OF HeLa CELLS AND OF ISOLATED NUCLEI. RELATION TO CELL SURVIVAL

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SUMMARY

The activity of DNA polymerase ∝ and β was assayed in heated HeLa S3 cells as well as in nuclei isolated from these cells. The enzyme activity as measured in cells and in nuclei has been compared with the extent of cell survival after the different hyperthermic doses. It was found that although the activity of the cellular DNA polymerases was related to cell survival after single heat doses, no correlation was found when thermotolerant cells were heated. When activity of the DNA polymerases was determined in nuclei of the heated cells, more polymerase activity was found in the nuclei of the heated cells. However, the heat sensitivity of DNA polymerase activity was the same for nuclei isolated from control, pre-heated and thermotolerant cells. Heat protection of polymerase activity by erythritol and sensitization by procaine was found when cells, but not when nuclei, were heated in the presence of these modifiers. It is concluded that (the nuclear bound) DNA polymerases are not to be considered as key enzymes in cellular heat sensitivity of HeLa S3 cells.

7.1 Introduction

The molecular mechanisms leading to cell death after hyperthermic damage are not well understood. Also, the synergistic action of hyperthermia and ionizing radiation has not yet been explained at the molecular level. Inhibition of repair of radiation-induced strand breaks by hyperthermia has repeatedly been shown (Corry et al. 1977, Lett and Clark 1978, Lunec et al. 1981, Bowden and Kasunic 1981, Mills and Meyn 1981, 1983, Dikomey 1982, Jorritsma and Konings 1983, 1984). A good correlation was demonstrated between the inhibition of strand-break repair and the extent of cell death after the hyperthermic treatment alone (Jorritsma and Konings 1983). It was also shown that if cells were made thermotolerant, then the rate of break rejoining was protected by tolerance (Jorritsma and Konings 1983). The group of Dewey et al. reported a correlation of the activity of DNA polymerase β, heat sensitivity and heat radiosensitization (Spiro et al. 1982, Denman et al. 1982, Dewey and Esch 1982). Although a key role for DNA polymerase activity in cellular heat sensitivity and radiosensitization by heat is suggestive, definite proof of the importance of these enzymes in the processes cited has not been given. It is the purpose of this paper to contribute to our knowledge concerning the role of DNA polymerase α and DNA polymerase β in hyperthermic cell killing. Because of the localization of polymerases in the cell (Kornberg 1980, 1982) and the findings by several investigators of an elevated protein to DNA ration chromatin isolated from heated cells (Roti Roti and Winward 1978, Tomasovic et al. 1978, Warters and Roti Roti 1979,1981, Clark et al. 1981), we also studied the effect of hyperthermia on polymerase activity in isolated nuclei. Procaine and erythritol may modify the heat response in mammalian cells at the level of cell survival (Yau 1979, Henle et al. 1983). These agents have been used in this study as tools further investigate possible molecular mechanisms underlying the process of cell killing by hyperthermia.

7.2 Material and methods

7.2.1 Cells and cell culturing

HeLa S3 ATCC no. CCL 2.2, Flow 03-157, Irvine,Scotland) tumour cells were grown in suspension cultures in Jokliks modification of minimal essential medium (Flow 10-323-22, Irvine, Scotland). The cells were cultured in a shaking incubator at 37^{o}C. The doubling time was about 25h. Exponentially growing cells were used in all experiments. Dye (trypan blue) was excluded by at least 95 per cent of the cells.

7.2.2 Preparation of cells and nuclei

About 5 x 10^{8} cells were pelleted by 250 g centrifugation for 5 min, washed twice in 250 ml phosphate-buffered saline (50 mM Na-phosphate buffer, pH=7.5; 0.9 M NaCl) and resuspended in a hypotonic Tris-buffer (TNM = 10 mM Tris HCL, pH = 7.5; 10mM NaCl; 1.5 mM $MgCl_2$) yielding a final concentration of 10^{7} cells/ml. The cells were allowed to swell at 0^{o}C fir 20 min and then homogenized in a 40 ml Dounce homogenizer (6 strokes, wide clearance d=0.16 mm, plus 6 strokes, narrow clearance d=0.05 mm). This homogenate was

used for the assays of polymerase activity. For some experiments, the cells were disrupted by freeze-thawing or sonication, instead of Dounce homogenization, which resulted in identical values (±5 per cent) for the polymerase activity. For the isolation of nuclei the cell homogenate obtained by the Dounce treatment was centrifuged for 10 min at 250 g (4°C). A crude nuclear pellet was obtained. The nuclei were resuspended in 10 ml of the TNM-buffer supplemented with 1.6 M sucrose (about 1 x 10^7 nuclei/ml). This suspension was layered over a discontinuous sucrose gradient of 1.9-2.5 M sucrose in TNM-buffer. The gradient consisted of 3 ml volumes of 1.9, 2.0, 2.1, 2.2, 2.3, 2.4 and 2.5 M sucrose in TNM-buffer. Centrifugation was performed for 1 h at 136 000 g in a Beckman SW28-rotor at 4°C (sucrose densities 1.25, 1.27, 1.28, 1.29, 1.30, 1.31 and 1.32 respectively). The nuclei present as a band in the gradient were gathered, diluted with about 5 volumes of TNM-buffer used for cell homogenization. These nuclei were essentially free of cytoplasmic contamination as judged by light and electron microscopy. For phasecontrast light microscopic observations the nuclei were stained with Trypan Blue dye. For the determination of the protein content of cells and isolated nuclei the method of Lowry et al. (1951) was used.

7.2.3 Assay of DNA polymerase activity

DNA polymerase activity was assayed in a medium containing 11.6 mM Tris-HCL (pH=7.9); 9.1 mM $MgCl_2$; 50mM KCL; 5 mM NaCl; 0.2 each of dATP (Sigma); 0.005 mM [^{3}H]dTTP (NEN, 56.0 Ci/mol); bovine serum albumin (BSA) 0.15 mg/ml (Sigma) and 100 μl/ml salmon sperm DNA (in 13.3 mM Tris-HCl (pH=7.9); 16.6 mM $MgCl_2$) activated as described by Schlabach et al. (1979). This procedure involves enzymatic digestion of the DNA by DNase I (Sigma, chromatographically pure) for 30 min at 37°C, followed by heating at 60°C for 15 min. For the determination of total polymerase activity 50 μl 34 mM dithiothreitol (DTT, Sigma) was added to 500 μl of cell homogenate (1 x 10^7 cells/ml TNM) or 500 μl of isolated nuclei (3 x 10^7 nuclei/ml TNM), followed after 20 min at room temperature by 450 μl of a mixture containing the other ingredients resulting in the medium composition as described above. Final concentration of DTT was 1.7 mM. For the assay of β polymerase activity, 50 μl 20 mM N-ethylmaleimide (NEM) was added instead of DTT, in order to inhibit α-DNA polymerase activity. The final concentration of NEM was 1.0 mM. One millilitre samples were incubated at 37°C up to 15 min, during which the incorporation rate was found to be constant. After different times of incubation 100 μl samples were taken and added to 1 ml 10 per cent cold TCA, 2 per cent $Na_4P_2O_7$. Salmon sperm DNA (0.05 mg, 1 mg/ml; Sigma) and BSA (0.3 mg, 6 mg/ml; Sigma) were added as carriers. To be able to correct for co-precipitation of non-incorporated label (blank values), samples were taken immediately after addition of the labelled solution to the samples. After overnight precipitation at 4°C the samples were centrifuged at 2000 g for 10 min. The precipitates were resuspended in 2 ml cold 5 per cent TCA, 2 per cent $Na_4P_2O_7$ and centrifuged again. The pellets obtained were washed twice with 2 ml cold 5 per cent TCA, 2 per cent $Na_4P_2O_7$ and once with 100 per cent ethanol. The pellets were dried and dissolved in 0.25 ml Soluene-350 (Packard). Hydroluma (10 ml: Lumac BV, Schaesberg, The Netherlands) was added and the samples were counted in a liquid scintillation counter. Corrections for quenching were made by the external standard channel ratio method. Polymerase α activity was calculated by subtracting the β-activity from the total activity. The nuclei isolated from HeLa S_3 cells usually contained about 5 per cent of the α- and 7.5 per cent of the β-polymerase activity present in the total cellular homogenate. These values are in ac-

cordance with data normally found after aqueous isolation of nuclei from HeLa S_3 cells (Weissbach et al. 1971).

7.2.4 Heating conditions

The cells were harvested by centrifugation for 10 min at 250g (20°C) and resuspended in fresh Jokliks medium plus 10 per cent foetal calf serum (FCS) at a concentration of 5 x 10^6 cells/ml. The suspension was placed in a shaking 37°C waterbath for 15 min before further treatment. Acute thermotolerance was induced by heating the cells (5 x 10^6 ml) for 5h. Chronic thermotolerance was induced after a 5-fold dilution of the suspension to a concentration of 1 x 10^6 cells/ml with fresh medium of 42°C. The cells were incubated at 42°C for 5h. Control cells were likewise diluted with 37°C medium and held at this temperature. Before heating at 45°C, control or thermotolerant cells were centrifuged (10 min, 250g, 20°C and resuspended in fresh medium at a concentration of 5 x 10^6 cells/ml. After 15 min at 37°C suspensions of 2-5 ml were heated in plastic tubes in precision waterbaths as previously described (Jorritsma and Konings 1983). Aliquots were taken and diluted immediately after the heat treatment for determination of cell survival. The remainder of the suspension was centrifuged (10 min, 250 g, 20°C) and the pellet was resuspended in TNM-buffer at a concentration of 1 x 10^7 cells/ml, homogenized by sonication and DNA polymerase activity was determined. When nuclei were to be isolated, a slightly different 45°C heating schedule for the cells was followed. Control or thermotolerant cells were spun down (10 min, 250 g, 20°C) and resuspended in fresh medium at a concentration of 5 x 10^7 cells/ml. After 15 min at 37°C the suspension (about 10 ml) was diluted 10-fold with fresh medium of the required temperature (37°C for control, 45°C for hyperthermic treatment) and maintained at that temperature for the time described in § 7.2.2. During all treatments the pH of the medium was 7.3-7.6 as judged by the colour of the phenol red indicator. Nuclei were heated in plastic tubes a suspensions of 1-2 ml at a concentration of 3 x 10^7 nuclei/ml of TNM-buffer. Before heating the nuclei were kept on ice. When procaine-HCL (10mM) was present during the heat treatment, samples were preincubated for 15 min (cells at 37°C, nuclei at 0°C). For erythritol (0.5 M) a 45 min preincubation was used.

7.2.5 Determination of cell survival

Colony forming ability of the suspension cells was assayed by applying 0.1 ml of an appropriate diluted sample to 0.5 per cent horse serum and streptomycin/penicillin. The plates were incubated in a humified CO_2-incubator at 37°C. Colonies (containing more than 50 cells) were counted after about 14 days.

7.3 Results

When HeLa S_3 cells are heated for 15 and 30 min at 45°C both cell survival and DNA polymerase activity decline. DNA polymerase β is somewhat more heat sensitive than polymerase α. This is shown in Table 1. These data suggest a possible key function for DNA polymerase in the molecular mechanism for heat sensitivity as suggested before by other workers (e.g. Spiro et al. 1982). In order to further test this hypothesis, the heat sensitivity of the DNA polymerases was compared in normal and thermotolerant cells. The cells were made thermotolerant either by an acute

method (15 min, 44°C; 5h, 37°C) or by a method involving chronic heat treatment (5h, 42°C). For the chronic heat treatment at 42°C, a plateau for cell survival as well as for the activity of the polymerases was found indicating thermotolerance on the level of the enzyme activity (figure 1). When DNA polymerase ∝ and β were assayed in normal and thermotolerant cells (chronic and acute) after a second treatment, little or no resistance to the second heat treatment was observed, although thermotolerance at the level of cell survival was expressed as expected. This illustrated in figure 2.

Heat dose	Survival (percent)	Activity of DNA polymerases ∝	β
None(100)(100)(100)			
15min, 45°C	40±4	67±11	42±4
30min, 45°C	5±2	49±2	34±4

Table 1: The effect of hyperthermia on cell survival and on the activity of DNA polymerase ∝ and β. Data are expressed as percentage of the control value. The specific activity of ∝- and β-polymerase in the untreated (control) cells was about 100 and about 11 pmol dTTP/mg Protein/min, respectively. Values are the mean ± s.e.m. of 3-9 experiments.

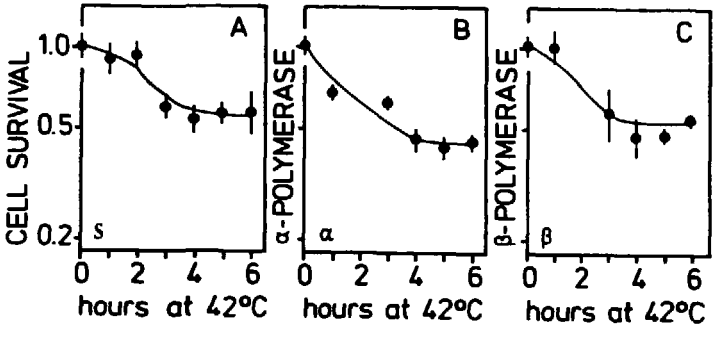

Figure 1. The effect of continuous heating of HeLa S_3 cells at a mild temperature (42°C) on surviving fraction (A) and on the fraction of specific activity of DNA polymerase ∝ and β (B,C) expressed as fraction of unheated cells (0h, 42°C = 1.0).

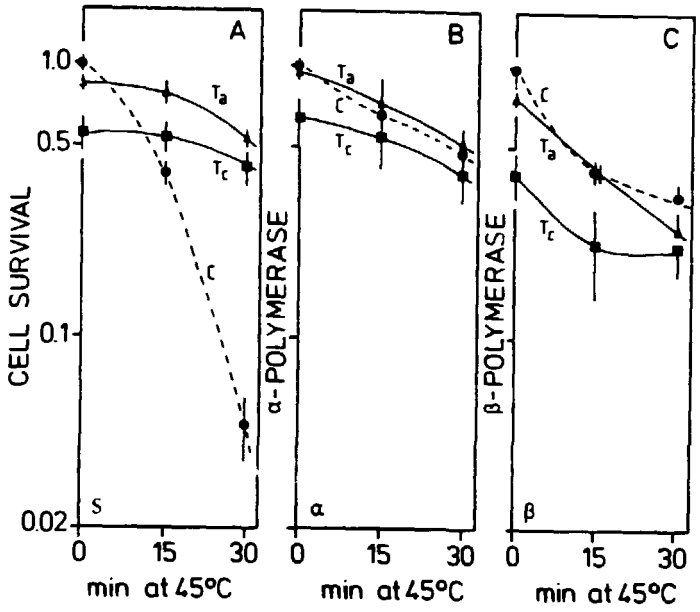

Figure 2. Comparison of the effect of hyperthermia on surviving fraction (A) and the fraction of DNA polymerase a and β (B,C) of normal and thermotolerant cells expressed as the fraction of unheated control cells (C, 0 min, 45°C=1.0). (circles) control cells (C); (squares) thermotolerant cells via a chronic heat treatment (Tc); (triangles) thermotolerant cells via an acute heat treatment (Ta). Mean ± s.e.m.; n≥3.)

It was of interest to investigate if the effect of hyperthermia on DNA polymerase activity as determined in whole cells, is also reflected at the level of the isolated nuclei. When cells are heated, structural changes in the nuclei can be observed (figure 3). The sedimentation distance of nuclei, analyzed by sucrose gradient centrifugation, decreased with the heat dose applied to the cells (figure 4). The decrease in nuclear sedimentation distance corresponded to an increase in protein content (see the legend of figure 4). A comparison of the effect of heating HeLa S_3 cells for 30 min at 45°C on polymerase activity as measured in cell homogenates and isolated nuclei is given in figure 5. The data are expressed as relative activities (as compared to homogenates from non-heated cells and to nuclei isolated from non-heated cells). As mentioned above, more protein is retained (bound) in nuclei isolated from heated cells than in nuclei isolated from control cells.

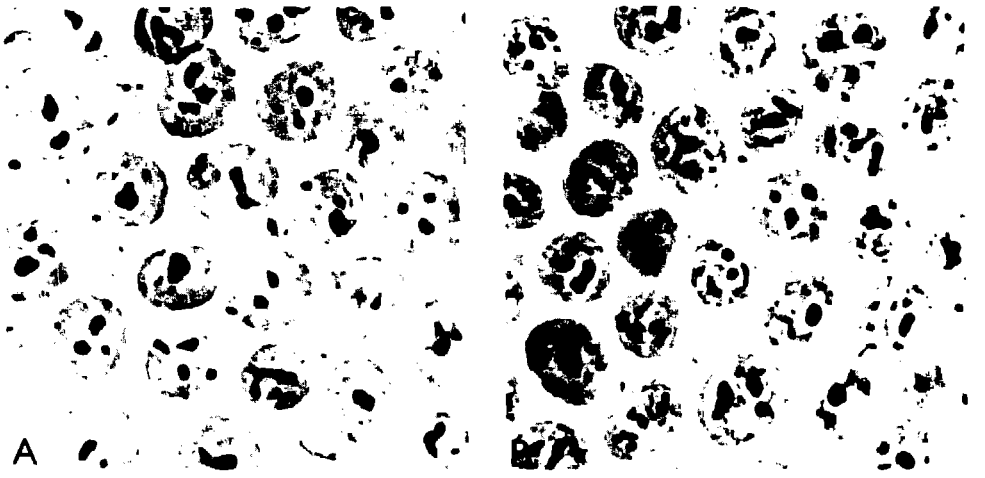

Figure 3. The effect of a hyperthermic treatment of HeLa S_3 cells on the morphology of isolated nuclei. Panel (A): nuclei isolated from normal cells. Panel (B): nuclei isolated from cells heated for 30 min at 45°C, showing a more coarse appearance. Phase-contrast light microscopy. The nuclei were stained with Trypan Blue.

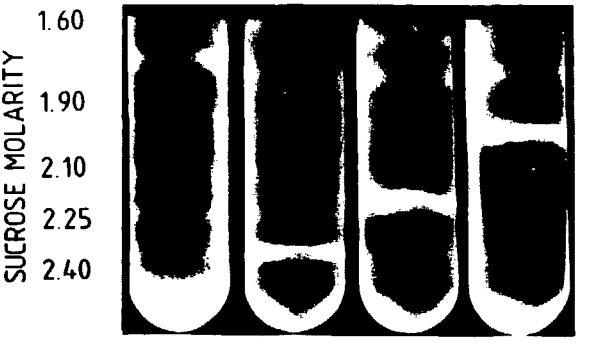

Figure 4. The effect of a hyperthermic treatment of HeLa S_3 cells on the sedimentation distance of the isolated nuclei. The relative content of protein per nucleus differs after the different heat treatments. A: nuclei of control cells with a relative protein content of 1.0 (bottom of the tube: 2.4-2.5 M sucrose). B: nuclei of cells heated at 45°C for 15 min and a relative protein content of 1.4 (band at 2.2-2.3 M sucrose). C: nuclei of cells heated at 45°C for 30 min and a relative protein content of 1.6 (band at 2.05-2.15 M sucrose). D: nuclei of cells heated at 45°C for 60 min and a relative protein content of 1.8 (band at 1.9-2.0 M sucrose). The molarity of sucrose present in the in the gradients is indicated on the ordinate.

Figure 5A shows that more polymerase ∝ is found in nuclei isolated from heated cells (30 min at 45°C) whereas the amount of polymerase β seems not to be altered, when compared to nuclei isolated from control cells. From figure 5B, in which specific activities are given, it can be concluded that more polymerase ∝ is bound to the nuclei from pre-heated cells. If it is assumed that DNA polymerase is localized in the nucleus (Kornberg 1980, 1982) we must conclude that there is less leakage of polymerases during isolation of nuclei when cells have been heated. When cells are heated for 30 min at 45°C, the fraction of cellular polymerase activity retained in the isolated nuclei increased 7-fold for ∝ polymerase (34 instead of 5 per cent) and 2-fold for β polymerase activity (15 instead of 7.5 per cent), whereas the protein content of the nuclei is increased only by a factor of 1.6±0.1 (mean ±s.e.m. of 9 experiments) after the same pre-treatment. So after heating of cells, not only polymerase ∝ but also polymerase β is specifically retained in the isolated nuclei, when compared to the bulk of the nuclear proteins.

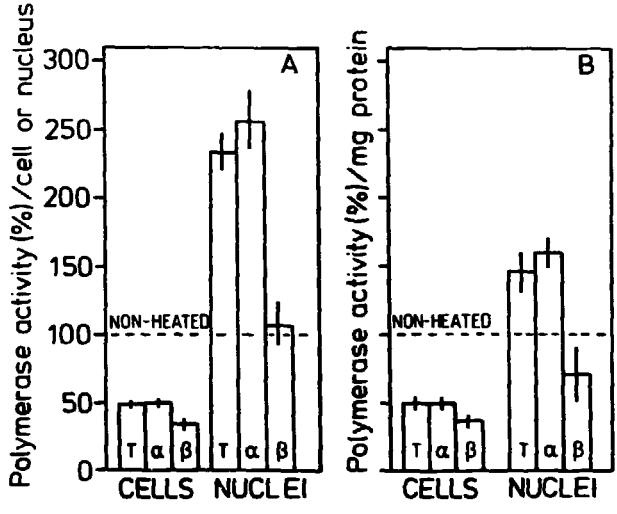

Figure 5. The effect of a hyperthermic treatment of HeLa S_3 cells during 30 min at 45°C polymerase activity in cells and isolated nuclei. Panel A: relative activity of the polymerases of heated cells compared to the non-heated control levels (100%) assayed in cell homogenates and isolated nuclei: T, total DNA polymerase; ∝, DNA polymerase ∝; β, DNA polymerase β. The activity in all three cases is expressed as a mean ± s.e.m. of the polymerase per cell of nucleus of 6-9 experiments. Panel B: as panel A but the activity is expressed per mg of cellular or nuclear protein respectively (specific activity).

The heat sensitivity of nuclear bound polymerase was determined by heating nuclei isolated form control and pre-heated cells. In figure 6 the heat sensitivity of DNA polymerase activity in nuclei isolated from control cells and pre-heated cells is given. In order to facilitate a comparison of the heat sensitivity of nuclear polymerase activity of pre-heated cells (30 min at 45°C or 15 min at 44°C plus 4h at 37°C), in figure 6B the polymerase activities of the heated nuclei (45°C) from the pre-heated cells are expressed relatively to the polymerase activity of the unheated nuclei form the pre-heated cells. The two pairs of bars in figure 6B show identical profiles to the first pair of bars in figure 6A; thus, nuclear polymerase activity of control, thermotolerant and heated (30 min at 45°C) cells show a comparable heat sensitivity.

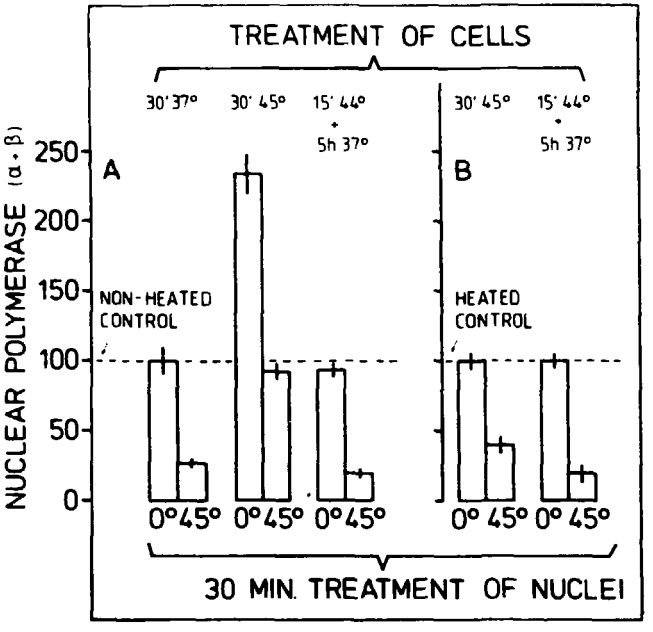

Figure 6: The effect of a hyperthermic treatment of cell nuclei isolated from heat treated and non-heated treated cells. Panel A: (∝ + B) DNA polymerase activity of unheated (0°C) and heated (45°C) nuclei isolated from (non-preheated or pre-heated) HeLa S_3 cells expressed as percentage of the activity present in unheated nuclei isolated from non-preheated cells (cells 30 min, 37°C, nuclei 0°C = 100 per cent). Panel B: as A but relative to the nuclei of preheated cells. For comparison of the heat sensitivity of nuclear (bound) polymerase activity, the data as used for the last two pairs of bars of A are taken and DNA polymerase activity is expressed as a percentage of the activity present in unheated nuclei (0°C) of pre-heated cells (cells 30 min 45°C, nuclei 0°C = 100 per cent and cells 15 min, 44°C plus 5h, 37°C, nuclei 0°C = 100 per cent).

Cells may be protected or sensitized during hyperthermia by the presence of erythritol or procaine respectively as shown in figure 7A. The modification of the heat response by these two agents was also present at the level of β polymerase when measured in whole cells. The effect of procaine-HCl was also expressed at the level of polymerase α, while only a slight protection by erythritol could be found (figure 7B and C). When procaine or erythritol was present during heating of isolated nuclei however, no effect of the "modulators" could be observed (figure 8).

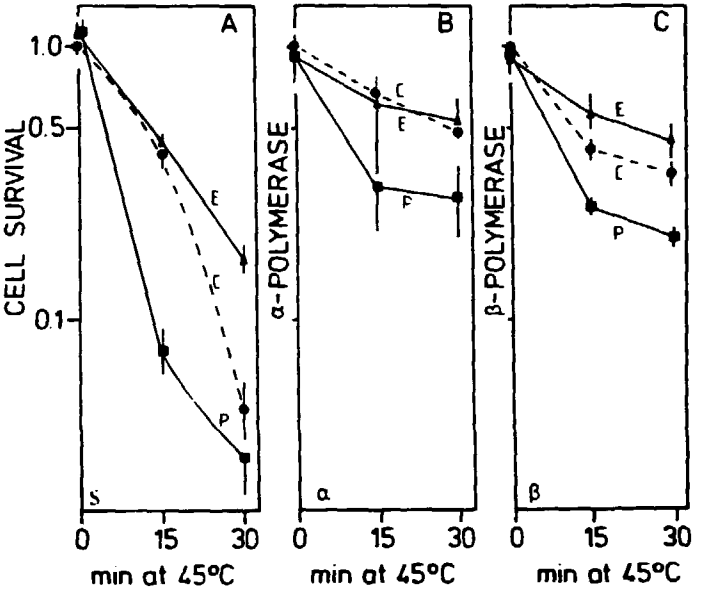

Figure 7. Effect of the present of erythritol (E) or procaine-HCl (P) during hyperthermic treatment of HeLa S_3 cells on survival (A) and on α (B) and β (C) DNA polymerase activity expressed as the fraction of unheated control cells (C, 0 min. 45°C = 1.0). (circles) control cells; (squares) 10 mM procaine; (triangles) 0.5 M erythritol. Procaine was added 15 min before the heat treatment and was present during the heat treatment. Erythritol was added 45 min before the heat treatment and was also present during the heat treatment. Cells were washed before plating or assaying polymerase activity. (Mean ± s.e.m.; n≥3.)

7.4 Discussion

The activity of DNA polymerase α as well as of DNA polymerase β is diminished after a hyperthermic treatment of cells (Denman et al. 1982, Dewey and Esch 1982, Spiro et al. 1982, this study). Prolonged heat treatments at a mild hyperthermic temperature (42°C) lead to heat resistance

(thermotolerance) for cell survival as well as for the activity of DNA polymerase (figure 2). This was also observed by Dewey and Esch (1982). The latter authors suggested a causal relationship between cell survival after heating and the inactivation of β-polymerase during the heat treatment. If DNA polymerase β is indeed playing an essential role in the mechanism of hyperthermic cell killing, heat resistance towards a second heat treatment, reflected in the extent of cell survival, should also be apparent at the level of β-polymerase activity. This, however, is not the case, at least not in the HeLa S_3 cells studied. When thermotolerant cells are heated at 45°C more cells survive compared to non-tolerant cells, while the decline in polymerase activity is essentially equal after the heating of normal and thermotolerant cells (figure 2). There is only one short report in the literature (Denman et al. 1982) suggesting thermoresistance of β-polymerase in CHO cells during a second heat dose at higher temperatures, although no correlation with cell survival is given. We are presently extending our studies to other cells lines (Ehrlich ascites tumour cells and mouse fibroblasts) in order to assess the general significance of the finding cited above.

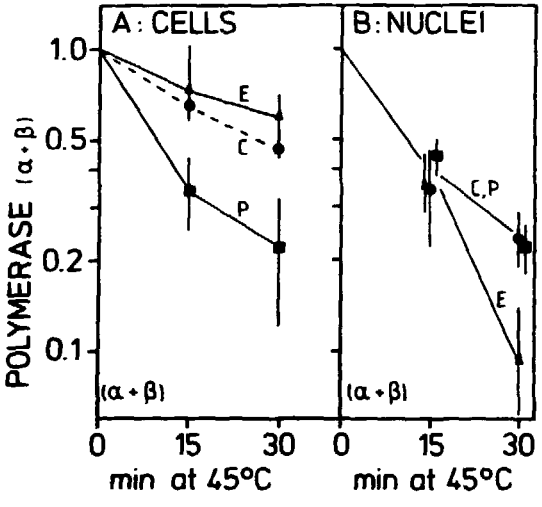

Figure 8. The effect of the presence of a heat sensitizer (P) and a protector (E) during hyperthermic treatment of whole cells (A) or isolated nuclei (B) on DNA polymerase ($\alpha + \beta$) activity expressed as fraction of unheated cells or nuclei (C,P,E, 0 min, 45°C = 1.0). (circles) control cells or nuclei, C; (squares) 10mM procaine present (sensitizer), P; (triangles) 0.5 M erythritol present (protector), E.

In the present study we found that the generally observed increased protein content in isolated nuclei from heated cells (Roti Roti and Winward 1978, Tomasovic et al. 1978, Warters and Roti Roti 1979, 1981, Clark et al. 1981, Roti Roti and Wilson 1984) correlated with a change in the structure of the isolated nuclei (sedimentation distance, morphology). The localization of α and β polymerases in vivo is thought to be in the cell nucleus (for a discussion see Kornberg 1980, 1982) although a perinuclear localization cannot be excluded (Brown et al. 1981). As more α as well as β polymerase activity is retained in the nuclear fraction when nuclei are isolated from heated cells (expressed as percentage of activity present in the cell homogenate), we conclude that there is probably less leakage of DNA polymerases during the (aqueous) isolation of nuclei from heated cells. Whereas for DNA replication a close association between DNA polymerase activity and the nuclear scaffold has been shown (Berezney et al. 1982, Vogelstein et al. 1982, Wanka et al. 1982), it is not known whether such an association is a prerequisite and whether it is also important for the function of DNA repair. If it assumed that especially the tightly (nuclear) bound polymerase is important for cell survival after heating, then it should be kept in mind that the availability of this enzyme is not diminished by the hyperthermic treatment of cells. In connection with this it is noteworthy that when nuclei isolated from pre-heated or thermotolerant cells are heated again, no resistance in polymerase activity could be observed (figure 6). This observation indicates that although relatively more α- and β-polymerase activity is bound tightly to nuclear structures after heating of the cells, this increase does not result in an increased resistance towards a second heat treatment. Cellular polymerase activity appeared to be heat-sensitized by procaine and heat-protected by erythritol when present during the heating of the cells, as shown by Dewey (1983). Nuclear bound polymerase activity is not influenced by the presence of these modifiers during the heat treatment of isolated nuclei (figure 8). Spiro et al.(1983) could not find an effect of procaine at the level of isolated polymerase β. Apparently the effects of procaine and erythritol upon DNA polymerase activity are cell-mediated, requiring extranuclear structures. The absence of the expression of thermotolerance on nuclear bound polymerase activity as well as the failure of procaine and erythritol to act at the nuclear level indicate that the primary effect of the heat insult leading to cell killing is not to be sought at the level of nuclear bound polymerase activity only. In our view the plasma membrane of cell might be a good candidate as a primary target for heat damage. Fluidizing the membranes of mammalian cells leads to an enhanced thermosensitivity in terms of clonogenic ability (Guffy et al. 1982, A.W.T. Konings, unpublished) and is related to changes in membrane permeability and membrane transport (Ruifrok et al. 1984). It is proposed that the modifying agents procaine and erythritol exert their action primarily on the plasma membrane and the cytoskeleton of the cell and thus influence the heat-induced changes in membrane permeability and transport. As a result of the membrane damage the intracellular environment is also modified,

including intracellular structures which are of vital importance for cell survival. Although the cellular activity of α- and β-polymerase is affected, this alteration seems not to be of essential importance for cell survival after heat treatment alone. It has to be kept in mind, however, that interactions between DNA polymerases and template may be different for endogenous template (damaged DNA) and added template (activated salmon sperm DNA) and may possibly be influenced by changes of the nuclear structures, as, e.g., the increased (nuclear) binding of DNA polymerase and other proteins. When hyperthermia is combined with radiation, DNA polymerase activity seems to be positively related to the capacity of the cell to repair radiation-induced damage of DNA as well as to cell survival after the combined treatment. The results of the latter experiments will be reported in a separate paper.

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CHAPTER 8

DIFFERENCES IN HEAT-INDUCED CELL KILLING AS DETERMINED IN THREE DIFFERENT CELL LINES DO NOT CORRESPOND WITH THE EXTENT OF RADIOSENSITIZATION

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SUMMARY

Three different cell lines, Ehrlich ascites tumour (EAT) cells, HeLaS_3 cells and LM mouse fibroblasts, were used to investigate whether or not the extent of heat killing (44°C) and heat radiosensitization (44°C before 0-6 Gy X-irradiation) are related. Although HeLa cells were the most heat-resistant cell line and showed the least heat radiosensitization, we found that the most heat-sensitive EAT cells (D_o EAT=8.0 min; D_o, LM=10.0 min; D_o, HeLa=12.5 min) showed less radiosensitization than the more heat-resistant LM fibroblasts ($TER_{HeLa} < TER_{EAT} < TER_{LM}$). Therefore, it is concluded that the routes leading to heat-induced cell death are not identical to those determining heat radiosensitization. Furthermore the inactivation of DNA polymerase α and β activities by heat seemed not to correlate with heat survival alone but showed a positive relationship to heat radiosensitization. The possibility of these enzymes being a determinant in heat radiosensitization is discussed.

8.1 Introduction

The mechanisms controlling cell killing after a combined treatment of heat and radiation are still unclear. As for killing with heat alone, the amount of heat radiosensitization seems to depend on time-temperature combinations (Dewey 1984). Lowering pH during heat and heat plus radiation increased both heat killing and heat radiosensitization (Freeman et al. 1981, Haveman 1983, Dewey 1984). Also, effects of heat modifying agents such as procaine or glycerol increased or decreased, respectively, heat killing and heat radiosensitization (Konings and van der Meer-Kalverkamp 1980, Djordjevic 1983, Dewey 1984). Furthermore, several studies involving the effect of thermotolerance on the slope and/or the shoulder region of radiation survival curves (Haveman 1983, Raaphorst and Azzam 1983, Holahan et al. 1984, Van Rijn et al. 1984) led Leeper (1985) to conclude that in general greater thermoradiosensitization is observed when greater thermal damage is achieved. This might imply that the routes leading to heat killing are identical to those determining heat radiosensitization. However, there are several reports which are in conflict with this hypothesis. Lunec and Parker (1980) reported no effect of lowering pH on heat radiosensitization, while heat survival levels were markedly reduced. Furthermore, Mivechi and Hofer (1983) showed that 5 per cent glycerol did increase heat resistance but had no effect on heat radiosensitization. In addition, several authors could not find significant effects of thermotolerance on the level of heat radiosensitization (Nielsen 1983, Hartson-Eaton et al. 1984, Jorritsma et al. 1985). The above studies deal with a comparison of heat killing and heat radiosensitization in only one cell line. In order to investigate whether or not the level of survival after heat alone determines the extent of heat radiosensitization we compared the relative differences in heat killing with the relative differences in effects of heat on radiosensitivity in three cell lines (Ehrlich ascites tumour (EAT) cells, HeLa S_3 cells and LM mouse fibroblasts). In order to determine parameters which might be related to the differences in heat sensitivity and heat radiosensitization of these three cell lines, we measured the levels of DNA polymerase α and β activities after the different heat treatments. It has been demonstrated that DNA polymerases can be inactivated by heat treatments of cells (Spiro et al. 1982, Dewey and Esch 1982, Denman et al. 1982, Kampinga et al. 1985) and that effects of lowering pH or effects of procaine, glycerol or erythritol on heat sensitivity (survival) correlated with inactivation of polymerase activities (Spiro et al. 1982, Mivechi and Dewey 1984, Kampinga et al. 1985). It has been suggested that these enzymes may have a determining role in cell survival after combined heat plus radiation treatment (Spiro et al. 1982, Dewey 1984, Mivechi and Dewey 1984, Jorritsma et al. 1985). When heat inactivation of DNA polymerase α and β activities are rate-limiting in the processes leading to heat-induced cell-killing and/or heat radiosensitization, the inactivation of the enzymes should occur concomitantly, not only with different conditions in one cell line, but also when comparing the three cell lines.

8.2 Materials and methods

8.2.1 Materials

Joklik's modification of minimal essential medium (MEM)and RPMI 1640 medium were purchased from Flow, Irvine, Scotland; foetal calf serum (FCS) was purchased from Gibco, Paisley, Scotland; [methyl-^{3}H]-thymidine-5'-triphosphate (^{3}HdTTP) from New England Nuclear, Dreieich F.R. Germany; N-ethylmaleimide (NEM) and salmon sperm DNA as well as nucleotide 3'-phosphates (dNTP) from Sigma Chemical Co., St Louis, Missouri, U.S.A. All other standard chemicals were from Merck, Darmstadt, F.R. Germany.

8.2.2 Cell culturing and determination of cell survival

The HeLa S_3 cells (ATTC no. CCL 2.2) were grown in suspension cultures in Joklik MEM supplemented with 10 per cent FCS. Doubling time was between 20 and 26 h. Ehrlich ascites tumour (EAT) cells were grown in suspension cultures in RPMI 1640 supplemented with 10 per cent FCS. Doubling time was between 11 and 15 h. The LM mouse fibroblasts (CCL 1.2) were adapted to growth in suspension culture in the serum-free, lipid-free, and protein free medium of Higuchi (Higuchi 1970) as described previously (Wolters and Konings 1982). Doubling time of these cells was between 24 and 30h. Asynchronously exponentially growing cells, with a viability above 95 per cent (trypan-blue assay) were used in all experiments. Cell survival was determined using 0 5 per cent soft agar plates with RPMI 1640 as described previously (Jorritsma and Konings 1983). Plating efficiency was always above 70 percent for all three cell lines. Triplicates of at least 50 colonies (containing more than 50 cells) for each experimental condition were counted after about 8 days for EAT cells and 14 days for HeLa cells and LM fibroblasts.

8.2.3 Hyperthermia and irradiation

Hyperthermia was performed in precision waterbaths(±0 05°C). For the determination of polymerase activities, requiring larger volumes of cell suspensions, cells were pelleted before heating (5 min at 250 g) and resuspended in prewarmed medium of the desired temperature in a final cell concentration of 5 x 10^6 cells/ml. After heating, samples were taken for determination of polymerase activities and for 0-6 Gy X-irradiation to determine cell survival. Time between heat and irradiation was less than 5 min. Irradiation was done with a Phillips-Müller MG 300 machine, operated at 200 kV and 15 mA, at room temperature. The X-rays were filtered by 0 5 mm Cu and 0 5 mm Al resulting in a dose rate of 6Gy/min. The thermal enhancement ratio (TER) was calculated from:

the linear part of the survival curves:

$$TER_{D0} = \frac{D_0 \text{ (X-rays alone)}}{D_0\text{(heat + X-rays)}}$$

or at 1 per cent isosurvival:

$$TER_{iso} = \frac{\text{Dose of X-rays (no heat)}}{\text{Dose of X-rays (+ heat)}} \text{ to acquire 1 per cent isosurvival}$$

8.2.4 Determination of DNA polymerase activity

After hyperthermia the cells were pelleted and resuspended in a buffer containing 10 mM Tris HCL (pH=7 5), 10 mM NaCl and 1 5 mM $MgCl_2$. After sonicating the cells, they were mixed with the appropriate DNA precursors and activated salmon sperm DNA as described previously (Kampinga et al 1985). The DNA polymerase activity was determined as the rate of incorporation of ^{3}HdTTP into 10 per cent TCA precipitable material. The DNA polymerase α activity was calculated by subtracting the β polymerase activity (NEM-insensitive) from the total activity measured.

8.3 Results

Figure 1 and table 1 show that EAT cells were more heat sensitive than the LM mouse fibroblasts (see also Konings and Ruifrok 1985). Heat radiosensitization was measured by assaying the clonogenic ability after subjecting the cells to different doses at 44°C prior to 0-6 Gy X-irradiation (figure 2, table 2). LM fibroblasts are more radiation resistant and HeLa S_3 cells are more radiation sensitive than EAT cells. The inverse of the thermal enhancement ratios (TER), based on the -*ln exp/slope* ($-D_0$) of the survival curves, as a function of heat dose prior to X-irradiation are shown in figure 3(a). In addition 1/TER based on 1 per cent isosurvival levels are plotted against the heat dose prior to X-irradiation (figure 3(b)).

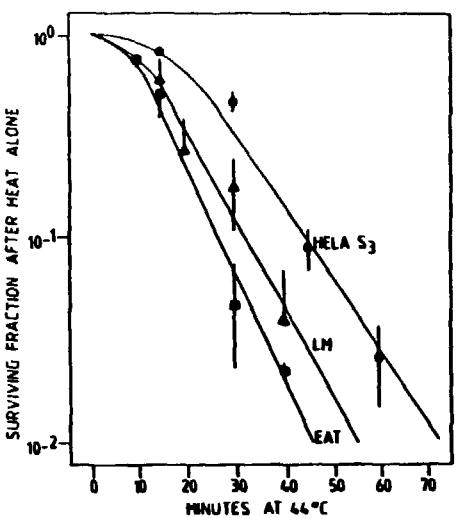

Figure 1. Surviving fractions after 44°C heat treatments of three mammalian cell lines. (Data are the mean ± s.e.m. of at least two independent experiments.) The lines were drawn by regression analysis.

	D_o (min.44°C)	n	D_q (min.44°C)	Isosurvival (min.44°C) 10 per-cent	1 per-cent
EAT	8.0	2.8	8.3	27.0	45.0
LM	10.0	2.5	9.3	32.4	55.5
$HeLaS_3$	12.5	3.5	15.7	44.4	73.1

Table 1. Parameters of heat sensitivity.Parameters of the heat survival curves of figure 1. The slopes of the linear part of the curves were determined by log-linear regression analysis and the D_o values (-ln exp/slope) were calculated. The shoulders of the curves are described by the parameters n (extrapolation number) and D_q (quasi-threshold dose, $D_q=D_o$ x ln n). Heat doses (in min. at 44°C) necessary to acquire 10 per cent and 1 per cent survival (isosurvival values) are also given. The Wilcoxon test revealed that heat sensitivities (based on D_o as well as isosurvival) were significantly different within 95 per cent confidence limits.

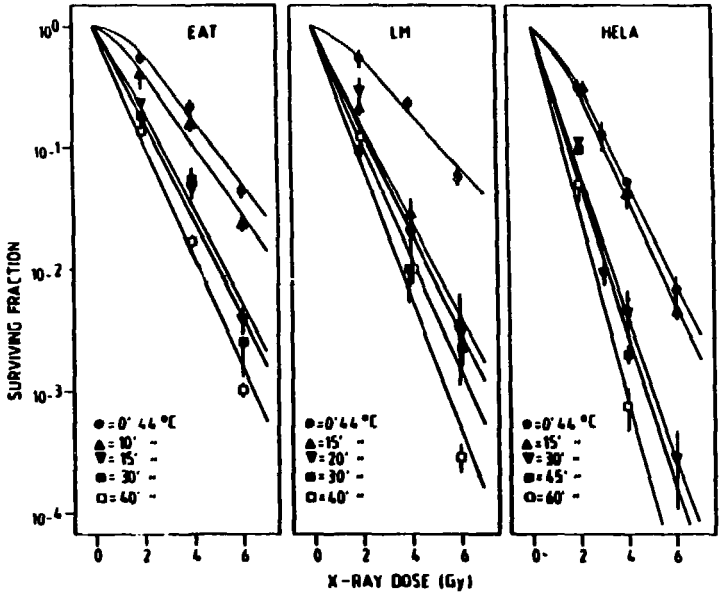

Figure 2. Surviving fractions after X-rays alone and after combined heat (44°C) + radiation treatment. (Data are the mean ± s.e.m. of at least two independent experiments.) The lines were drawn by regression analysis.

Table2(a). EAT-cells.

Heat dose (min.) prior to X-irradiation	D_o (Gy)	n	D_q(Gy)	Isosurvival (Gy)	
				10 per cent	1 per cent
Unheated	1.67±0.10	2.1±0.2	1.2±0.2	5.0±0.3	8.8±0.5
10 44°C	1.42±0.10	1.9±0.2	0.9±0.2	4.2±0.3	7.5±0.5
15 44°C	1.19±0.12	1.2±0.1	0.2±0.1	2.9±0.2	5.6±0.5
30 44°C	1.10±0.11	1.1±0.06	0.1±0.05	2.7±0.3	5.2±0.5
40 40°C	0.9±10.10	1.1±0.2	0.1±0.2	2.2±0.3	4.3±0.5

Table 2 (b). LM-fibroblasts

Heat dose (min.) prior to X-irradiation	D_o(Gy)	n	D_q (Gy)	Isosurvival (Gy)	
				10 per cent	1 per cent
Unheated	2.02±0.17	1.7±0.3	0.7±0.4	5.4±0.2	10.00±0.4
15 44°C	1.00±0.13	1.3±0.2	0.2±0.1	2.5±0.2	4.9±0.5
20 44°C	0.93±0.08	1.5±0.1	0.3±0.07	2.5±0.2	4.7±0.4
30 44°C	0.86±0.13	1.0±0.2	0	1.9±0.2	3.9±0.5
40 44°C	0.74±0.10	1.4±0.2	0.3±0.2	2.0±0.2	3.5±0.5

Table 2 (c). HeLa S_3 cells.

Heat dose (min.) prior to X-irradiation	D_o(Gy)	n	D_q (Gy)	Isosurvival (Gy)	
				10 per cent	1 per cent
Unheated	1.03±0.05	2.5±0.3	0.9±0.1	3.2±0.1	5.6±0.2
15 44°C	0.98±0.03	2.7±0.2	1.0±0.05	3.1±0.05	5.3±0.1
30 44°C	0.70±0.03	1.1±0.2	0.04±0.12	1.7±0.07	3.3±0.06
45 44°C	0.65±0.03	1.3±0.2	0.2±0.1	1.7±0.05	3.2±0.1
60 44°C	0.56±0.04	1.2±0.2	0.1±0.1	1.4±0.05	2.7±0.1

Table 2. Parameters of radiation sensitivity and heat radiosensitization. Parameters of survival curves shown in figure 2. See footnote to table 1. The X-ray doses (Gy) to acquire 10 per cent and 1 per cent survival (isosurvival values) after radiation with or without prior heating are given. Values given are the mean ± s.e.m. of at least two independent experiments.

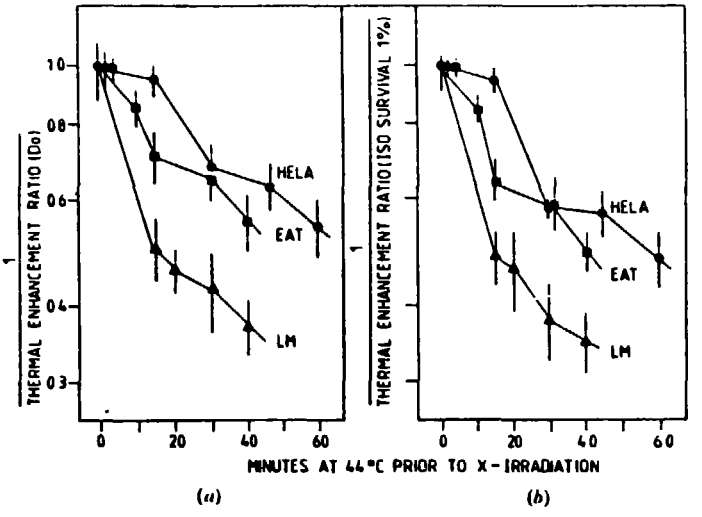

Figure 3. Heat radiosensitization by 44°C heat treatments in three mammalian cell lines. Heat radiosensitization ex-pressed as the inverse of the thermal enhancement ratio (TER), is plotted versus the heat dose given prior to 0-6 Gy X-irradiation. (a) TER_{Do}; (b) TER_{iso} (see text). Data from figure 2 and table 2.

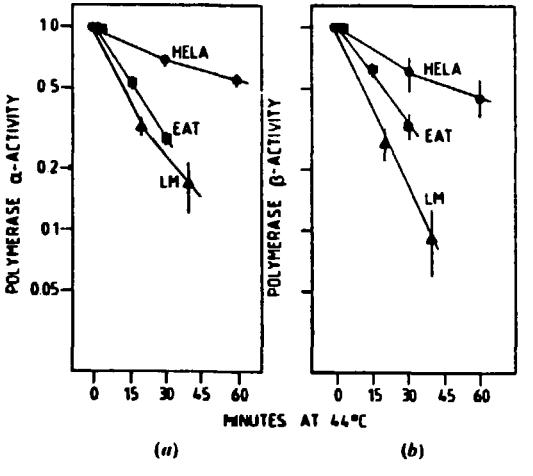

Figure 4. Heat inactivation of DNA polymerase α (a) and polymerase β (b) in three mammalian cell lines. Polymerase activities were expressed relative to the unheated controls (=1.0). (Data are the mean ± s.e.m. of at least three independent experiments).

It can be seen from figures 1, 2, and 3 that HeLa S_3 cells were the most heat resistant and showed the least heat radiosensitization. However, the level of heat radiosensitization in LM fibroblasts was higher than in EAT cells, although a lower heat resistance was observed for EAT cells compared to LM fibroblasts. This result indicated that the absolute level of survival after heat alone may not generally be considered as an indication of the extent of heat radiosensitization in the cell lines tested. Because of the possible involvement of DNA polymerases in the radiosensitivity of cells, DNA polymerase α and DNA polymerase β (NEM-insensitive) activities were assayed in the three cell lines after the different heat treatments. For both polymerase α and β it was observed that the other of heat sensitivity in the three cell lines was the same as that found for heat radiosensitization, being the highest in LM fibroblasts and the lowest in HeLa S_3 cells (figure 4).

8.4 Discussion

8.4.1 Heat sensitivity versus the radiosensitizing effect of heat

Many data, based on different heat treatments in one cell line, using altered pH, heat modifying agents or development of thermotolerance have suggested a causal relationship between heat killing and heat radiosensitization (Konings and v.d. Meer-Kalverkamp 1980, Freeman et al. 1981, Djordjevic 1983, Haveman 1983, Raaphorst and Azzam 1983, Dewey 1983, 1984, Holahan et al. 1984, van Rijn et al. 1984) although other data do not show this relationship (Lunec and Parker 1980, Mivechi and Hofer 1983, Nielsen 1983, Hartson-Eaton et al. 1984, Jorritsma et al. 1985). The different results of these investigations may possibly be explained by the differences in cell lines used and in the different heat schedules employed. The experiments presented in the current study, where three different cell lines with different heat sensitivities are used, are not hampered by possible difficulties related to fractionated heat treatments or non-specific effects of heat modifiers. Our results show that heat resistance to radiosensitization by heat are not always correlated. Similarly, Miyakoshi (1981) showed that hyperthermic radiosensitization assayed in a rather heat sensitive fibroblast cell line was much less compared to three other far more heat resistant cell lines. Raaphorst and Azzam (1983) found that transformed CH3-10T$\frac{1}{2}$ cells were of similar heat sensitivity to normal CH3-10T$\frac{1}{2}$ cells, whereas sensitivity to combined radiation and heat treatment was different. We like to conclude that the mechanisms leading to heat killing might not be identical to those determining heat radiosensitization.

8.4.2 Heat radiosensitization and inactivation of DNA polymerase activities

As observed earlier (Jorritsma et al. 1985) and in the experiments presented here, there seems to be no direct relationship between heat inactivation of

polymerase activities and the level of survival after heat alone. Heat inactivation of polymerase α and β was less in EAT cells compared to LM fibroblasts whereas EAT cells were more heat sensitive. Effects of heat on the repair of radiation-induced DNA damage have been described (e.g. Corry et al. 1977, Warters and Roti Roti 1978, 1979, Dikomey 1982, Jorritsma and Konings 1983). This might be caused by either an inactivation of enzymes involved in the DNA repair process such as DNA polymerases α and β, topoisomerase, ligase and/or ADP-ribose transferase or by an altered accessibility of the DNA for the repair enzymes, for example, by an increased protein content of the chromatin (Tomasovic et al. 1978, Roti Roti and Winward 1978), or by a combination of both. Comparing the three cell lines we found that heat radiosensitization occurred concomitantly with heat inactivation of polymerase α and β activities. The results are in accordance with earlier studies in HeLa S_3 cells (Jorritsma et al. 1985 a, b). Also, Dewey (1983, 1984) comparing inactivation of DNA polymerase and heat radiosensitization in CHO cells, using lowered pH, glycerol or procaine, suggested a relationship between these parameters. In addition, the data of Spiro et al. (1982) relating recovery from heat damage of polymerase β activity with loss of synergism between heat and radiation, support the possibility of polymerase activities being determinants in heat radiosensitization. However, one has to be cautious in the interpretation of these correlations (Jorritsma et al. 1985). A functional relationship between the parameters studied (DNA polymerases and heat radiosensitization) has still to be established. Moreover our recent experiments (Jorritsma et al. 1986) in which fractionated heat treatments were employed, led to the conclusion that α and β DNA polymerase inactivation is not always the (only) critical cellular process responsible for heat radiosensitization. We propose that because of the complexity of DNA repair processes and the possible differential vulnerability of these processes to different heat treatments (thermotolerance, thermosensitization) in different cell lines, there is probably no sole enzymatic or structural cellular component which is responsible for the extent of heat radiosensitization. The effects of heat on the DNA repair enzymes as well as on chromatin structure may lead to an inhibition of repair of X-ray induced DNA damage, thus causing radiosensitization.

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CHAPTER 9

INHIBITION OF REPAIR OF X-RAY INDUCED DNA DAMAGE BY HEAT. THE ROLE OF HYPERTHERMIC INHIBITION OF DNA POLYMERASE α ACTIVITY

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SUMMARY

HeLa S3 cells growing in suspension have been used to investigate possible mechanisms underlying the inhibitory action of hyperthermia (44°C) on the repair of DNA strand breaks as caused by a 6 Gy X-irradiation treatment. The role of hyperthermic inactivation of DNA polymerase ' was investigated using the specific DNA polymerase α-inhibitor, aphidicolin. It was found that both heat and aphidicolin (> 2 μg/ml) could inhibit DNA repair rates in a dose dependent way. When the applications of heat and aphidicolin were combined, each at non maximal doses, no full additivity in effects were observed on DNA repair rates. When the heat and radiation treatment were separated in time by post-heat incubation at 37°C, restoration to normal repair kinetics were observed within 8 hours after hyperthermia. When heat was combined with aphidicolin addition, restoration of the aphidicolin effect to control level was also observed about 8 hours after hyperthermia. It is suggested that although DNA polymerase α seems to be involved in the repair of X-ray induced DNA damage, and although this enzyme is partially inactivated by heat, other forms of heat damage have to be taken into account to explain the observed repair inhibition.

9.1 Introduction

Hyperthermic inhibition of repair of radiation damage has been suggested as the essential factor causing the synergistic cell-killing effect of X-rays and hyperthermia (1). Heating cells before X-irradiation has been shown to inhibit the repair of alkali labile sites in DNA, including DNA strand breaks (2-5), as well as the excision of base damage (6,7). This hyperthermic repair inhibition might be caused by either an altered template (DNA) accessibility or by a decreased activity of the DNA repair enzymes or by a combination of both (8). Several investigators found an elevated protein to DNA ratio in nuclei and chromatin isolated from heated cells (3,4,7,9-11). This protein elevation seemed responsible for the hyperthermic inhibition of the excision of t'-type damage (7) and the decreased rate of nuclease digestion of chromatin (12). It was suggested that these extra proteins altered DNA-accessibility thus causing the observed repair inhibition by heat. For as far as the role of hyperthermic inactivation of repair enzymes as possible cause for heat radiosensitization is concerned, most emphasis has been put on DNA polymerase -α and -β activities (8,11-14). No clear insight has emerged as yet for the precise involvement of either one or both of these DNA polymerases, in the hyperthermic inhibition of repair. In order to obtain more insight in the mechanisms of repair inhibition by heat we started focussing on DNA polymerase α. This enzyme is believed to play a major role in DNA replication (for review see 16-18). Its activity can be very specifically inhibited by the tetracyclic diterpene tetraol, aphidicolin (APC). This agent inhibits DNA polymerase α in a competitive way with respect to dCTP, in a non-competitive way with respect to other dNTPs and uncompetitive with respect to DNA (17,19,20). On the role of polymerase α in repair processes (and its inhibition by APC) conflicting reports exist. The results vary from no effect of APC on DNA repair synthesis after UV-irradiation (21,22) to significant inhibition of DNA repair processes (23-33). DNA polymerase α might act jointly or sequentially with DNA polymerase β in the UV-repolymerization (30,31). Others suggest that the fraction of repair synthesis mediated by either DNA polymerase α or β is dependent on the nature of the DNA damaging agent (32,33). The experiments reported in the current communication were initiated to investigate whether APC has an inhibitory effect on the repair of X-ray induced DNA alkali labile sites, and if heat inactivation of polymerase α plays a role in hyperthermic inhibition of repair.

9.2 Materials and methods

9.2.1 Cell culturing and cell labelling conditions

HeLa S3 cells (ATCC no. CCL 2.2) were grown in suspension cultures in Jokliks modification of minimal essential medium (Flow, Irvine, Scotland) supplemented with 10 per cent Fetal Calf Serum (Gibco, Paisley, Scotland). The doubling time of the cells was about 26 hours. Asynchronously exponentially grown cells in suspension were uniformally DNA-

labelled (42-48 hours) in a medium containing 2 μM [methyl-^{3}H]thymidine (NEN, Dreieich, W. Germany) with a specific activity of 20 Ci/mol. After labelling, the cells were chased for at least 1.5 hours at 37°C in complete medium containing 2 μM unlabelled thymidine. Before the experiment the cells were centrifuged and resuspended in fresh medium buffered with 20 mM Hepes (Sigma, St. Louis, MO) pH = 7.3, instead of $NaHCO_3$, at a concentration of 1.1 x 10^6 cells/ml.

9.2.2 Conditions for hyperthermia, X-irradiation and incubations

Hyperthermia was performed in a precision waterbath (± 0.05°C); the cells were heated in plastic tubes (0.5 ml samples). Aphidicolin (APC) stock-solution (1 mg/ml) was dissolved in 10% DMSO. It was always added 45 min. prior to X-irradiation. A 50 μl solution of the desired APC concentration was added to 450 μl cell suspension in Joklik-Hepes (1.0 x 10^6 cells/ml, final concentration). During X-irradiation cells were cooled on ice. Immediately after hyperthermia (t < 5 min.) X-irradiation was done with a Philips-Muller MG 300 machine operating at 200 kV and 15 mA. X-rays were filtered with 0.5 mm Cu and 0.5 mm Al. The dose rate was 6 Gy/min, as measured with a Phillips Universal Dosimeter. After X-irradiation the 0.5 ml samples were reincubated at 37°C to allow repair. At various times, repair was stopped by putting the samples on ice. Three samples, each 0.1 ml, were taken for strand break determination.

9.2.3 Determination of DNA damage

For detection of DNA damage we used the alkaline unwinding method followed by hydroxylapetite column chromatography as described previously (5). By this method strand breaks and alkali labile sites (e.g. apurinic sites and some types of sugar damage) can be detected. We will refer to this as "DNA strand breaks" being the majority of the damage detected by this method. Shortly, the cooled 0.1 ml samples (10^5 cells) were treated with an alkaline buffer and unwinding was allowed for 30 min. at 20°C. After rapid neutralization and addition of sodium dodecyl sulphate the samples were stored at -20°C. Prior to column chromatography the cells were thawed and sonicated (20 sec., 50 Watts). The samples were brought to 60°C and 2 ml of each sample was applied to a hydroxylapetite column and maintained at 60°C in an aluminum block. After washing the column, single stranded DNA was eluted with 2 x 2 ml 0.125 M and 2 x 2 ml 0.150 M phosphate buffer (pH = 6.8) and double stranded DNA was eluted with 2 x 2 ml 0.4 M phosphate buffer (pH = 6.8). All buffers were preheated at 60°C before applying to the columns. The logarithm of the double stranded DNA fraction (log (DS/(DS + SS) x 100)) was used as a measure for the amount of alkali labile sites (5).

9.2.4 Measurement of DNA synthesis

For measurements of DNA synthesis heated and non-heated cells, treated in the presence of 0-20 μg/ml APC (30 min. preincubation at 37°C) were incubated at 37°C with 7 μM [methyl-^{3}H]thymidine for 0-20 min. During this period the incorporation rate was found to be constant. Label incorporation was stopped by bringing the cells on ice and adding 2 ml of a cold TCA (10%) - $Na_4P_2O_7$ (2%) solution. Salmon sperm DNA (0.05 mg) and BSA (0.3 mg) were added as carriers. After overnight precipitation at 4°C, the cells were centrifuged (10 min. at 1000 g, 4°C) and washed twice with cold TCA (5%) - $Na_4P_2O_7$ (2%) and once with 100% cold ethanol. Dried pellets were dissolved in 0.25 ml Soluene-350 (Packard) and label incorporation was determined by scintillation-counting. The relative incorporation rate towards untreated control cells (= 100%) was used as a parameter for the effects of both heat and APC on replicative DNA synthesis.

9.3 Results

The effect of heat or APC on the rate of replicative DNA synthesis is shown in figure 1. As can be seen, hyperthermia at 44°C (triangles) reduced DNA replication in a dose-dependent way as was shown before (34). APC (circles) at a concentration of 0.2 μg/ml already inhibited 85% of DNA replicative synthesis, while 2 μg/ml and 20 μg/ml nearly completely (> 98%) inhibited DNA replication. This is in accordance with the proposed role of polymerase α in DNA replication (16-18). Increasing doses of hyperthermia (44°C) showed progressively more repair inhibition as shown in figure 2a. In figure 2b one can observe that for 0.2 μg/ml APC no inhibition of the DNA repair is observed, although already 85% of DNA replicative synthesis was inhibited (figure 1). Higher concentrations of APC (> 2 μg/ml) however, substantially inhibited DNA repair, suggesting a possible role for DNA polymerase α in the repair of X-ray induced DNA damage. Because APC was dissolved in DMSO, we measured the effect of DMSO on DNA repair kinetics as a control. Up to concentrations of 0.5% DMSO no effects upon DNA repair kinetics were observed (not shown).

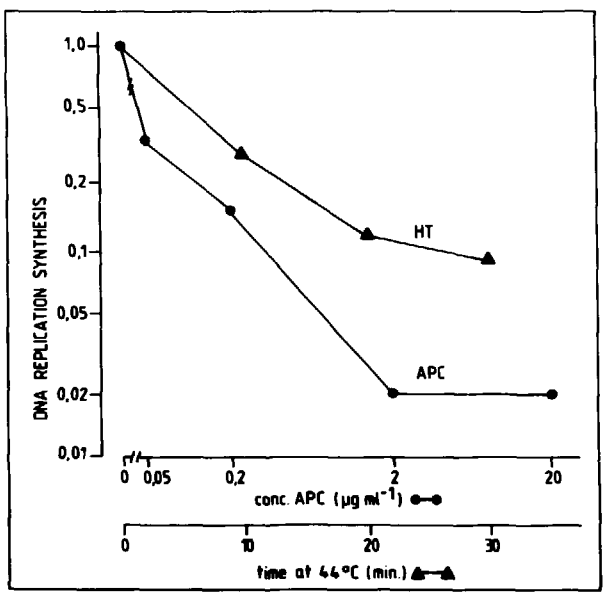

Figure 1; The effect of heat and aphidicolin on DNA replicative synthesis. DNA synthesis was measured as the incorporation of ^{3}H-thymidine into TCA-precipitable material. Data are expressed as values relative to that in untreated controls (= 1.0) and as a function of heating time at 44°C (triangles) or concentration of aphidicolin in μg/ml (circles).

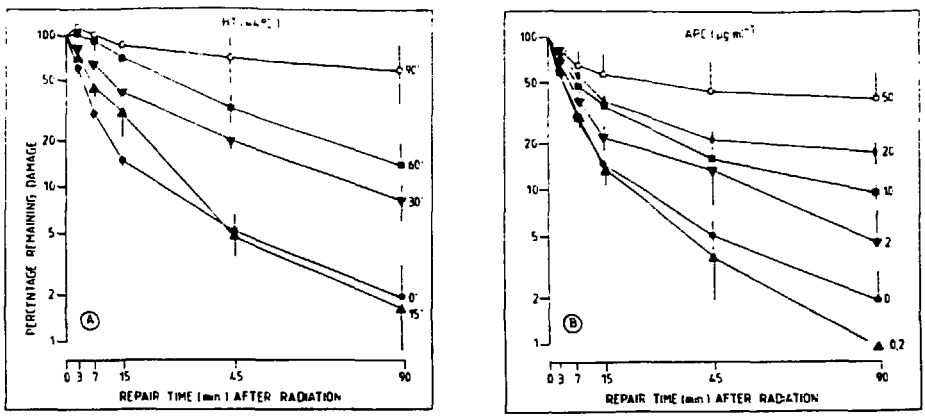

Figure 2: Time course of rejoining X-ray-induced DNA alkali labile sites. HeLa S3 cells were exposed to 6 Gy of X-rays at 0°C. After various times of post-irradiation incubation at 37°C, to allow repair, the number of alkali labile sites were determined. The relative amount of alkali labile sites (towards cells for which no repair time was allowed = 100%) was plotted versus the various repair times. Values given are the mean ± SEM of 2 to 11 experiments. A) The effect of 44°C hyperthermia, given prior to X-irradiation, on DNA repair kinetics. B) The effect of aphidicolin (APC: µg/ ml) given 45 min. prior to X-irradiation on repair kinetics.

The curves of figure 2 were evaluated by least squares analysis to fit the model:

$$Y = A \exp^{(-\alpha t)} + B \exp^{(-\beta t)} + C \exp^{(-\gamma t)} + \ldots$$

in which Y is the fraction of damage remaining after t minutes of repair and α, β, γ and A, B, and C are constants. The data were fitted with either mono- or biphasic computer-simulated curves. The results of these analyses are shown in table 1. In order to compare the obtained mono- and biphasic curves quantitatively we took the area under the curve (AUC) as a measure for the inverse of the repair capacity (15). The "weighted mean half time of repair" is then represented by: AUC ln 2 = A $t_{\frac{1}{2}}(\alpha)$ + B$t_{\frac{1}{2}}(\beta)$.

Treatment	Monophasic fit ∝-rate ± SD	A ± SD	$T_{\frac{1}{2}}$	Biphasic fit ∝-rate ± SD	A ± SD	$T_{\frac{1}{2}}$	β-rate ± SD	B ± SD	$T_{\frac{1}{2}}$	AUC ln 2 Mono	Bi	Mean
Control				0.200±0.006	85.5±12.2	3.47	0.0217 ±0.0028	14.1±13.8	31.9	-	7.5	7.5
15'44°C				0.210±0.039	95 1.0±15.7	3.31	0.0518 ±0.0040	49.0±18.4	13.4	-	8.3	8.3
30'44°C				0.103±0.030	464.7± 2.6	6.71	0.0152 ±0.00084	35.3± 4.3	45.6	-	20.5	20.5
60'44°C	0.0249 ±0.00165	100±8.1	27.8	0.079±0.048	18.9±58.7	8.77	0.0206 ±0.0018	81.1± 9.2	33.7	27.8	29.1	28.4
90'44°C	0.00875±0.00192	100±11.2	79.2							79.2	-	79.2
0.2 μg APC/ml				0.205±0.003	784.9± 6.8	3.38	0.0302 ±0.0	15.1± 0.0	22.9	-	6.4	6.4
2 μg APC/ml				0.195±0.016	878.9± 6.5	3.55	0.0128 ±0.0043	21.1± 21.4	54.1	-	14.2	14.2
10 μg APC/ml				0.123±0.017	572.3±17.7	5.61	0.0113 ±0.00291	27.7±19.9	63.1	-	21.0	21.0
20 μg APC/ml				0.147±0.006	264.0± 4.8	4.72	0.0129 ±0.00129	36.0± 3.5	61.8	-	25.3	25.3
50 μg APC/ml				0.251±0.003	836.3± 1.4	2.76	0.00727 ±0.0001	63.7± 0.3	95.3	-	61.8	61.8

Table 1: Effect of heat (44°C) and APC (μg/ml) on the DNA repair kinetics after a 6-Gy X-irradiation treatment. For each treatment the repair curv fitted to the multiphasic model by computer-aided weighted least-squares analysis. When a biphasic model fitted the experimental values better (P<0 biphasic model is given. Otherwise both models or monophasic models are given. The rates of repair, ∝ and β, calculated from the mean repair curve ar halftimes of repair ($T_{\frac{1}{2}}$) were calculated from these repair rates (ln 2/∝ and ln 2/β, respectively). The relative weight factors A and B (±SD) are also gi parison of mono- and biphasic curves, the AUC ln 2 parameter is introduced (see Results, paragraph 3).

Plotting the AUC ln 2-repair parameter versus the heat dose or versus the APC concentration, we observe (figure 3) that for both heat and APC higher doses are needed to inhibit repair than to inhibit replicative synthesis to comparable degrees (figure 1). Computer analysis of data (table 1) concerning repair inhibition by heat and aphidicolin generally reveals qualitative differences. While heat mostly altered the α-rate and increased the relative contribution (B) of the slow component (β) in the biphasic models, APC mostly affected the a-rate. The relative contributions of the fast and slow components were altered to a minor extent only. These differences in the mode of inhibition kinetics between heat and APC may already point to different molecular mechanisms of inhibition. It is however important to realize that this type of computer analysis has its limitations with respect to the interpretation of the results.

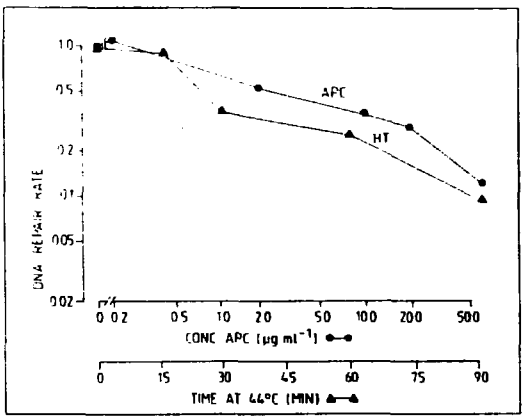

Figure 3: The effect of heat and aphidicolin on DNA repair. Relative DNA repair rates, AUC ln $2_{(control)}$/AUC ln $2_{(treated)}$, were plotted as a function of the heating time (triangles) or concentration of aphidicolin (circles).

If hyperthermic repair inhibition would be totally determined by the observed (11) hyperthermic inhibition of DNA polymerase α, one could assume that heat and APC would substitute for each other in repair inhibition and would also have additive effects on the inhibition of repair of X-rays DNA damage when applied each at non maximal doses. In figure 4A, experiments are illustrated where a fixed APC dose (20 μg/ml) was combined with various heat doses and its effect on the "mean half time of repair" (AUC ln 2) was

compared to the effect of heat alone. In figure 4B a similar comparison between a fixed heat dose (30 min. at 44°C) and various APC concentrations is shown. In order to determine whether heat and APC are additive in inhibiting DNA strand repair we used the curves obtained in figure 4 (from regression analysis) to determine additivity levels. This is illustrated in figure 5. If data derived from the combination studies (figure 4) were below these lines, this would suggest synergistic action. If these values were on these lines it would suggest additivity. If the values of the combinations studies would fall above the corresponding additivity lines this would suggest less than additive action of heat and APC.

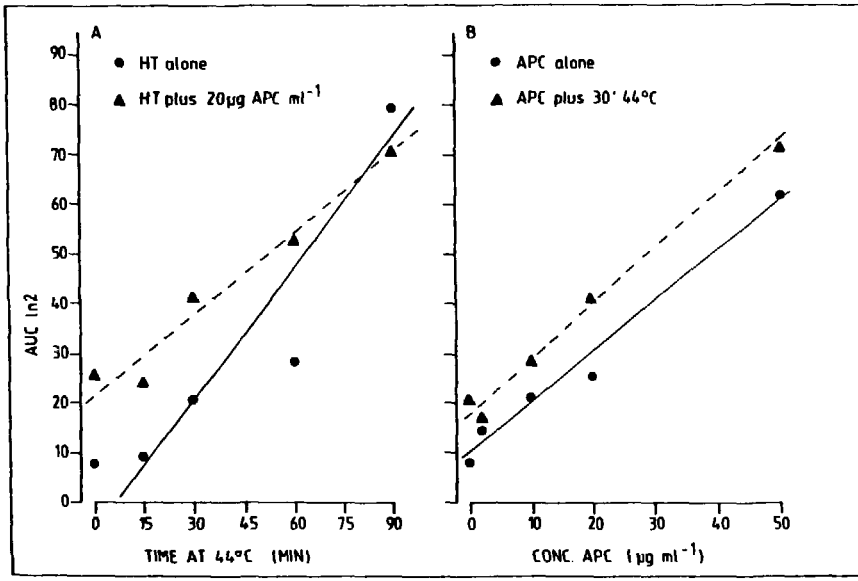

Figure 4 The effect of combinations of heat and aphidicolin on DNA repair. Panel (A): The effect of heat alone (circles) or combined with a fixed dose of APC (20 µg/ml: triangles) on "the weighted half time of repair" (AUC ln 2) is plotted versus the time of heating at 44°C. Panel (B): The effect of APC alone (circles) or combined with a fixed heat dose (30 min. at 44°C: triangles) on "the weighted half time of repair" (AUC ln 2) is plotted versus the APC concentration (µg/ml) used. AUC ln 2 values are calculated from repair curves similar as shown in figure 2 (from at least 2 experiments). The lines are drawn using regression analysis (r = 0.8622 for heat alone; r = 0.9615 for heat plus 20 µg/ml APC; r = 0.9803 for APC alone; r = 0.9897 for APC plus 30 min. 44°C).

Examples of the 30, 40 and 50 values (AUC ln 2) are shown. Repair inhibition resulting in an AUC ln 2 = 40, can be obtained by a 53 min. at 44°C heat treatment alone or 29 μg/ml APC treatment alone. So an AUC ln 2 = 40 "additivity line" can be drawn between these points (inverted triangles). Combining heat and APC revealed (again calculated from figure 4) that, when using 20 μg/ml APC, a heat dose of 35 min. at 44°C (20 min. above additivity) is needed to obtain an AUC ln 2 = 40 (inverted triangles[40]). Also when using the 30 min. 44°C treatment in combination with APC, a value (20.5 μg/ml APC: inverted triangles[40]) is found which is above the value for additivity (12.5 μg/ml APC). The less than additive effects of heat and APC as found for various combination treatments suggest that although DNA polymerase α is involved in repair of X-ray damage, hyperthermic inhibition of this repair cannot solely be explained by hyperthermic inactivation of DNA polymerase α.

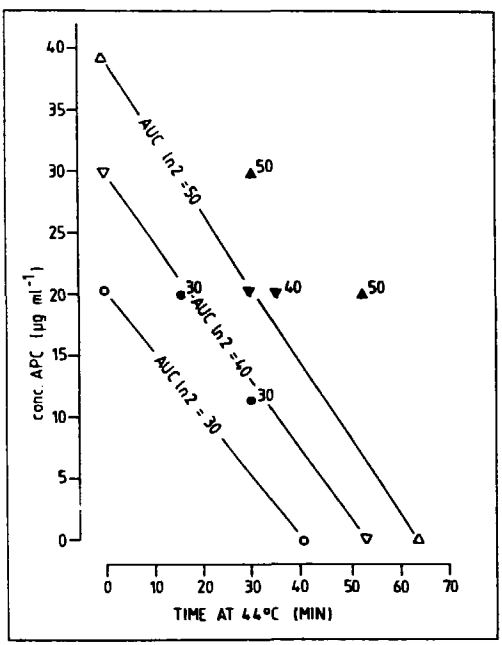

Figure 5: Graphic demonstration of non-additivity effects by heat and aphidicolin on DNA repair. Heat dose (time at 44°C) was plotted versus APC dose (μg/ml). Data of figure 4, were deduced to obtain AUC ln 2 values of 30, 40 and 50 respectively for the case that the agents were used separately to inhibit DNA repair. Between these values co-called "additivity lines" were drawn. In this figure the values representing the effect of the combined treatment (also derived from figure 4) are given in closed symbols (resp. 30 (circles), 40 (inverted triangles) and 50 (triangles) and may be compared to the additivity levels (lines) for the action of the agents alone.

In order to investigate whether hyperthermic inactivation of DNA polymerase α is involved at all in repair inhibition by heat we performed a different experiment. When we separated (δt) heat (30 min. 44°C) and X-irradiation (6 Gy) by post-heat incubations at 37°C, we observed recovery of repair upon longer post-heat incubations (figure 6). It may be deduced from this figure that when radiation is given directly after hyperthermia ($\delta t = 0$) most of the repair inhibition is caused by the heat treatment as such and a minor part by the direct inhibition of polymerase α by APC. The heat effect vanishes when δt is increased. After 8 hours the repair parameter (AUC ln 2) for heat alone was almost back to the value observed in the non-treated situation and the relative inhibitory action of APC is nearly maximal (almost the same as in the non-heated situation).

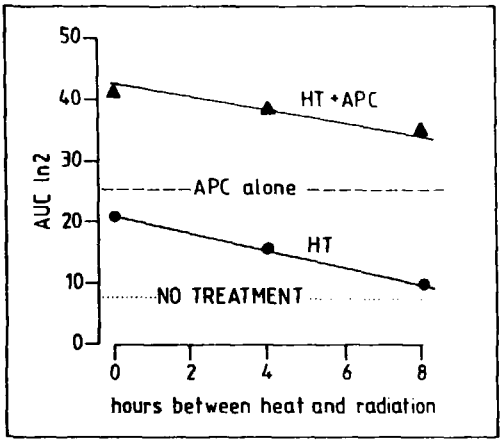

Figure 6: The effect of post-heat incubation at 37°C upon the "weighted mean half time of repair" (AUC ln 2) in APC (triangles) and non-APC (circles) treated cells. AUC ln 2 was plotted versus the time between heat (30 min. 44°C) and X-irradiation (6 Gy). The dotted line represents the AUC ln 2 value for untreated cells and the dashed line represents the AUC ln 2 value for cells treated with APC (20 µg/ml) only. Mean ± SEM of at least 2 experiments are given.

9.4 Discussion

9.4.1 Effect of heat alone on repair of X-ray induced DNA damage

The observed hyperthermic inhibition of repair of X-ray induced DNA strand breaks (figure 2a, table 1) is in accordance with previous reports in the literature (2-6,15). We found more or less the same qualitative inhibition kinetics as described earlier (15). Heat causes repair inhibition mainly by

altering the weight ratio and the α-rate of the fast component. This resulted in an increase in the weighted mean half time of repair, AUC ln 2 (figure 4, table 1).

9.4.2 Effect of aphidicolin alone on repair of X-ray induced DNA damage
We observed that APC could inhibit the repair of X-ray induced DNA strand breaks (figure 2b, table 1), which might be an indication for DNA polymerase α being involved in repair of this kind of DNA damage. However, an APC-concentration of 0.2 μg/ml that decreased 85% of DNA replication synthesis (figure 1), was not enough to inhibit the repair (figure 2b). Only concentrations above 2 μg/ml APC could inhibit repair. Comparison of figure 1 and 3 revealed that for both heat and APC repair inhibition occurred at higher doses as compared to inhibition of replicative synthesis. Iliakis and co-workers (35) concluded that the inhibitory effects of APC at these high concentrations might be due to non-specific effects rather than to inhibition of polymerase α. It has extensively been shown though that APC is a very specific DNA polymerase α inhibitor (17,18,20). These observations were extended in DNA-polymerase assays performed in our laboratories. In these experiments up to 50 μg/ml APC did not affect DNA polymerase β activity (measured as 1 mM NEM insensitive activity). In addition the APC concentration could only inhibit 85% of DNA polymerase α activity (1 mM NEM sensitive activity (11)). Furthermore there are at least 4 other explanations possible to explain the differences in APC sensitivity of replication and repair processes. Firstly, the amount of polymerase α necessary for repair is probably much smaller than the amount needed for replication. So even at high concentrations of APC some non inhibited polymerase α might still be available for the repair synthesis. Secondly, it is possible that DNA polymerase α might occur in different enzyme complexes (e.g. replitase) which may vary in function and APC sensitivity (36,37). In the third place, it was suggested by Mattern (38) that DNA polymerase α and β may substitute for each other in a number of pathways when one is inhibited. This might be the case for repair and not for replication, since polymerase β seems not involved in replication (17,18). In the fourth place, as suggested by Collins (39) replication might be carried out by the enzyme complex "replitase". APC might leave other components such as the ribonucleotide reductase enzyme (40) active thus augmenting the pool of free dNTP's. Augmentation of this pool, especially dCTP (which acts competitive to aphidicolin (19,20) could explain the less effective inhibition of aphidicolin of non-replitase- associated polymerase α, involved in repair. So, rather than ascribing the APC effects on DNA repair as non specific (35) we like to suggest with Waters et al. (25) and Lonn and Lonn (29) that DNA polymerase α is involved in the repair of X-ray induced DNA damage. The involvement of polymerase α in repair might not be determined by the repair patch size (41,42) which is thought to be small for X-irradiation. Rather the amount (32,33,43) or actual type of damage (44) might determine with polymerase (α or a) is involved in the repair process. Our experiments do not rule out the

possibility that DNA polymerase β is involved in this repair process too, either jointly or sequentially which polymerase α as was suggested by Keyse and Tyrrell (30) and Licastro (31). The mechanism of repair inhibition might be via an enlargement of the patch-sizes, as demonstrated for UV-repair[2] thus remaining patches unligated. This model of inhibition as discussed by Collins and co-workers (28,39) might also explain the observations of some investigators, especially those using HeLa S3 cells (21,22) who could not find an inhibitory effect of relatively high concentrations APC on unscheduled DNA synthesis after UV-irradiation.

9.4.3 The role of DNA polymerase α in hyperthermic repair inhibition

The differential inhibitory kinetics of heat and APC on DNA repair (figure 2a,b, table 1) as well as the less than additive effects as found for various combinations of these agents on repair (figure 4,5, table 2) show that the hyperthermic inactivation of DNA polymerase α (8,11,13-15,45) cannot solely be responsible for the inhibition of repair by heat. Other alterations in the cell must be involved. The less than additive effects cannot be explained by the possibility that APC reacts with polymerase α molecules that had been heat-inactivated ("double" inactivation of the same molecule). If this were the case we would not have observed the recovery effect on the inhibiting capacity of APC at increasing post heating times (figure 6). It has been reported by other workers (13,14) that DNA polymerase α activity was more or less constant during the first hours of post-heating incubations at 37°C (13,14). If this also holds for our cell line, it provides additional arguments for the conclusion that some other component after heat must have become rate limiting; this component recovers after the heat treatment, thus allowing DNA polymerase α becoming rate limiting again.

9.4.4 Enhanced binding of nuclear protein as possible cause for heat radiosensitization

The altered protein to DNA ratio in nuclei and chromatin from heated cells may possible account for the less than additive inhibitory effects of heat and APC as discussed above. Restricted DNA-accessibility (caused by heat-induced protein binding) for DNA repair enzymes such as polymerase α might become rate limiting when higher heat doses are applied. It is known from experiments of the group of Roti Roti (6,46) that reincubation at 37°C after heating cells resulted in restoration of normal protein to DNA ratios. This would implicate for our experiments where we separated heat and X-irradiation by incubation at 37°C, that repair capacity as well as the inhibiting

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[2] J.G. WALKER, J.P.H. TH'NG and D. LEE. The effect on inhibitors of DNA polymerase α on the size of the excision repair patch. Abstract in Proceedings of Meeting of the British Photobiology Society on the Molecular Biology of DNA repair (Manchester) Poster Abstract E19. 1986

effect of APC should restore upon post-heating times, which was exactly what we observed (figure 6).
In conclusion, inhibition of DNA polymerase α by aphidicolin is found to inhibit DNA repair under normal conditions. Though after heat treatment of cells, other cellular targets such as an impaired DNA accessibility or decreased enzymatic activity of DNA- repair enzymes (other than DNA polymerase α) have become a limiting factor in determining the mean repair rate of X-ray induced DNA damage.

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CHAPTER 10

INTERACTION OF HYPERTHERMIA AND RADIATION IN TOLERANT AND NONTOLERANT HeLa S3 CELLS. ROLE OF DNA POLYMERASE INACTIVATION

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SUMMARY

The activities of DNA polymerase ∝ and β were measured in tolerant and nontolerant HeLa S3 suspension cells. The heat-inactivation of the enzymes and their recovery when cells were incubated at 37°C after the heat challenge, was compared to the synergistic action of heat and radiation and its disappearance at the level of cell survival. Thermotolerant cells were radiosensitized by heat similarly to nontolerant cells, but the sensitization decreased more rapidly in the tolerant cells when time at 37°C was allowed between the two treatments. For polymerase activities the extent of inactivation as well as the kinetics of recovery were similar in tolerant and nontolerant cells. The results show that the activities of DNA polymerase ∝ and β do not always correlate with the extent of heat radiosensitization. It is concluded that heat inactivation of these enzymes may not be taken as a general cause for the synergistic effect of hyperthermia and radiation. As an alternative mechanism, changes in nuclear protein binding due to cellular heating are suggested, since these correlate well with effects observed for radiosensitization under different experimental conditions, including the use of thermotolerant cells.

10.1 Introduction

Heat and radiation act synergistically, probably because hyperthermia inhibits the repair of radiation-induced damage on the level of DNA (Ben Hur et al. 1974, Leeper 1985). Thermal inactivation of DNA repair enzymes, especially DNA polymerases α and β is postulated as a mechanism to explain radiosensitization (Leeper 1985). Although not conclusive, it is very likely that DNA polymerase α and β are involved in repair of damaged DNA (Fry and Loeb 1986). These enzymes are either acting independently, each dealing with specific types of DNA damage (Miller and Chinault 1982, Miller and Lui 1982) or sequentially and/or jointly on the same type of damage (Keyse and Tyrell 1985, Licastro et al. 1985). It might even be that both polymerases can substitute for each other in repair (Mattern 1985). Therefore it is of interest that heat partially inactivates DNA polymerase α and β (Spiro et al. 1982,) and that this might be related to heat radiosensitization (Jorritsma et al. 1985,1986, Kampinga et al. 1986, Mivechi and Dewey 1985; Chu and Dewey 1987,1988; Dikomey and Jung 1988). However, both step-up and step-down heating procedures of Ehrlich Ascites Tumor cells were experimental conditions that led to poor correlations between polymerase inactivation and thermal radiosensitization (Jorritsma et al. 1986). Furthermore polymerase α recovery after cellular heating did not parallel the disappearance of the synergism between heat and radiation (Spiro et al. 1982; Mivechi and Dewey 1985). For DNA polymerase β, recovery kinetics were to some extent similar to the pattern for heat radiosensitization. However, for DNA polymerase β activities a recovery in 24 hours to value above control (110-140%) was found, whereas at this time point the cells still have enhanced radiosensitivity (Spiro et al. 1982; Mivechi and Dewey 1985). These observations cast doubt on the generality of DNA polymerase inactivation being the determining step in heat radiosensitization. In the current study we tried to establish more definitely whether or not DNA polymerase inactivation by heat can be held responsible for thermal radiosensitization. We, therefore, investigated the effect of thermotolerance on heat radiosensitization in HeLa S3 cells. Radiation was given directly after hyperthermia or after various intervals at 37^{o}C to allow the cells to recover from heat damage. The extent of radiosensitization was compared with the extent of DNA polymerase α and β activity at the various time points after cellular heating.

As an alternative approach, we also investigated the role of heat induced alteration of the nuclear structure in hyperthermic radiosensitization, as revealed by increase in the protein content of nuclei isolated from heated cells. Nuclear protein content in thermotolerant and nontolerant cells was analyzed after various post heating periods. Earlier data (Kampinga et al. 1987) already showed that changes in nuclear protein mass were not different for tolerant and nontolerant cells directly after the cellular heating, but that in tolerant cells recovery to a normal protein content was enhanced.

10.2 Materials and Methods

10.2.1 Cell culturing, hyperthermia and X-irradiation

HeLa S3 cells (ATCC no. CCL 2.2, Flow 03-157, Irvine, Scotland) were grown asynchronously in suspension cultures in Joklik-MEM (Flow, Irvine, Scotland) supplemented with 10% foetal calf serum (Hyclone, Logan, Utah). Exponentially growing cells (doubling time about 26 hours) were used in all experiments. For heating, cells were harvested by centrifugation (5 min 260g) and resuspended in prewarmed medium of the desired temperature in a concentration of 5.10^6cells/ml. After heating, samples were taken for X-irradiation immediately (interval < 5min) to determine cell survival (clonogenic assay on soft agar: Kampinga et al. 1985) as well as for DNA polymerase and nuclear protein determinations. For recovery studies, the heated cells were 5-fold diluted in 37°C-medium and incubated up to 6 hours at 37°C. After these various time points, again cells were irradiated and assayed for their clonogenic ability; at the same time points samples were taken for polymerase- and nuclear protein determinations. Always extra samples were taken for unheated tolerant and nontolerant controls. Thermotolerance was induced by heating the cells (5.10^6/ml) for 15 min at 44°C. After 5-fold dilution in fresh 37°C-medium the cells were incubated for 5h at 37°C before the test heat dose was given. The pH was held at 7.4 under all experimental conditions. Hyperthermia was performed in precision water baths (±0.05°C) X-irradiation took place with a Phillips-Muller MG 300 machine operating at 200 kV and 15 mA. X-rays were filtered with 0.5mm Cu and 0.5mm Al. The dose rate was 6 Gy/min. During irradiation the cells were kept on ice.

10.2.3 Determination of cellular DNA polymerase α and β activity

The activity of DNA polymerase α and β was assayed according to the principals described before (Kampinga et al. 1985). Shortly, cells were centrifuged and washed in phosphate-buffered saline (PBS) and resuspended (2.10^7 cells/ml) in a 10 mM Tris/10 mM NaCl/1.5 mM $MgCl_2$ buffer (pH 7.5). After disruption of the cells by sonication, the polymerases were assayed for their ability to incorporate ^{3}H-dTTP onto exogenous added activated salmon sperm DNA (Schlabach et al. 1971). For the assay of DNA polymerase β, N-ethylmaleimide (NEM) was used to a final concentration in the assay of 1 mM, to inhibit DNA polymerase α activity (Kornberg 1982). Polymerase α activity was calculated by subtracting the NEM-resistant (β) activity from the total activity. Aliquots of all samples were taken for protein determination (Lowry et al. 1951) and activities were always expressed per mg protein. The results are plotted as the fractional activity as compared to the activity of unheated nontolerant control cells.

10.2.4 Isolation and staining of cell nuclei flow cytometric analysis

Nuclei were isolated according to the method of Blair et al. (1979). This method is based on washing the cells in phosphate buffered saline (PBS; three times), followed by washing two times in a detergent solution (1% Triton X-100; 0.08 M NaCl; 0.01 M EDTA; pH 7.2) and washing once in TNMP (10 mM Tris-base; 10 mM NaCl; 5 mM $MgCl_2$; 0.1 mM PMSF; pH 7.4). All procedures were done on ice. After staining the nuclei (1.10^7/ml) with fluorescein isothiocyanate (FITC; 30 μg/ml) and propidium iodide (PI; 35 μg/ml) overnight, they were analyzed on a FACS 440 (Becton Dickinson(BD), Mountain View, CA) equipped with an argon laser emitting 300 mW at 488 nm. Green and red fluorescence signals were separated with a DM 560 (BD and collected using a 530/30 BP (FITC (BD)) and a 630/20 BP (PI (BD)) respectively. Five thousand events were collected using linear amplification, without compensation. The relative nuclear protein content was analyzed by computing the mean

of the FITC fluorescence distribution (using Consort 30 (BD) software) and plotting the mean of the treated samples relative to the mean FITC fluorescence of nuclei from control cells (Blair et al. 1979, Roti Roti et al. 1982, Roti Roti et al. 1986 heat). Cell cycle analysis was done with cell cycle analysis software (BD) using the polynomial model.

10.3 Results

When HeLa cells are treated for 15 min at 44°C followed by a 5h incubation at 37°C after the heat challenge, they developed thermotolerance for a subsequent heat challenge at 44°C (figure 1). For the purpose of our investigations, these tolerant and nontolerant cells were X-irradiated either immediately after the heat challenge or after various post-heat incubations at 37°C. The Thermal Enhancement Ratios (TERs) for the various treatments were calculated as:

$$TER = \frac{\text{survival after 4 Gy of the control cells}}{\text{survival after 4 Gy of the treated cells}}$$

It can be seen that the tolerant cells, that are not heated again still have an enhanced radiosensitivity (figure 2: , TER = 1.9 ± 0.2). Directly after a heat treatment of 30 min at 44°C the extent of radiosensitization (TER) was not significantly different for tolerant and nontolerant HeLa cells (figure 2) which is in accordance with earlier observations from our department (Jorritsma et al. 1985).

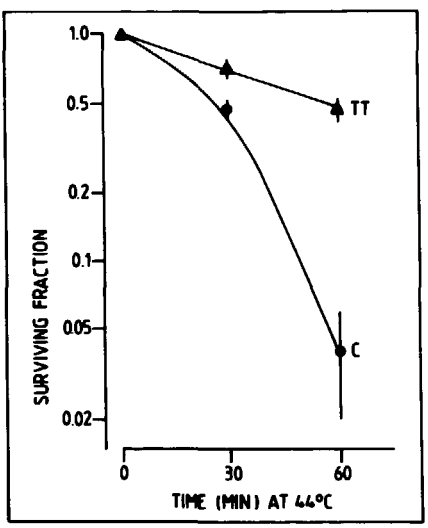

Figure 1: Effect of hyperthermia (44°C) on cellular survival of control (C) and thermotolerant (TT) HeLa S3 cells. Thermotolerance was induced by a 15 minutes at 44°C plus 5 hours at 37°C pretreatment. (mean ± s.e.m.; n=4)

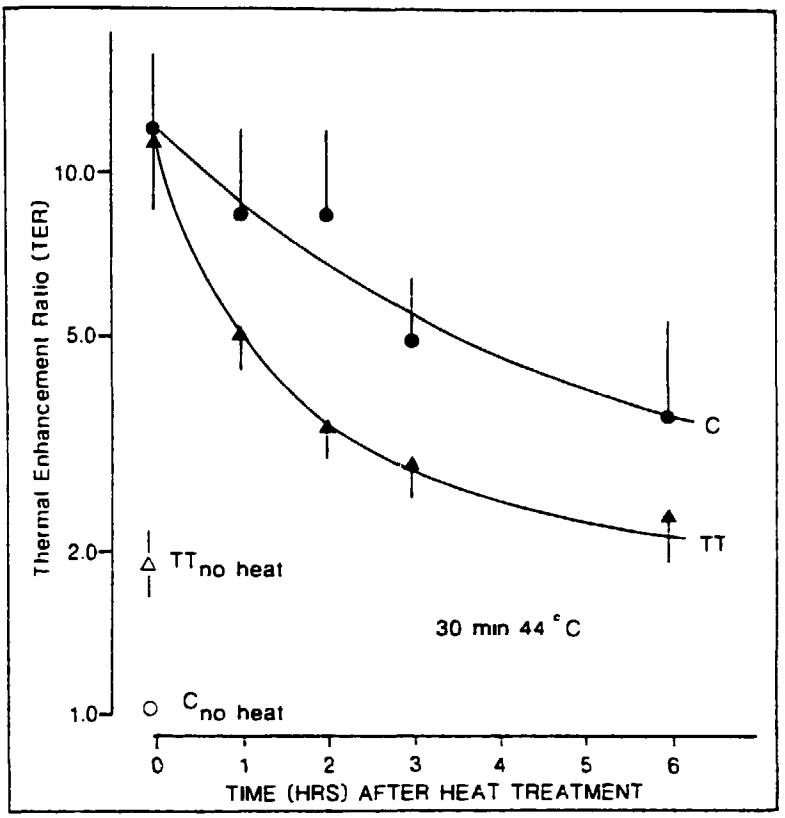

Figure 2: Effect of hyperthermia (30 min. 44°C) and post hyperthermic incubations at 37°C on radiation (4 Gy) sensitivity of control (C) and thermotolerant (TT) HeLa S3 cells. Thermal radiosensitization (TER) for each time point is expressed as the mean TER obtained from the TERs of each individual experiment.

$$TER = \frac{\text{survival after 4 Gy of control cells}}{\text{survival after 4 Gy of treated cells}}$$

The TER-value for unheated tolerant cells (open triangle) is 1.9 ± 0.2 (mean ± s.e.m.; n=4)

When the cells were allowed to recover from heat damage, the synergistic action between heat and radiation disappeared more rapidly in thermotolerant cells as compared to the nontolerant cells as can also be seen in figure 2. This effect can not be attributed to cell cycle redistributions since no major changes were observed during the time span of the experiment (figure 3). Only small changes in the fraction of G_1 and S-phase cells were observed 6 hours after heating the cells, the effect being slightly more pronounced in the tolerant cells.

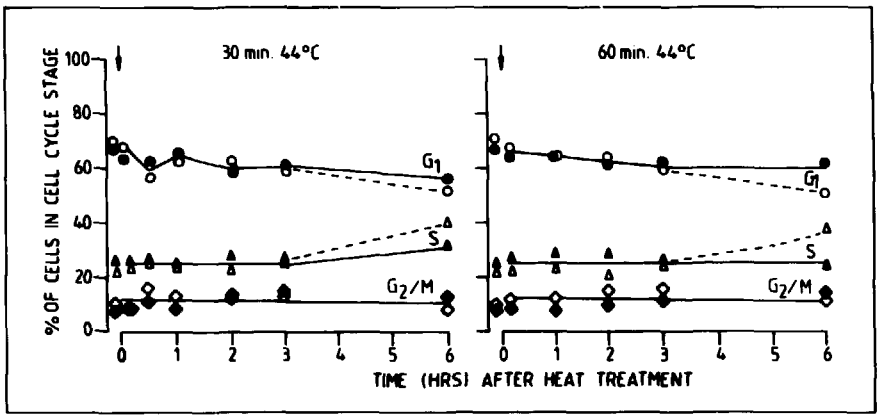

Figure 3: Effect of hyperthermia and post hyperthermic incubations at 37°C on cell cycle distribution of control (C, closed symbols) and thermotolerant (TT, open symbols) Hela S3 cells. Left panel: 30 min. 44°C; right panel: 60 min. 44°C. Arrow indicates heat treatment. (circles) % cells in G_1; (triangles) % cells in S; (diamonds) % cells in G_2/M

If DNA polymerase inactivation is the determining process in heat radiosensitization, the extent of hyperthermic inactivation of these enzymes should be the same in tolerant and nontolerant cells directly after the heat treatment and restoration of activity should be enhanced in the tolerant cells. In figure 4, it can be seen that both DNA polymerase α (figure 4A,B) and -β (figure 4C,D) are inactivated similarly in tolerant and nontolerant cells directly after a 30 (figure 4A,C) or 60 (figure 4B,D) min 44°C heat treatment and that both α and -β activities recover with similar kinetics in tolerant and nontolerant cells upon post-heating times at 37°C (figure 4). So, the recovery of DNA polymerase activity after heat treatment of tolerant and nontolerant cells does not parallel the decrease in heat radio- sensitization (figure 2). Besides hyperthermic inactivation of DNA repair enzymes as a cause for thermal radiosensitization, it has been suggested (Warters and Roti Roti 1979, Jorritsma et al. 1986, Kampinga et al. 1988) that heat causes structural alterations of the chromatin organization that might render the damaged DNA less accessible for repair. This change in chromatin organization seems related to an increase in the protein content of nuclei isolated from heated cells. It therefore seemed worthwhile to investigate the changes in nuclear protein binding in relation to thermal radiosensitization in tolerant and nontolerant HeLa cells. Earlier data from our laboratory (Kampinga et al. 1987) had indicated that tolerant HeLa cells showed similar

increase in nuclear protein mass as compared to their nontolerant counterparts after heating at 45°C and that these tolerant cells recovered faster from heat-induced damage. As can be seen in figure 5 a similar heat-dose dependent increase of nuclear protein was observed for tolerant and non-tolerant cells when measured directly after cellular heating (30 or 60 minutes at 44°C). However in the tolerant cells the recovery to normal protein content was enhanced, in accordance with the enhanced recovery of thermal radiosensitization in tolerant cells (figure 2).

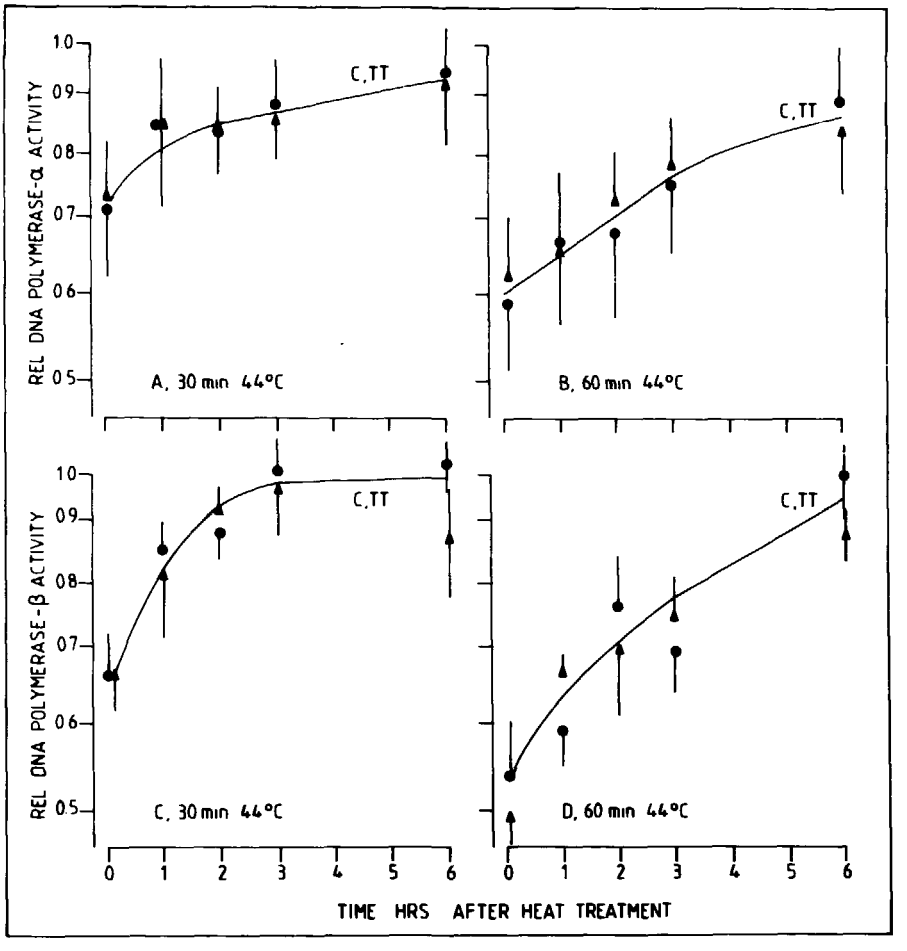

Figure 4: Effect of hyperthermia and post hyperthermic incubations at 37°C on DNA polymerase α (A,B) and β(C,D) inactivation and recovery in control (C, circles) and thermotolerant (TT, triangles) cells.
Panel A,C: 30 min. 44°C; panel B,D: 60 min. 44°C. DNA polymerase activities are plotted relative to unheated samples (C = 1.0 and TT = 1.0 ± 0.05 for both α and β polymerase activity) (mean ± s.e.m.; n=4)

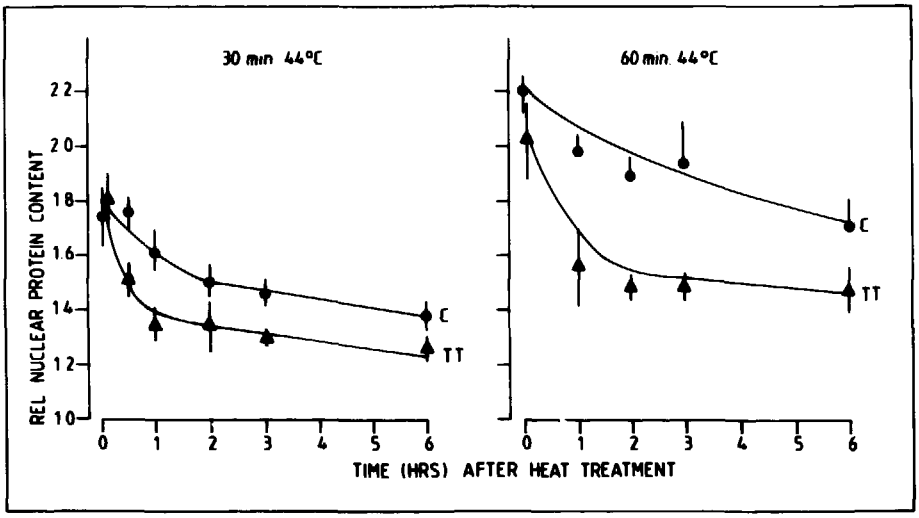

Figure 5: Effect of hyperthermia and post hyperthermic incubations on nuclear protein mass and recovery in control (C) and thermotolerant (TT) HeLa S3 cells. Left panel: 30 min. 44°C; right panel: 60 min. 44°C. Nuclear protein mass is plotted relative to unheated samples (C = 1.0 and TT = 1.12 ± 0.06) (mean ± s.e.m.; n=3)

10.4 Discussion

10.4.1 Heat radiosensitization: the effect of thermotolerance

The effect of thermotolerance on thermal radiosensitization has been rather contradictory (Konings 1987). Although some reports have shown that thermotolerant cells are less sensitized by heat for radiation in comparison to their nontolerant counterparts (Henle et al. 1979, Raaphorst and Azzam 1983, Haveman 1983, van Rijn 1984, Holahan et al. 1986), this was not observed by others (Nielsen 1983, Hartson- Eaton et al. 1984, Jorritsma et al. 1985, Majima et al. 1985). In our experiments no effect of tolerance was found for heat radiosensitization when the radiation treatment immediately followed hyperthermia (figure 2). This is in accordance with earlier findings for HeLa S3 cells (Jorritsma et al. 1985). In the tolerant cells however, this synergistic action of heat and radiation disappeared more rapidly than in nontolerant cells when the treatments were separated by incubation at 37°C. For comparison of the extent of heat radiosensitization in tolerant and nontolerant, it therefore seems of essential importance to keep the interval between heat and radiation as short as possible. Inconsistency of working conditions, cell line dependent differences, and/or possible cell cycle effects

in the various cell lines, might be responsible for the differences observed for the effect of thermotolerance on heat radiosensitization. Our data were not affected by major cell cycle disturbances (figure 3). Only a small change is seen 6 hrs after the heat treatment. Since the differences on radiosensitization were already present 1-3 hours after the heat dose, a time period during which no cell cycle changes were observed in both tolerant and nontolerant cells, cell cycle disturbances can be excluded as an explanation for our observations.

10.4.2 Role of DNA polymerase inactivation in heat radiosensitization

DNA polymerases are inactivated to a similar extent in tolerant and nontolerant cells when measured directly after the heat shock, which correlates with heat radiosensitization as observed earlier (Jorritsma et al. 1985). However, the enzymes recover with the same kinetics in tolerant and nontolerant cells, while heat radiosensitization recovers faster in the tolerant cells. Since DNA polymerases are known to fluctuate during the cell cycle (Kornberg 1982; Mivechi and Dewey 1985) it was important to be sure that our data were not affected by cell cycle redistributions, which was found not to be the case (figure 3). Also it can be seen that unheated tolerant cells were still more sensitive to radiation than control cells (TER = 1.9) while DNA polymerase activities of unheated tolerant and nontolerant cells were the same, similar to data found earlier for HeLa cells (Jorritsma et al. 1985) and Ehrlich Ascites tumor cells (Jorritsma et al. 1986). This in itself is already a prove for the non-correlation of both parameters. Since the detectibility of changes in DNA polymerase activities seems less sensitive than that for differences in radiosensitivity, the recovery data for tolerant and nontolerant cells (using higher heat doses to clearly inactivate the polymerase activities) are more conclusive. On the basis of these results it must be concluded that DNA polymerase α and β inactivation by heat cannot be solely responsible for heat radiosensitization in HeLa S3 cells. For DNA polymerase α inactivation this conclusion was already indicated before (Spiro et al. 1982, Kampinga and Konings 1987). Most investigators have suggested good correlations between polymerase β inactivation and heat radiosensitization (Spiro et al. 1982, Jorritsma et al. 1985, Mivechi and Dewey 1985, Chu and Dewey 1987, 1988, Dikomey and Jung 1988), although comparing these parameters in Ehrlich Ascites Tumor cells revealed a far less good correlation (Jorritsma et al. 1986). Often however these relations were not quantitated. Quantitative analysis and comparison of the extent of heat radiosensitization in CHO cells after heating in the presence of glycerol (Mivechi & Dewey 1985) or cycloheximide (Chu and Dewey 1987), after pretreatment with arsenite (Mivechi and Dewey 1985) or after low pH (Chu and Dewey 1988) with the extent of DNA polymerase inactivation under the same conditions revealed a poor correlation. The correlation coefficient relating the thermal enhancement ratios (based on the Do of the radiation survival curves) and polymerase activities after combining all the data (same cell line, same polymerase assay, same laboratory) were as low as 0.61 for polymerase

∝ and 0.30 for polymerase β. So, in accordance with doubts expressed earlier (Jorritsma et al. 1986), our data presented here more definitively show that it is justified to state the general conclusion, that heat inactivation of total cellular DNA polymerase activities is not the cause of heat radio-sensitization.

10.4.3 Changes in nuclear protein binding and heat radiosensitization

The present report shows that alterations in nuclear protein binding seems to correspond with differences in heat radiosensitization. This relation also holds for cells in a thermotolerant state. The relative protein content at the 6 hour point seems somewhat high in relation to the TERs at this time point (figure 2). The small increase in S-phase cells and decrease in G_1 cells at this time point (figure 3) may in part account for this, since it is known that S-phase cell nuclei have about 1.4 fold more nuclear protein than G_1 nuclei as revealed by FITC distributions (Roti Roti et al. 1982). Alterations in nuclear (matrix) protein mass have already previously been suggested to be related to hyperthermic inhibition of repair (Warters and Roti Roti 1979, Mills and Meyn 1981, Warters et al. 1987, Kampinga et al. 1988). A decreased accessibility of radiation induced DNA damage for repair enzymes is indicated by results of recent studies (Kampinga et al. 1988), using the fluorescent halo assay (Roti Roti and Wright 1987). Alternatively, it is possible that heat treatment of cells leads to binding of repair enzymes to sites in the nucleus so that they cannot be functional at the sites of damage in the DNA. An indication for this stems from earlier studies of our group (Kampinga et al. 1985), where it was shown that (in spite of cellular inactivation) DNA polymerase activities in nuclei isolated from heated cells was higher as compared to the activities in nuclei from non heated cells. So, the heat-induced increase of total protein binding in the nucleus goes along with enhanced binding of enzyme activity.

In summary, it is concluded that heat-inactivation of <u>total</u> cellular DNA polymerase ∝ and β activities as measured as incorporation on <u>exogenously</u> added gapped DNA can <u>not</u> be <u>solely</u> responsible for heat radio- sensitization. No information so far exists about <u>functional</u> activities on the <u>endogenous</u> (damaged) DNA. Changes in nuclear protein binding might either have changed the accessibility of the damaged DNA (e.g. for DNA polymerases) and/or have caused a fixation of repair enzymes (again maybe polymerases) that therefor cannot repair the damaged DNA. Moreover the thermal radiosensitization might be a complicated function of both DNA accessibility and DNA repair enzyme activities.

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CHAPTER 11

THE INTERACTION OF HEAT AND RADIATION AFFECTING THE ABILITY OF NUCLEAR DNA TO UNDERGO SUPERCOILING CHANGES

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SUMMARY

DNA damage (putatively strand breaks) from ionizing radiation inhibits the ability of intercalating dyes to induce right-handed supercoils in the DNA loops of HeLa nucleoids (Cook and Brazelle, 1976, Roti Roti and Wright, 1987) while heat-induced changes in the nuclear matrix enhance this ability (Roti Roti and Painter, 1982). Since heat and radiation interact synergistically or additively on most cellular functions which they affect, the rewinding of DNA supercoils is unusual in that these agents alone affect it in an antagonistic manner. When HeLa cells were exposed to 45°C for 30 min and immediately irradiated with 10 Gy of ^{137} Cs-γ-rays, the rewinding response was intermediate between that for cells which had been exposed to to 10 Gy only and control. When repair of this damage was assayed in control cells, 97% of the initial damage had been repaired at 30 min post irradiation, at the same time only 10% of the initial damage had been repaired in the heat-shocked cells. This apparent dose reduction effect and the inhibition of repair were interpreted to indicate that heat-induced changes in nuclear structure were masking DNA damage from the assay and the repair system. These effects correlated with the amount of heat-induced excess protein associated with the nucleus and the nucleoid.

11.1 Introduction

One of the cellular effects of heat which has contributed to the rationale for the clinical use of hyperthermia is that heat plus radiation is more effective in cell killing than either agent alone. By and large hyperthermic treatment (at temperatures 41-46^{o}) does not significantly alter the amount of initial DNA damage induced by radiation (see Discussion). However, hyperthermic treatment greatly inhibits the repair of radiation-induced DNA damage. Using alkaline unwinding (4) and alkaline elution (5,6) it was shown that the rate of repair of alkali labile sites (predominately single strand breaks, ssb) was inhibited by heat. Also "double strand break (dsb) repair", measured by "neutral" filter elution (7) was inhibited as well as the repair of DNA-protein crosslinks (8). Furthermore, it was shown that excision of 5'-6'-dihydroxyhydrothymine (t') was inhibited by heat (9). Thus, it is clear that repair inhibition is a major component of heat radiosensitization. The cause for repair inhibition, however, remains unclear. Two possible mechanisms can be considered for the heat-induced inhibition of DNA repair, 1) inactivation of repair enzymes, and 2) reduced access of the enzymes to the damaged sites. DNA polymerase (α and β) inactivation by heat was shown to correlate with heat radiosensitization (10, 11, 12) but not under all conditions (13). DNA polymerase α inactivation by heat certainly cannot solely explain hyperthermic repair inhibition (14) and/or heat radiosensitization (13,15). The inactivation of repair enzymes involved in excision of t' type damage could not explain heat-induced repair inhibition (9). In the latter study it was shown that the observed repair inhibition was due to an alteration in chromatin structure and it correlated with an increase in protein content of chromatin. In the current study we have obtained results consistent with the idea that inhibition of DNA repair by hyperthermia may be due to masking of the damaged sites. However, a related, but different question provided the central motivation for the current study.

In the study presented here, we used a DNA supercoiling assay as described by Roti Roti and Wright (2) which was based on the method of Vogelstein et al (16) for detecting hyperthermic action on repair of X-ray induced DNA damage. The assay allowed the measurement of repair in individual unlabelled cells. The method involves the visualization of DNA loops, titrated with the fluorescent intercalating dye, propidium iodide (PI). DNA damage from ionizing radiation inhibits the ability of intercalating dyes to induce right handed supercoils in DNA loops (2) while hyperthermic damage enhances this ability (3,17). We believed it useful to investigate the effects of the two agents together on this process. Heat and radiation interact synergistically or additively on most cellular processes. However, the rewinding of DNA supercoils is an unusual process in that these agents act antagonistically. It was, therefore, of interest to determine which, if either, of these effects predominate.

11.2 Materials and methods

HeLa S3 cells were maintained in exponential growth in suspension culture (doubling time 17-22 hrs) by daily subculturing in Joklik-MEM (GIBCO, Grand Island, NY) supplemented with 3.5% calf and 3.5% fetal bovine serum (GIBCO). For heat treatment, cells were sedimented for 5 min at 150 x g and resuspended in prewarmed 45°C complete medium. Cells were maintained at 45°C in a precision controlled water bath regulated to ± 0.1°C. Thereafter heated cells and control cells were 5-fold diluted in 37°C to allow repair.

The fluorescent halo assay was performed as described earlier (2,17). Briefly, cells were washed and resuspended in Eagle's spinner salt solution (2.0 M NaCl, 10 mM EDTA,2 mM Tris pH 8, 0.5% Triton x100, plus various concentrations of PI). After 45 min. of lysis the resulting "fluorescent halos" (nuclear core plus extending DNA loops) were measured using a Model 3000 Image Analyzer (Image Technology, Corp., New York) (17). The images were visualized via a SIT TV camera and monitor and analyzed by the IBM PC based image analysis system. Exciting light intensity was set for each specific PI concentration to compensate for the variable fluorescence intensity resulting from different dye concentrations. The same setting used for each experimental condition. Background light emission was measured and automatically subtracted during measurements. The threshold (grey level) was set either high for overall halo or low for core measurement. Each field measured was selected for uniformity of focus. The system was programmed to select all of the image pixels above the grey level setting and measure the diameters of the ensuing shapes. Size calibration was performed using a stage micrometer. The image analysis system measurements were standardized against measurement of photographic images of the nucleoids (2); which in turn had been calibrated against ocular micrometer measurements. Upon addition of increasing PI concentration the endogenous left-handed DNA supercoiled domains start to unwind. After full extension (relaxation point) rewinding starts and the DNA rewinds into right-handed, supercoiled domains. These processes are dependent on the topological constraints on the DNA loop, providing the basis for the use of the assay to detect DNA damage and its repair.

To study the associated proteins, nucleoids were prepared as described above by dilution of cells with lysis buffer except that lysis was done in 15 ml Corex centrifuge tubes and after 45 min of lysis sedimented at 10,000 rpm in a Beckman 2JB with a JS-13 rotor. The resulting pellet was dissolved in TNMP (10 mM Tris pH 7.4, 5 mM $MgCl_2$, 10 mM NaCl, 0.1 mM PMSF) and prepared for gel electrophoresis by digestion with 25 μg/ml electrophoretically pure DNaseI (Cooper Biomedical) at room temperature for 1 hour or until the pellets became dispersed. An equal volume of SDS sample buffer (125 mM Tris, pH 6.8, 2.0 M Glycerol, 100 mM DTT, 7 mM SDS) was then added and the samples boiled for 5 min. SDS-polyacrylamide (12.5%) electrophoresis was performed by the method of Laemmli et al (18). The resultant Comassie blue stained electrophoretograms were scanned using a LKB 2202 Ultrascan laser densitometer.

Colorimetric immunodetection of nucleoid proteins immobilized on nitrocellulose membranes was performed as described previously (Towbin et al, 1979) (19). Briefly, nucleoid proteins separated by SDS=PAGE (see above) were electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell, Keene, NH) (20) and probed with antisera detected against mouse mastocytoma HSP70 (Ohtsuka et al, 1986). Immune complexes were detected using immunoperoxidase techniques (Hawkes et al, 1982) (21) using 4-chloro-naphtol (Sigma, St. Louis, MO) as a reaction substrate.

11.3 Results

To investigate the ability of DNA to undergo supercoiling changes we have employed image analysis and the fluorescent halo method. Cells are lysed in the presence of Triton X100, 1 M NaCl and varying concentrations of PI. As the DNA unwinds with increasing PI concentration, the diameter of the fluorescent halo around the nuclear lamina increases until the DNA is relaxed fully. As the PI concentration continues to increase, the DNA rewinds and the fluorescent halo diameter decreases (see figure 1). Using a semi-automated image analysis system, (see Materials and Methods) 4 diameters per nucleoid were measured per nucleoid. Approximately 100 nucleoids were analyzed and averaged per experimental point and all experiments were repeated 3-4 times with the experimental variation expressed as the standard error of the mean. (SEM). Each sample was coded prior to measurement so that the image analysis machine operator did not know the experimental history of any given sample.

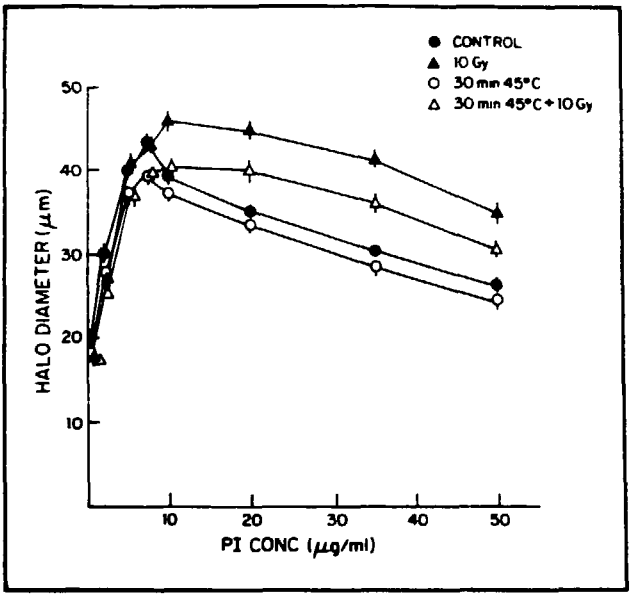

Figure 1: The effect of Hyperthermia and Irradiation on the Ability of DNA Loops in Nucleoids to Undergo Supercoiling Changes. HeLa cells were exposed to hyperthermia (45°C for 30 min) and irradiated with 10 Gy of ^{137}Cs-γ-rays. Aliquots of the cell culture were given either or both treatments as indicated on the figure. The resulting ability of DNA to undergo supercoiling changes was analyzed by the fluorescent halo method after 45°C min lysis. Overall nucleoid diameter was plotted as a function of PI concentration. The plotted points represent the mean of 3 experiments and the bars represent one SEM.

When HeLa cells were irradiated with 10 Gy and then assayed for the ability of their DNA to undergo supercoiling changes in the presence of PI, the ability of the DNA to rewind was inhibited while DNA unwinding was unaffected (Figure 1). If cells were heated at 45°C for 30 min prior to irradiation the ability of the DNA to be rewound was enhanced in nucleoids from both non-irradiated and irradiated heated cells. Rewinding was enhanced to a greater extent in the nucleoids from irradiated cells relative to those from control. To quantify the above effects as a function of radiation dose and eventually repair time, we used the arbitrary parameter of excess halo diameter which was computed by adding the halo diameters measured at 10, 20, 35 and 50 μg/ml PI for the nucleoids from the irradiated cells and subtracting the equivalent sum for the control (2). When this procedure was done for radiation doses up to 10 Gy a multiphasic dose response function was observed (figure 2). When cells had been heated prior to irradiation a reduction in the excess halo diameter was observed at all of the doses studied (figure 2). Thus, the effect of heating is apparently a reduction in radiation-induced damage. However, numerous previous observations and considerations (see Discussion) and the remaining experiments in this study suggest that the effect of heat is to mask a fraction of the DNA damage from the assay and form the repair system (described below).

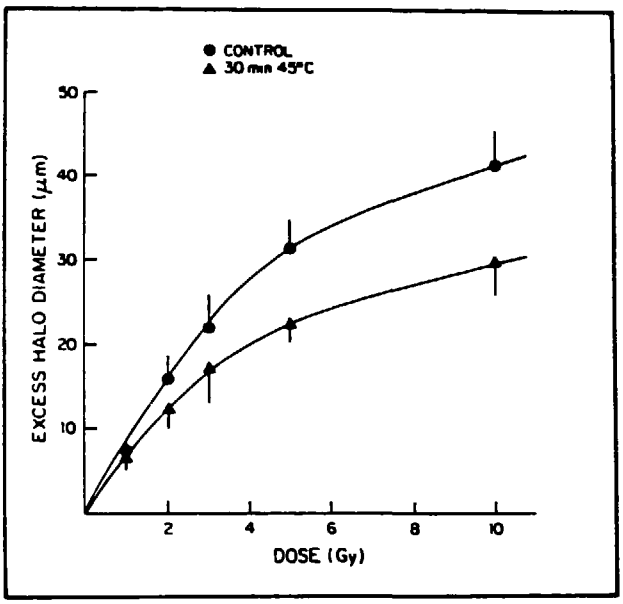

Figure 2: *The Dose Response for the Radiation-Induced Inhibition of DNA Rewinding. The excess halo diameter (as described in the text) is used as a measure of the extent of radiation-induced inhibition of the ability of PI to rewind DNA loops (i.e. the loss of topological constraints). This parameter is plotted as a function of γ-ray dose for both control and cells exposed to 45°C for 30 min. The plotted points represent the mean of 3 separate experiments and the bars are ± one SEM.*

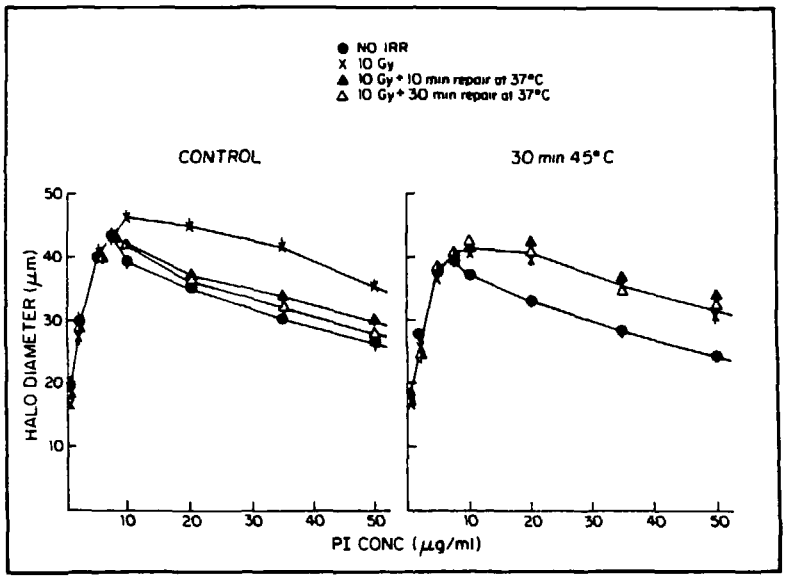

Figure 3: The Effects of Hyperthermia on the Post-Irradiation Restoration of the Ability to Rewind DNA loops. Hela cells were exposed to 45°C for 30 min (right panel) or maintained at 37°C (left panel) and then irradiated with 10 Gy of ^{137}Cs-γ-rays. Cells were analyzed for DNA supercoiling changes immediately after irradiation or after 10 or 30 min of post-irradiation incubation at 37°C as indicated in the figure. Each point represents the mean of 3 separate experiments and the bars are one SEM.

When cells were reincubated at 37°C after irradiation, they were able to restore the ability to rewind DNA supercoils within 10 min of post-irradiation incubation (figure 3A). However, cells that had been heated 30 min of post-irradiation incubation only a slight restoration of the ability to rewind DNA supercoils was observed (figure 3B). Our hypothesis was that these observed heat effects were due to increased protein content in the nucleoids, as was observed for inhibition of t' repair in chromatin from heated cells (9). Increased protein content has been observed in nuclei (22-24) and nuclear matrices (25-26) which form the core of the nucleoid. We therefore isolated nucleoids from control and heated cells immediately after heat exposure of 45°C for 30 min which increased the nuclear protein content 1.72 ± 0.10 fold above control as measured by the method of Blair et al (25). As can be seen in figure 4, there is an increase in protein content of nucleoids from heated cells as seen by the increased staining of many bands, in particular those above 60 kD molecular weight. Among others a subset of proteins in the 68-73 kD range was increased; Western blot

analysis with an antibody to HSP72/73 demonstrated the presence of the HSP 72/73 antigen in this band. Upon post-heat incubation at 37°C the protein content of the nucleoids was returned to near control levels. Less protein was found in nucleoids from heated cells at 3 hrs after heat and by 6 hours the nucleoids had almost the same polypeptide pattern a those from unheated cells except that they were enriched in HSP72/73 (figure 4). The relative nuclear protein content was 1.43 ± 0.10 at 3 hr and 1.02 ± 0.02 at 6 hr showing nuclear protein content had returned to near control levels after 6 hr of recovery in parallel to observed changes in protein composition of the nucleoid.

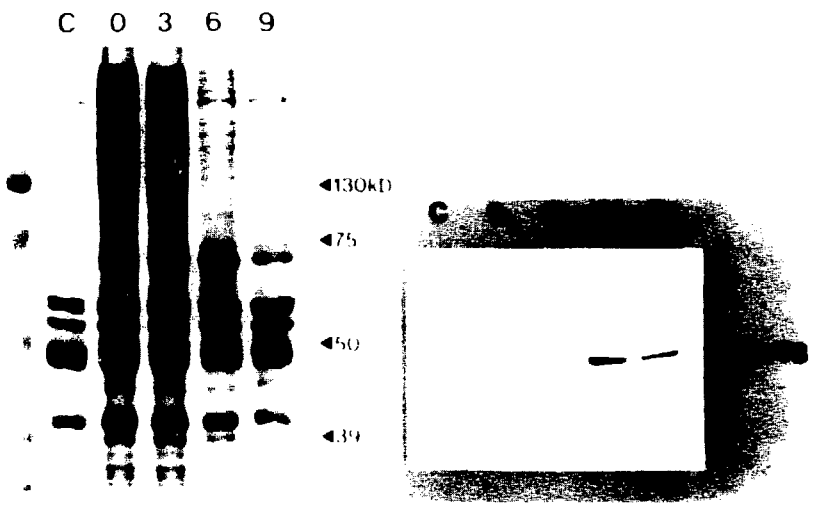

Figure 4: One Dimensional Polyacrylamide Gel Electrophoretograms and Western Blots of the Proteins Associated with Nucleoids at Various Time Intervals After Hyperthermia (45°C, 30 min) nucleoids were prepared from the treated HeLa cells and proteins recovered for electrophoretic analysis as described (see Materials and Methods). In the left panel from left to right the lanes show the polypeptides associated with nucleoids form control cells; (c) cells immediately after hyperthermia, (0); and cells at 3, 6 and 9 hours after hyperthermia (indicated as 3, 6 and 9, respectively). Lanes are loaded with protein from equal numbers of nucleoids (5×10^5). Molecular weight standards are shown in the left most lane. The right hand panel shows the western analysis of this electrophoretogram (see Materials and Methods) using a polyclonal antibody to HeLa HSP 72/73 (21). Only the 72/73 band demonstrated binding with the antibody. The lanes are identified in the same manner as in the left panel.

If the increased protein content of nucleoids was indeed responsible for the observed effects (masking radiation damage and repair inhibition) it would be expected that upon post-heat incubation these effects should disappear. In figure 5, it can be seen, using the excess halo diameter at and above 10 μg PI/ml as a measure, that the rate of repair recovered to near control levels within 6 hours post-heating times (figure 5A) and that the heat-masking effects also disappeared (fig. 5B). The rate of recovery of these effects paralleled the removal of excess nuclear (described above) and nucleoid protein (figure 4).

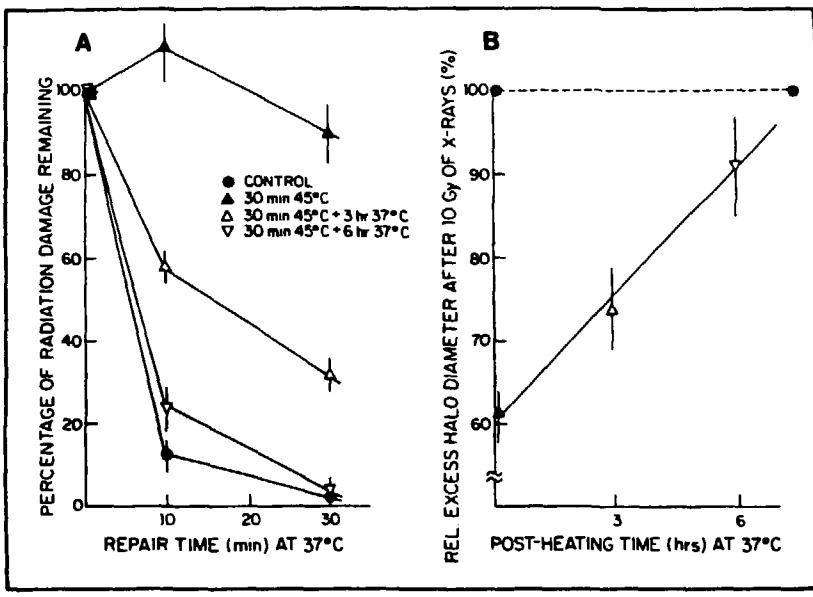

Figure 5: The Effect of Post-Hyperthermia Incubation on the Heat-Induced Inhibition of Post-Irradiation Repair and Masking of Radiation Induced Damage. Panel A shows the effects of heat and post-heat incubation at 37°C on the ability of cells to repair the radiation damage that results in inhibition of the ability to rewind DNA supercoils. The percent of initial damage remaining (ordinate) is plotted against post-heat incubation on the masking of radiation-induced DNA damage. The relative excess halo diameter after 10 Gy as a percent of the unheated, but irradiated cells (ordinate) is plotted against post-heat incubation time. The various experimental conditions are indicated on the figure and the symbols apply to both panels. For both panels the plotted points represent the mean of 3 separate experiments and the bars are one SEM. The abscissa for panel A is time after irradiation while that for panel B is time after hyperthermia.

11.4 Discussion

The fluorescent DNA halo method (2) enables one to measure DNA damage and repair in cells individually which need not be radioactively labelled. The results of this assay following irradiation with 10 Gy differs from those observed in nucleoid sedimentation studies (1) in that the DNA supercoil the unwinding phase (PI concentrations 0.5 - 7.5 μg/ml) is unaltered. Since the nuclear lamina appears to remain intact following lysis (2) it is possible that this organelle constrains the DNA within the nuclear matrix regardless of the strand breaks introduced by radiation in the 1-25 Gy dose range. Conversely, sedimenting this particle through viscous sucrose (15-30%) used in the sedimentation technique could force the broken DNA out of the nuclear lamina thereby slowing sedimentation. Thus. it may be that the fluorescent DNA halo technique is a less disruptive procedure than sedimentation. The fluorescent DNA halo assay is sensitive enough to detect 1 Gy of X-ray-induced DNA damage (figure 2) which makes it nearly as sensitive as the alkaline unwinding method (29). The assay is more sensitive than the flow cytometric analysis of nucleoids (30) or the "neutral" elution method (31). Since there is no need for incorporation of any labelled DNA precursors prior to the assay, it can be applied to in vivo systems such as cerebellar neurons (32) or to plateau phase cells. Therefore, we believe that this assay has many potential uses. Previously published considerations of the kinetics of the process of restoring the ability of DNA to supercoil (2) have led us to assume that the reduction in the ability to rewind DNA supercoils is due to the presence of DNA strand breaks. Acute exposure to heat was shown using a wide variety of techniques to inhibit the repair of radiation-induced DNA strand breaks and alkali labile sites (4-8, 33). After combining heat and radiation several investigators found more stand break induction for the combination than for radiation alone (4-8). In some cases no difference was observed (33,34). However, Jorritsma and Konings (35) using the alkaline unwinding method in HeLa S_3 cells, found a small reduction of the initial amount of radiation-induced DNA lesions. Using the fluorescent halo method we found an apparent reduction in the initial amount of radiation-induced damage. This notion is reinforced by the observations that these proteins may be inhibiting DNA supercoiling changes (3,17). Further it is likely that the heat-induced changes which could as DNA strand breaks from the assay could also make these lesions from repair systems. The heat-induced masking effect disappears with the recovery to normal protein levels of the nucleoids (figure 4, figure 5b) and with the removal of heat-induced inhibition of DNA supercoiling (17). Concomitant with the foregoing, the repair of radiation-induced damage recovers to control levels (figure 5a). It can be hypothesized therefore that the increased amount of protein at the nuclear core inhibits the DNA from full unwinding, anchors some of the radiation-induced strand breaks, and reduces the accessibility of the damaged DNA for repair enzymes. Also, since hyperthermia clearly inhibited the DNA relaxation by irradiation (figure 1 and 2) and since DNA unwinding seems to be a

necessity for DNA repair (35), this might be an additional mechanism by which heat inhibits DNA repair. The next logical question would be which of the heat-induced protein changes in nucleoids might be responsible for anchoring the radiation-induced DNA breaks. One major change seen in figure 4 is the increase of HSP 72/73 which is in accordance with observations of Welch and Feramisco (36) and Ohtsuka et al (21). Other changes include increases in polypeptides of 160, 140, 125, 95, and 87 kD (apparent molecular weight). The content of these polypeptides in the nucleoid has returned to near control levels at 6 hours after the heat shock when the masking and supercoiling effects has disappeared; whereas HSP 72/73 content in nucleoids remains elevated. Thus, it is unlikely that HSP 72/73 is responsible for these effects. In terms of the kinetics of removal of proteins from the nucleoids from heat-shocked cells, the higher molecular weight proteins (160, 140, 125, 95 and 87 kD) appear to be more likely candidates for playing a role in the heat effects on DNA supercoiling masking of DNA damage, and possibly repair of DNA damage.

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CHAPTER 12

GENERAL DISCUSSION

Hyperthermia can lead to the loss of reproductive capacity of cells and to an enhanced sensitivity to ionizing radiation. The search for the molecular pathways leading to both heat killing and -radiosensitization has been the aim of this study.
With regard to heat killing the attention was focussed on the possible role of heat-induced changes in (sub)nuclear protein binding. The studies indicate that such changes are important, as far as they are characterized by both the extent and the duration. Analysis of DNA loop organization using various approaches has led to a better insight in the possible localization of the altered protein binding and its consequences for thermal death and radiosensitization.
Some information was obtained on the kind and nature of the proteins involved in the increased intranuclear protein binding. Also, different types of nuclear protein binding were detected and analyzed for their relation to cytotoxicity.
Radiosensitization by heat was initially suggested to be related to the loss of cellular DNA polymerase α and/or β activity. During the course of the investigations described in this thesis, it became clear that a loss of polymerase activity could not, under all circumstances, be the cause of radiosensitization. Decreased accessibility of the damaged DNA to repair enzymes as a consequence of the changed protein binding to (sub)nuclear structures is probably a better explanation of radiosensitization observed upon hyperthermic treatment.

12.1 Heat killing

12.1.1 DNA

Damage to DNA can be observed immediately after heating of cells using high "heat doses". Up to 2 Gy equivalents of DNA damage was found with the alkaline unwinding assay after treating HeLa S3 and EAT cells (Chapter 6) with "heat doses", resulting in a cell survival of less than 0.05%.

Irradiation with 2 Gy of X-rays, however, leads to survival levels above 50% (Chapter 8). Moreover, no protection or sensitization for heat-induced DNA damage was found when cells were heated in the presence of polyols or procaine respectively (1,2). This fact and other arguments (see also 1.6.4) led us to conclude that cell death by hyperthermia is not caused by direct damage to the DNA. Furthermore, DNA damage did not trigger the thermotolerance development in mammalian cells (3).

12.1.2 Plasma membrane

It becomes less and less likely that, as has been suggested (4), heat damage at the level of the plasma membrane is the primary cause of hyperthermic cell death. As reviewed in 1.6.4, neither changes at the level of membrane lipids, nor of the intracellular pH (Na-H-exchanger), nor of the intracellular concentration of potassium (Na-K-ATPase) or calcium (Ca-ATPase) ions, nor changes in the architecture of the level of the cytoskeleton (that interacts with the plasma membrane) seem related to heat killing. Whether putative damage at the level of the plasma membrane might be a trigger for other reactions in the cell that ultimately lead to cell death after hyperthermia, has yet to be elucidated.

A principle role in thermal killing is probably played by the nuclear matrix (this thesis). It has been suggested that the observed heat-induced increase in nuclear protein binding might be a result of primary damage to the plasma membrane (5,6). It has been reported (5) that if cells are treated with membrane-permeabilizing agents, such as Tween-80, the chromatin, subsequently isolated from these cells, contains more protein. In an attempt to further investigate this observation in our laboratory, we indeed observed an increase in the nuclear protein mass per nucleus; however the percentage of intact nuclei that could be isolated from cells treated with the detergent was much lower than that from untreated control or heated cells (Stege et al., unpublished results). So the observed increase may be due to a selection (during isolation) of nuclei with a higher nuclear protein content (e.g. from G_2-phase cells). Treatment of cells with the calcium ionophores A23187 and ionomycin did not affect the nuclear protein content, nor the heat-induced increase in nuclear protein binding, nor the "efficiency" of heat killing.

12.1.3 Increased nuclear protein binding

One of the earliest detectable molecular changes after heating cells is seen at the level of the cell nucleus: an increased protein mass of isolated (sub)nuclear structures. Since the nuclear matrix is, in some way, involved in the organization, expression and duplication of the cell's genetic information, and since all these functions are impaired after heating of cells, structural alterations of the nuclear matrix might be connected with heat-induced cell death.

12.1.3.1 Relation to cell survival

Initially it was found that the extent of increased nuclear protein binding, as determined immediately after heating of cells, closely reflected the extent of hyperthermic cell killing. This was also seen when cells were heated in the presence of procaine, ethanol or glycerol. Also the development of chronic thermotolerance corresponded with changes in intranuclear protein binding: the heat-induced increase in nuclear protein binding levels of after prolonged heating at relatively low hyperthermic temperatures (7). A nice log-linear correlation (see figure 1) is found when the relative increase in nuclear protein binding (linear) is plotted against survival from hyperthermic treatment (log). The correlation curve in figure 1 resembles a heat survival curve in which the extent of the increase in nuclear protein binding replaces the "thermal dose"; a larger increase correlates with less survival (independent of the time-temperature protocol).

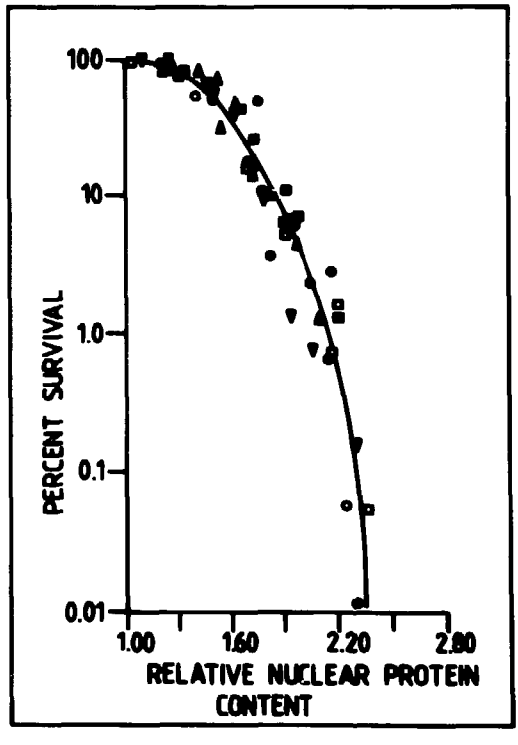

Figure 1: Correlation between survival from hyperthermic treatment and initial increase in nuclear protein binding, as determined for various heating procedures (redrawn after Roti Roti and Laszlo (7))
Open symbols represent data obtained from heat-treatment alone; closed symbols refer to treatment with heat and some modifying treatment. (closed symbols: ethanol = circles, procaine = squares, glycerol = triangles, and thermotolerance = inverted triangles).

Thus, the extent of nuclear protein binding seems to be a good predictor for the extent of heat killing and might be causally related to thermal death. However, the data presented in this thesis (Chapters 2 and 3) clearly reveal that measurement of just initial nuclear protein mass increase is not enough. Especially the data on acute thermotolerance indicate that also the rate of recovery from the increased protein binding has to be taken into account. The parameter "excess nuclear protein mass hours (ENPH)", including both initial nuclear protein binding increase and its recovery with post-heating time, shows a nice correlation to thermal killing under all experimental conditions tested so far. Low temperature storage (5), procaine (Chapters 2 and 3) and step-down heating (8) inhibited (or delayed) this recovery in accordance to their heat sensitizing potential (1.6.2) and inhibited (or delayed) thermotolerance development (Chapter 1, table 7), while the recovery from increased nuclear protein binding is not affected by inhibitors of DNA-, RNA- and protein synthesis nor by inhibitors of oxidative phosphorylation or microtubulus assembly (5). These observations prompt the speculation that increased nuclear protein binding and its recovery might be a prerequisite for heat-induced development of thermotolerance. The use of other inhibitors of heat-induced thermotolerance as well as the use of chemical inducers of thermotolerance may lead to further insight in the significance of the increase of nuclear protein binding and its (post-heat) recovery with respect to the actual cause of heat-induced cell death.
One study (9) showed somewhat conflicting data on nuclear protein mass increase and its post-heat recovery in relation to heat killing. In accordance with our findings regarding the analysis of nuclei from mid-S phase cells only (Chapter 3) no cell cycle stage dependency of both heat- induced, increased intranuclear protein binding and its recovery was observed, although cells display a clear cell cycle stage dependent heat sensitivity (1.6.3). This seems to argue against the hypothesis according to which the increase in intranuclear protein binding would be directly related to the extent of thermal death. It is, however, possible that during the different phases of the cell cycle a similar intranuclear change in heat-induced protein binding and its recovery has a different impact on cells in different cell cycle stages, because various DNA-associated processes are cell cycle stage specific. Other, possibly conflicting, results come from experiments (9) showing that the rates of recovery from different heating protocols leading to the same initial protein mass increase are dependent on the duration of the heat treatment used rather than on the hyperthermic temperature or temperature-time combination applied. The heating protocols were designed as to cause isosurvival levels, but no survival was measured, and heating-up problems (especially affecting the shorter treatment schedules) may have contributed to the differences in recovery rates. Simultaneous measurements of survival, increase of nuclear protein binding, and recovery from the latter are, therefore, indispensable.

12.1.3.2 Identification of proteins participating in heat-induced enhanced, intranuclear binding

Considerable progress has been made with respect to the localization and identity of some of the proteins involved in the heat-induced (sub)nuclear protein binding.

As indicated by the early data of Wheeler and Warters (10), the heat-induced excess proteins were found to be associated mainly with the nuclear matrix and with the DNA. We and others also found a considerable heat-induced increase in protein binding to the nuclear matrix or nucleoids of mammalian cells (Chapters 4,5,12; refs. 8,10-17), but also of Drosophila cells (18) and yeast cells (19). The indirect evidence obtained with the topoisomerase II inhibitor mAMSA (Chapter 6) clearly indicates that the heat-induced increased protein binding is (at least partially) located at the basis of the DNA loops, which provides the attachment to the nuclear matrix (1.3). The decreased accessibility of these sites upon hyperthermic treatment of cells (Chapter 6) suggests that the excess protein is bound near to or at the attachment points. The higher rewinding efficiency upon titration of nucleoids (matrices with associated DNA) with intercalating dyes after heating of cells (11; Chapter 5) is in accordance with this suggestion. The nucleosomal structure seems unaffected by hyperthermia (20-22).

A number of different proteins are involved in the heat-induced, increased intranuclear binding. These proteins have some properties in common. It has been shown that the excess nuclear proteins are not enriched in lysine (like histones are) but enriched in leucine and tryptophan (23). This is in accordance with earlier findings (24,25) that the proteins involved in the increased nuclear protein binding are non-histone proteins. Higashikubo et al. (23) showed that the proteins involved in the increased binding are present in the cell already before and during the heat shock (the addition of actinomycin-D and cycloheximide 1 hour prior to the heat exposure did not affect the increase), and that a fraction of these proteins has a high turnover rate. Furthermore, the increased binding does not seem to be the result of a cytoskeletal collapse, since no increase in the amount of nucleus-associated actin, vimentin or cytokeratin was observed upon western blotting (26). Also, we (Chapters 5 and 12) and others (8,14) did not find any significant increase in the amount of actin bound to nuclear matrices or nucleoids from heated cells. So, it seems evident that the increased nuclear protein binding as measured in these isolated structures is not due to an enhanced, non-specific binding of cytoskeletal elements, in spite of the fact that indirect-immunofluorescence analysis using anti-vimentin antibodies and electron microscopic analysis of whole cells suggested that such a collapse takes place. The "collapsed cytoskeleton" is probably removed during isolation of the nuclei. When isolated nuclei are treated with high-salt solutions, this results a subsequent loss of several proteins and yields nuclear matrices (see 1.3). As discussed exposure of cells to hyperthermic (> 41 ^{o}C) temperatures leads to increased protein binding to the nuclear matrix. Interestingly, treatment of nuclei (isolated from unheated cells) in buffer at non-hyper-

thermic, elevated temperature ($37^{o}C$ compared to $4^{o}C$) also leads to an enhanced binding of, previously high-salt-soluble, proteins to the nuclear matrix (12,18,19). These studies show that increases in intranuclear protein binding can occur in the absence of a cytoskeleton (or a plasma membrane). They also indicate that nuclear proteins aggregate in reaction to (non-hyperthermic) temperature elevation under non-physiological conditions. Different temperatures are necessary to induce changes in intranuclear protein binding (above $41^{o}C$ ("heat dose" dependent) for cells and (already maximal?) after $37^{o}C$ treatment of isolated nuclei (no "dose-response" has been established as yet)). Furthermore it was shown (18) that heating cells leads to enhanced protein binding to the nuclear matrix that is not identical (protein pattern) to the enhanced nuclear matrix binding induced by a $37^{o}C$ treatment of nuclei.

Two proteins that might be involved in the increased nuclear protein binding are the DNA polymerases α and β (Chapter 7). These enzymes also leak from the nucleus of a heated cell, to a lesser extent, during the isolation procedure. It has to be kept in mind, however, that the increase in DNA polymerase binding to the nucleus is based on measured activity and not on the number of molecules present in the nucleus (Chapter 7). It is possible that altered binding of the enzymes might have changed their specific activity. Furthermore, the differences in the extent of nuclear protein binding induced by procaine-HCl (Chapters 2 and 3) were detected after isolation of nuclei using detergent or non-detergent extraction procedures. Further evidence for less leakage during isolation of nuclei, because of increased binding due to thermal stress may be deduced from studies in which isolated nuclei, treated at elevated temperatures showed an increase in intranuclear binding of previously high-salt-soluble polypeptides (12,18,19). All these studies show that data on nuclear (matrix) protein composition (and alteration of this composition by any agent) have to be interpreted with great caution, and have to be viewed in the light of the type of isolation procedure used, of which even the duration might influence the outcome of the results (Chapter 5; 18).

Procaine, at low (non-toxic) concentrations, is able to induce a rather weak (triton-sensitive) nuclear protein binding (Chapters 2 and 3) and at higher (toxic) concentrations, a triton-in-sensitive binding (6). It can be speculated that, rather than through acting via the plasma membrane (4), procaine sensitizes for heat killing by a direct action on the nucleus; induction of a weak intranuclear protein binding might facilitate and enhance the extent and/or tightness of the heat-induced intranuclear protein binding. This may then cause a retardation of the post-heat recovery of increased intranuclear protein binding (Chapters 2 and 3) and a delay in thermotolerance development (27,28). It, therefore, seems worthwhile to investigate whether other sensitizers and/or inhibitors of heat-induced thermotolerance such as ethanol (that, at high concentrations, induces a triton-resistant protein mass increase (6)) also, at lower concentrations, induces an increased intranuclear protein binding, when nuclei are isolated using a non-detergent method. Furthermore,

the effect of heat protectors and heat sensitizers during treatment of isolated nuclei at elevated temperatures could be investigated,, to find out whether or not they can protect or sensitize against the heat-induced changes in the protein binding to subsequently isolated subnuclear structures. Apart from DNA polymerases, a variety of polypeptides were found in increased amounts in nuclei and subnuclear structures isolated from heated cells; these included various undefined polypeptides with molecular masses usually over 45 kD (8; Chapters 5 and 12) as well as the human oncogene c-myc product (12) and topoisomerase II (18). The significance of the enhanced binding of each of these individual polypeptides remains, as of yet, unclear.

12.1.3.3 Relation to heat shock proteins

Interestingly, our studies have shown an increased appearance of HSP70 proteins in isolated nucleoids (Chapters 5 and 12), as was also shown by others for nuclei (26) using immunoblotting with anti-HSP70. This is in accordance with immunological studies, in which, using fluorescent microscopic analysis of whole cells, translocation of HSP70 from the cytoplasm to the nucleus was observed upon heating of cells (Chapter 1, Table 12). This indicates that, besides a decreased loss of nuclear proteins during the isolation, also translocation of proteins from the cytoplasm to the nucleus occurs after exposure of cells to heat. This HSP-increase accounts for only a very small fraction of the total increase in intranuclear protein binding. Cycloheximide and actinomycin-D did not measurably affect the heat-induced increase in intranuclear protein binding (23). The total increase can certainly not be explained by an increase of the amount of heat-inducible HSP70 in the nucleus. The amount of "constitutive" HSP70 in cells is also too low to explain for all of the increase in intranuclear protein binding. If we assume that (at most) 6% of the total protein content of cells may consist of HSP (29) and HSP70 is half of that, and that all of this protein is bound to nuclei in heated cells and none to nuclei from unheated cells (that contain, in our hands, about 20% of total cell protein), we calculate that, at the most, a 1.15 increase in nuclear protein mass can be caused by the translocation of HSP70. For all HSPs together a maximum increase of 1.3 can be calculated; however, not all HSPs are translocated into the nucleus (Chapter 1, Table 12). The calculation shows that, most likely, HSP70 (constitutive or inducible) does not significantly contribute to the heat-induced nuclear protein mass increase; the same seems true for other HSPs. HSP70 (constitutive and/or inducible) is present in the nucleus and in nucleoids, and in enhanced quantities 6-9 hours post-hyperthermia (Chapters 5 and 12). At this time point, thermotolerance is expressed in HeLa S3 cells (see e.g., Chapters 2,3,7, and 10). These findings, together with the putative function of HSP70 in mediating disaggregation of protein complexes (1.7, figure 12), lead us to propose a model for the involvement of HSP70 in the restoration of the heat-induced, increased intranuclear protein binding. This model is depicted in figure 2. The model proposes that hyperthermia (directly or indirectly)

induces an aggregation of nuclear proteins (intranuclear protein binding). This leads to saturation of the ubiquitin degradation pathway (1.7.5, figure 13), causing HSTF activation (and HSTF translocation to the nucleus), and activation of HSP gene expression through interaction between HSTF and the HSE elements of the HSP genes according to the model of Burdon (30; see Chapter 1, figure 13). Since abnormal protein aggregates probably trigger HSP synthesis (1.7.5), an aberrant binding of proteins to the nuclear matrix might, likewise, induce HSP synthesis. An increased HSP -and especially HSP70- synthesis, followed by translocation of the protein into the nucleus may then assist the cell in recovering from the aberrant intranuclear protein-protein binding. Since HSP70 expression seems to be self-regulatory, we propose a negative feedback on HSE-HSTF binding; this negative feedback will subsequently lead to a decrement in HSP70 gene activity. The capacity of HSP to mediate disaggregation of abnormal protein-protein aggregates (31; Chapter 1, figure 12) in an ATP consuming reaction, supports this idea. The increased amount of nuclear HSP70 at the time thermotolerance has developed (6-9 hours post heating, Chapters 5 and 12), in relation to the enhanced recovery of tolerant cells from the (same) initial increase in intranuclear protein binding (Chapters 2 and 3) further substantiates the hypothesis. Moreover, preliminary data from our laboratory (Kampinga et al., unpublished observations) showed that in vitro incubation of nuclei with ATP (0.1mM) reduces the increased intranuclear protein binding.

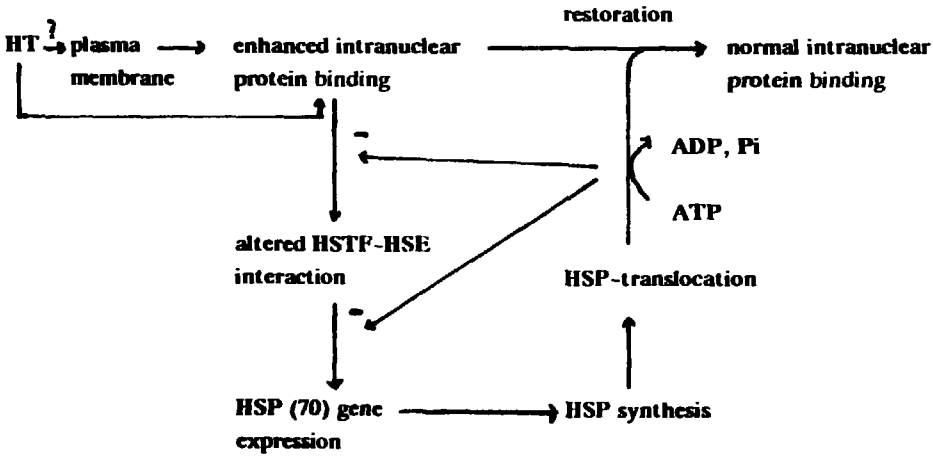

Figure 2: Hypothetical model for the triggering of HSP induction and the role of HSP70 (translocated into the nucleus) in the disaggregation of abnormal protein-protein binding due to exposure of cells to heat. (see the text for further details).

HSP70-like proteins are bound tightly to the nuclear matrix and nucleoli (see the review by Bienz and Pelham (32)) and this binding can be reversed by ATP but not by its non-hydrolyzable analogues (33,34). Although absolute HSP levels and rates of HSP synthesis do not always correlate with the degree of thermosensitivity (1.7.4), the ability of cells to translocate (various amounts) of HSP70 to damaged (intracellular) sites, particulary the nucleus, may be crucial in the defense against thermal death. The literature provides additional data suggesting that the appearance of HSP70 in the nucleus might sustained to an enhanced rate of recovery (see also Chapter 3). The presence of HSPs in the nucleus (nucleolus) has, for instance, been related to the nuclear matrix-associated function of RNA-processing (35-37). A recent study by Laszlo (38) showed that thermotolerant cells and heat-resistant cells recovered faster from (the same) hyperthermic inhibition of, for instance, RNA synthesis (a nuclear matrix related process); this finding was related to the kinetics of HSP70 translocation to and from the nucleus. Apparently, cells are unable to deal with a relatively mild heat shock after microinjection with antibodies against HSP70 (the antibodies impair the translocation of HSP70 to the nucleus upon a heat shock). This finding shows that HSP70 translocation into the nucleus is important in the cell's defense against hyperthermia. Nevertheless, more data will be needed to establish a causal relationship between HSP70 translocation (such as a better quantification of the extent of translocation), recovery from the increase in intranuclear protein binding, and thermal killing. Induction of thermotolerance by sodium-arsenite might serve as a tool in such studies. Arsenite by itself causes no or only a slight nuclear and nucleolar translocation of HSP70 (see 1.7.4). We suggest that the translocation of HSP70s is related to an enhanced recovery of increased intranuclear protein binding in heat-induced thermotolerant cells. Arsenite-induced tolerance might be different in this respect, since arsenite seems to be unable to induce nuclear translocation of HSP70, and thus seems to induce thermotolerance via a different route than heat. The finding that arsenite induced tolerance differs -with respect to the sensitivity to cycloheximide treatment- from the heat-induced tolerance (39,40), taking into account the different abilities of heat and arsenite to cause translocation of HSP70, is intriguing in this respect.

12.1.3.4 Mechanisms of toxicity

The data obtained so far suggest a couple of possibilities by which a heat-induced increase in intranuclear protein binding may cause thermal cytotoxicity:

1. The nuclear matrix (1.3) is structurally altered changed. A structural change of the matrix may affect the organization and function of the DNA. The data on enhanced recovery of DNA polymerase activities in the nucleus may indicate that important nuclear proteins like the polymerases α and β (involved in DNA replication and/or repair; see 1.4.5) become abnormally bound within the

nucleus, and may thereby loose their normal mobility and dynamic function.

2. The excess nuclear proteins may be involved in the heat-induced changes in DNA supercoiling as revealed by the halo assay (Chapter 5). It is not known -at this point- whether these proteins are responsible for the inhibition of matrix-associated processes or whether they, by their presence, just influence the results of the biochemical assay used. Studies on the digestion kinetics of loop DNA (Chapter 4) and the results of the halo assay (Chapter 5) show that although heat does not alter the number of loop attachment sites, but it rather changes the nature of these sites, causing an unnatural stabilization of the anchorpoints (see Chapter 5 for a specific discussion). These studies supported earlier ideas based on experiments using the nucleoid sedimentation assay (11). The reduced accessibility of the anchorpoints (topo II sites: Chapter 6) and decreased antibody-staining for topoisomerase II (18) seem in accordance with this idea. The data also fit with the finding (16) that the topoisomerase II inhibitor novobiocin can enhance both thermal killing and intranuclear protein binding. Changes in the matrix structure at the level of the topoisomerase II sites, due to an aberrant protein binding, may lead to disregulation of the degree of supercoiling and a non-efficient opening-up of the DNA structure, needed for proper replication, transcription and/or repair.
3. Restriction of the accessibility of regulatory sites at the matrix-basis of the DNA loops, as discussed in Chapter 6, might decrease gene activity. This, in turn, may lead to "fatal" deprivation of essential proteins.

   All three possibilities mentioned above, either by themselves or in combination, finally may have the same result: nuclear matrix associated functions are greatly impaired by hyperthermia. The persistence of loss of such functions might then determine whether a cell is destined to die. This might be reflected in the outcome of the calculations of "excess nuclear protein hours (ENPH)" (Chapter 3).
4. Finally, an abnormal structural organization of the DNA may lead to the appearance of "late" damage to the DNA, especially in S-phase cells; the detection of DNA fragments with minimal sizes corresponding to DNA loop sizes (120-140S) after heating of cells (41) indicates potential heat damage at the basis of the loops (by the altered protein binding at these sites). On the other hand, it might reflect an inhibition of matrix-associated replication. As indicated by Wong et al. (42), the duration of heat effects on the various subprocesses during DNA synthesis (persistent single stranded regions or inhibition of chain elongation into replicon clusters) correlates with the time needed for the restoration of

the heat-induced increase in intranuclear protein binding. This long-term effect on DNA replication increases the probability of DNA exchange, leading to chromosomal aberrations that might -in turn- promote thermal killing of, especially, S-phase cells. This also would explain why the same, heat-induced increase in intranuclear protein binding could have more "impact" on S-phase cells, in the sense that S-phase cells are more heat-sensitive.

In general, hyperthermia causes an inhibition of transcription. If it is the heat-induced increase in intranuclear protein binding that is responsible for the inhibition of transcription, then how do HSPs escape from this inhibition? Transcriptional control of HSP genes is probably mediated by interaction of the HSTF with the HSE of the HSP genes (1.7.1). The binding of HSTF to the HSE element is induced or changed after heat shock, possibly via enhanced local unwinding of the gene, and better exposure of regulatory sequences. Since these regulatory elements are often found to be closely associated with the nuclear matrix (1.3), heat shock might affect these sites directly or indirectly via an effect on increased intranuclear protein binding on DNA supercoiling. Transcription of normal genes might be inhibited in this way, but, via the HSTF-HSE interaction, HSP genes might bend thuslike that they escape from this transcription block and their TATA promotor is still accessible to transcription factors. Since the primary transcripts of most HSP genes don't need to be spliced (the splicing process is also inhibited by hyperthermia (see reference 7), they can directly be used for translation. The preferential translation of HSPmRNAs over nonHSPmRNAs, perhaps regulated at the level of mRNA stability (43) or via the availability of initiation specific factors (see 1.7.2), then will lead to the observed increased HSP synthesis. Detailed analysis of HSP and nonHSP gene structure before and after heat shock and their association with the nuclear matrix (in terms of e.g., accessibility to transcription factors, polymerases, etc.) is needed to understand whether the above described ideas about transcriptional control are valid or not.

12.2 Heat-radiosensitization

12.2.1 Heat killing versus heat-radiosensitization

The observed differences in heat sensitivity of various cell lines do not correspond with the differences in the extent of radiosensitization (Chapter 8). This is not surprising, since heat-induced radiosensitization only concerns cells that survive heat treatment. The impact of thermotolerance on the extent of heat-radiosensitization was re-evaluated and an important observation was made in HeLa cells. Thermotolerance ("acute") not always affects radiosensitization when radiation is given immediately after (or simultaneously with) the heat treatment. But when the interval between heat and radiation increases, tolerance may be expressed at the level of radiosen-

sitization (Chapter 10). This tolerance effect was already apparent for a 1 hour interval after a relatively high (30 minutes at 44°C) "heat dose". With lower heat doses, the interval to show a tolerance effect for radiosensitization is probably even much shorter (may be only a few minutes) for lower heat doses. This might, at least in part, explain the differences reported concerning the effect of thermotolerance on heat-radiosensitization even within the same cell line (1.8.2). The data stress the importance of accuracy in measuring and describing heat radiosensitization effects especially with respect to interval times.
It seems that the mechanisms leading to heat killing and to radiosensitization are only partially identical. In both phenomena, the heat-increased nuclear (matrix) protein binding might play a crucial role. In heat killing, the increase plus recovery (ENPH) seems important (12.1) while for radiosensitization the actual status of the increase at the moment of application of radiation may be an (the) determining factor (see below).

12.2.2 Possible mechanisms for heat radiosensitization

12.2.2.1 Initial DNA damage

As discussed in 1.8.3 (Table 14), for the induction of DNA damage after a combined treatment of cells with heat and radiation, no clear-cut relationship with the synergistic action of the two agents at the level of survival was observed. More, less, or the same amount of initial DNA damage was observed for the combination in relation to radiation alone. The data in Chapter 11 show that the heat-altered protein-protein and/or protein-DNA interactions causes decreased detectability of damage. The conflicting reports on the effect of heat on radiation-induced initial DNA damage may have been to be ascribed to the different assays used. In some assays damage may be masked, as in our halo assay. Also, since the incorporation of pyrimidine analogues does not significantly increase thermal radiosensitization (44), heat-induced alterations of initial damage to the DNA do not seem to be primarily responsible for the synergistic action of heat and radiation. "Damage accessibility" to the repair machinery (as, for instance, revealed by some biochemical assays) seem to be a more important factor in heat radiosensitization.

12.2.2.2 Repair of damaged DNA

Repair of damaged DNA and its impairment by heat is an important process to be considered when relations with increased radiation killing are sought. Heat inhibits repair of damaged DNA, as measured by all DNA-damage-assays available (1.8.3). This is confirmed by the results described in this thesis (Chapters 9 and 11). In this context, there are two questions that will be discussed below:

A. Is hyperthermic inhibition of DNA repair functionally related to radiosensitization by heat?
B. What is the mechanism of heat-induced inhibition of DNA repair?

A. Hyperthermia not only inhibits the rate of repair of radiation-induced DNA damage, but may also lead to an increase in DNA repair rates (as judged by alkaline unwinding and elution assays). The latter has been observed when cells were exposed to heat at hyperthermic temperatures around 41-42°C (45,46), where a clear-cut radiosensitization occurred. This indicates the fact that impaired repair rates as such are not sufficient to explain radiosensitization as such. Data obtained in our laboratory (47) show that for HeLa cells thermotolerance is expressed at the level of hyperthermic repair inhibition (alkaline unwinding assay) while it is not expressed at the level of heat-radiosensitization when heat is directly followed by radiation (Chapter 10). The above, together with the finding that repair rates (as generally assayed) are not sufficient to fully explain radiation-induced killing (1.4) suggests that besides repair rates (and the inhibition by heat) other factors are involved in radiosensitivity (and sensitization by heat). Pre-irradiation hyperthermia leads to an increased number of chromatin aberrations (50); this is an indication that other factors are involved in heat radiosensitization at the level of the DNA. The following processes have to be studied in more detail in the near future :

1. Fidelity of DNA repair. It is possible that heat causes error-prone repair. Faulty nucleotide insertion by DNA polymerases may have increased, especially when the accessibility of the DNA to repair enzymes has been changed. Interestingly, Raaphorst and Azzam (49) found that thermotolerant cells, in comparison to their non-tolerant counterparts, show an increased frequency of transformation when treated with heat and radiation. This suggests that there is an increased error-prone repair in tolerant cells. In some cell lines this error-prone repair might result in a higher chance of cell death, being reflected in a similar heat-radiosensitization for tolerant and nontolerant cells, in spite of a higher repair rate in tolerant cells (as, for instance, detected in HeLa cells). Also, the enhanced repair at relatively low hyperthermic temperatures (resulting in radiosensitization (45,46)) can be explained by a hyperthermic induction of error-prone repair.
2. Modification of radiation-induced damage. Heat may modify the nature of damage induced by radiation, rendering it less repairable. If and how this occurs is unclear at the moment, but the possibility cannot be ruled out.
3. Radiation damage will become irreparable when it has been "fixed" in S- or M-phase. Cell cycle progression and its delay caused by hyperthermia needs to be investigated in relation to the extent and duration of repair inhibition. If cell cycle progression re-starts before full recovery of the repair capacity has taken place, the balance between repair and "fixation" becomes in favour of the latter, causing enhanced cell death.
4. Active, gene-specific repair. As reviewed in Chapter 1, transcription and repair may be highly intertwined processes. The extent of

repair of active genes, rather than that of the overall genome, inversely correlates with cell death after UV-irradiation. The preferential repair of UV-induced dimers in active genes (that are supposed to be matrix-associated) might also take place in response to damage induced by ionizing radiation. With regard to ionizing radiation, some studies (50) suggest that also in that case, active, gene-specific repair may occur. The repair of active and inactive genes, the effect of heat on gene activity (increased HSP gene activity versus decreased activity of most other genes), repairability of various genes, and their accessibility to macromolecules in general (alterations in matrix structure), are all subjects for future research.

B. Although hyperthermic inhibition of DNA repair rates does not seem to be sufficient to explain the increase in radiation sensitivity, lower repair rates may certainly contribute to an increased radiation sensitivity. It remains important, therefore, to know what causes the change in DNA repair potential after heating of cells. It is still unclear which type of DNA damage, detected by any of the available DNA damage assays, is crucial in radiation-caused cell death. Heat may not only inhibit repair, as measured by the various DNA damage assays, but also affect the fidelity of repair, the modification of damage, the fixation, and gene-specific repair. Thus, in exploring potential targets for hyperthermic radiosensitization, heat-induced, molecular changes in the structure and dynamics of such putative targets must be directly related to radiosensitization (survival), and not to DNA repair rates only.

Our initial approach was to test a possible role of heat-induced loss of cellular DNA polymerase activity in hyperthermic radiosensitization[1]. The nuclear enzymes, DNA polymerase α and β, are thought to be involved in the repair of damaged DNA (1.4.5; 51). Spiro et al. (52) were the first to observe that DNA polymerases were partially inactivated after heating of cells. The extent of inactivation of both enzymes was found to correlate fairly well with the extent of heat-radiosensitization in HeLa S3 cells (47,53). Figure 3 shows a log-log correlation between the reciprocal of the thermal enhancement ratio (1/TER) and the extent of polymerase inactivation under various experimental conditions. A similar correlation is observed when CHO cells are heated in the presence of glycerol, or after pretreatments with sodium-arsenite, to induce thermotolerance (54). Dikomey and Jung (55) found a fair correlation between DNA polymerase β inactivation and heat-radiosensitization for CHO cells that had been made thermotolerant or that had been thermosensitized (step-down heating). Our experiments show that the

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[1] part of the discussion on the role of DNA polymerase inactivation in thermal radiosensitization is published in: Kampinga H.H. and Konings A.W.T.Proc. of the 5th Int. Symp. Hyperth. Onc., Kyoto 1988.

correlation even holds when 3 different cell lines with different heat sensitivities are compared (Chapter 8).

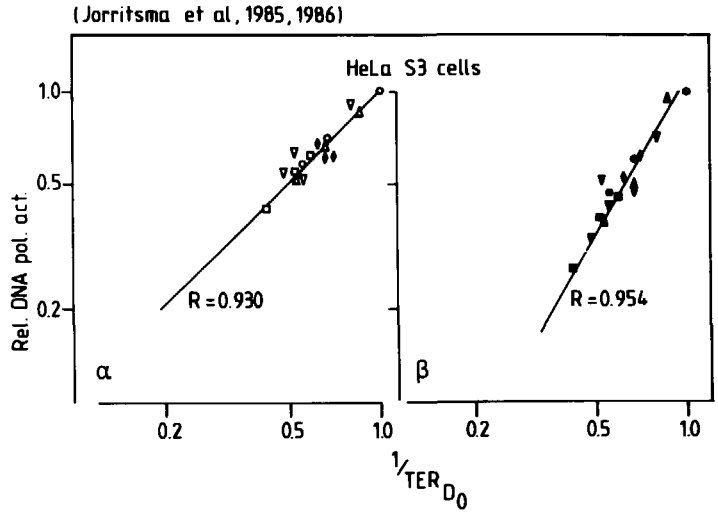

Figure 3: Log-log correlation between the inactivation of DNA polymerases α and β and the reciprocal of the TER_{Do} of HeLa S3 cells.
circles= 44°C; diamonds= 42°C; triangles= TT_a,44°C; squares= TT_c,44°C; inverted triangles= TS,44-42°C (Data derived from Jorritsma et al. (47,53))

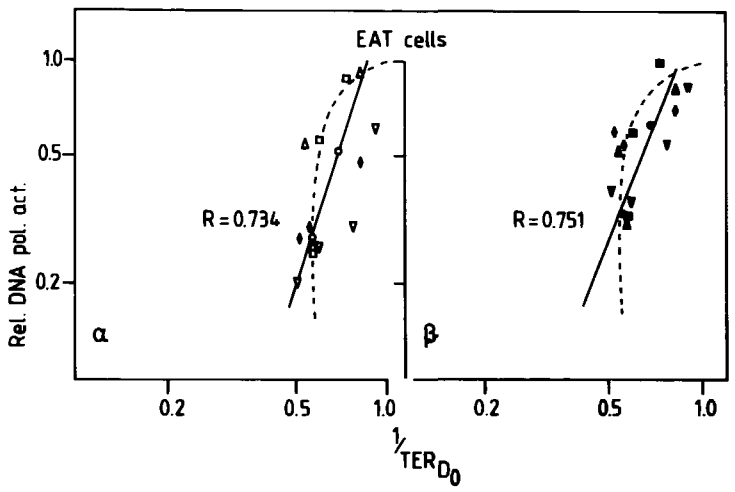

Figure 4: Log-log correlation between inactivation of DNA polymerase α and β and the reciprocal of the TER_{D^0} of EAT cells.
circles= 44°C; diamonds= 41°C; triangles= TT_a,44°C; squares= TT_c,44°C; inverted triangles= TS,44-41°C (Data derived from Jorritsma et al. (53).

A comparison between the TER and extent of polymerase inactivation in EAT cells (figure 4), under conditions comparable to those applied in the experiments shown in figure 3, revealed lower correlation coefficients between the indicated parameters than observed with the HeLa S3 cells (figure 3). The lower correlation coefficients can especially be attributed to the radiosensitizing effect of the thermotolerance-inducing heat dose and the apparent saturation of the TER. Because both the latter two effects were not found for polymerase inactivation (53), the actual curve (connecting the data points, dotted line) deviates from the log-log correlation. Another reason why polymerase inactivation is probably not a decisive factor in heat-radiosensitization comes from recovery studies where heat and radiation treatments were separated by post-heat incubations at 37°C and polymerase activities were determined at different time points after the application of hyperthermia (52,54). The TER recovered with clearly different kinetics than did DNA polymerase α. Also, the data in Chapter 9 cast doubt on the relation between α polymerase activity and hyperthermic inhibition of repair. Polymerase β seems to recover with kinetics similar to that of radiosensitization. However, 24 hours after the heat treatment, polymerase β had recovered to above-normal levels, while radiosensitization was still apparent. The experiments described in Chapter 10 were designed to try and more conclusively elucidate the role of hyperthermic inactivation of DNA polymerases. Tolerant and nontolerant HeLa cells were heated for 30 minutes at 44°C and either irradiated with 4 Gy immediately or after various times of subsequent incubation at 37°C. In parallel experiments, cells were assayed for DNA polymerase activity. The data showed a non-correlation between the recovery of DNA polymerase α and β activity and the recovery of TER in tolerant and non-tolerant cells. We re-analyzed some data from the literature. Data from one laboratory (same cell line, same DNA polymerase assay) were pooled. Figure 5 shows that for the data from Mivechi and Dewey (54, line 1) as well as for the data from Chu and Dewey (56,57, lines 2 and 3) good log-log correlations exist between DNA polymerase α and β inactivation and radiosensitization by hyperthermia. As correctly indicated by the investigators, the individual experiments suggested a relation between polymerase inactivation and radiosensitization by hyperthermia. Figure 5 also shows, however, that the slopes of the curves drawn for the different data points under different experimental conditions display substantial variation, resulting in rather low inter-experimental correlation coefficients (r=0.61 for α and 0.29 for β). It seems justified to conclude that there is no general, clear-cut relation between DNA polymerase inactivation and heat-radiosensitization. The general conclusion from all these studies must be that the total activity of polymerase α and β, as measured by the rate of incorporation of nucleotides into exogenously added, gapped DNA, is not exquisitely correlated to the extent of heat-radiosensitization. It needs to be emphasized though, that the data shown do not provide information with respect to the repair activity of the enzymes with respect to an endogenous (damaged) DNA. In this respect, it is interesting to note that the endogenous chromatin

structure is changed after hyperthermia, and that this change affects the binding of the polymerases (Chapter 7). It seems that neither the loss of cellular DNA polymerases (this thesis), nor the loss of DNA glycosylases, exonucleases, exinucleases or 5'endonucleases (58; see 1.8.4), nor the loss of topoisomerase II (Chapter 6) activity can be considered ("individually") responsible for heat-radiosensitization. It should be noted that the heat-sensitivity of other enzymes, especially those involved in the reversal of DNA damage (e.g., DNA-ligase) has not been investigated so far.

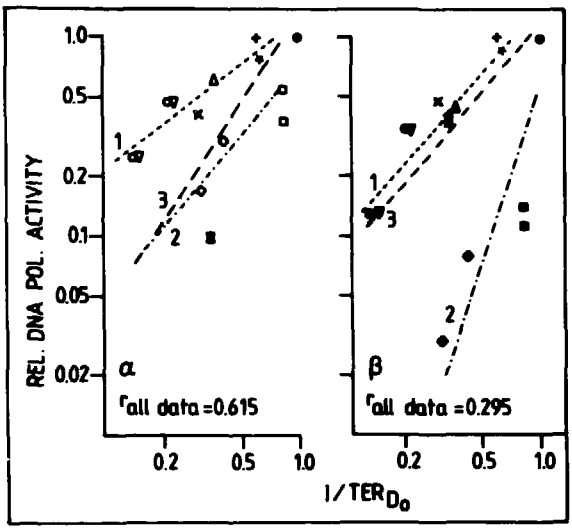

Figure 5: Log-log correlation between inactivation of DNA polymerase α and β and the reciprocal of TER_{D_0} for CHO cells.
Data re-analyzed from Mivechi and Dewey (54, line 1), Chu and Dewey (56, line 2) and Chu and Dewey (57, line 3).

The data presented in this thesis indicate that the observed increase in intranuclear protein binding is a more likely mechanism to explain heat radiosensitization. Heat-induced, increased intranuclear protein binding relates to:

- the hyperthermic inhibition of repair of t'type damage (58; 1.8.4, Figure 16)
- the hyperthermic inhibition of repair of lesions detected with the alkaline elution technique (59).
- the hyperthermic inhibition of detection and repair of lesions assayed by the "halo" assay (Chapter 11). Recently, this correlation was shown to hold under conditions of thermotolerance (same initial heat effect but enhanced recovery upon post-heating (60).
- the extent of hyperthermic radiosensitization (Chapter 10).

We are aware of only one report in literature (61) that notices a non-correlation between increased nuclear protein binding and DNA repair rates (alkaline sucrose gradient assay). These investigators found that repair was still inhibited while the increased protein binding had already been completely abolished. Their data on protein binding and repair inhibition (measured in asynchronous cells) should, however, not directly be compared to their data on heat-radiosensitization (measured in synchronized G_1 and S-phase cells).

A mechanism by which an altered nuclear (matrix) protein binding may cause inhibition of DNA damage repair may comprise the following elements:

- altered stability of the (normally) malleable nuclear matrix and a decreased ability of the matrix to deal with radiation damage at the moment of radiation (see 12.1). This implies that repair of damaged DNA is matrix-mediated. Although there is no clear evidence that DNA repair is strictly matrix-associated (1.3.5), the observation that hyperthermia alters the association of putative DNA repair enzymes (DNA polymerases α and β: Chapter 7 and topoisomerases: 18) with the nucleus/nuclear matrix may support this idea. On the other hand, altered binding characteristics of DNA repair enzymes could just result in a lower availability of normally soluble, nuclear repair enzymes, which might result in decreased repair rates. It is important to know how the binding characteristics of repair enzymes change upon heating of tolerant cells and whether or not this binding is more rapidly restored during post-heating period in the tolerant cells, in accordance with what we observed for heat-radiosensitization (Chapter 10). So, although the total cellular activity of these enzymes does not seem to be rate-limiting in repair inhibition, their distribution (functional activity) and its disturbance by hyperthermia might be restrictive.
- decreased accessibility of the damaged sites to enzymes involved in the repair process. This idea is substantiated by the experiments of Warters and Roti Roti (58: Chapter 1, Figure 16) in which the excision of t'type damage has been studied. Also the decreased rate of DNA digestion using micrococcal nuclease (10,20,21) might be explained by a decreased accessibility. Furthermore, the data in this thesis, with respect to "masking of radiation damage in heated cells" are in favour of the existence of such a mechanism (Chapter 11). A better insight into the extent of non-accessibility of damaged sites in the DNA could come from experiments in which isolated nuclei from heated and unheated cells that are irradiated with ultraviolet light to randomly induce dimers, are investigated. T4-endonuclease V (a small enzyme specifically nicking at pyrimidine dimers: 62) induces strand breaks that can be used as a measure of accessibility of the damaged DNA. If accessibility of damaged DNA for such a small enzyme is impaired, than this is

even more likely to be the case for bigger eukaryotic repair enzymes such as the polymerases. Similar types of experiments using X-irradiated nuclei as "endogenous" templates for DNA polymerases might shed additional light on the (potential) significance of accessibility of damaged sites to repair enzymes. The decreased accessibility of damaged sites in the DNA may either be the direct result of a general increase of protein binding to the nuclear matrix or to a more specific phenomenon such as a decreased accessibility of the topoisomerase II sites (Chapter 6). The function of this enzyme, which catalyzes DNA relaxation/re-coiling, may be a key to repair (1.4.3, 1.4.4) and impairment of this function by hyperthermia may, therefore, affect DNA repair. Heat effects on DNA repair might also be a result of a depressed transcriptional activity after exposure of cells to heat. Hyper-thermic inhibition of repair and of transcription might be tightly coupled via hyperthermic alteration of the structure of the nuclear matrix. DNA repair was found to be enhanced in (nuclear matrix-associated) active genes (1.3, 1.4); this could result from a more open conformation of the DNA during the process of transcription or from a coupled repair-transcription process (1.3.5). Parallel measurements of thermal effects on transcription, DNA repair and radiosensitivity in relation to matrix alterations (again using the induction of thermotolerance as a tool) will be done in the near future.

12.3 Implications of the obtained results for clinical hyperthermia

Studies on the effect(s) of heat shock give more insight how a cell deals with stress situations. Especially the function of the (evolutionary) highly conserved "stress proteins" (HSPs) under both stress and physiological situations may be elucidated in this way. Apart from extending our know-ledge of thermobiology, the reported experiments have a relevance for clinical applications of hyperthermia. Successful use of combinations of hyperthermia and radiation depends on a number of factors (see Chapter 1). Preferential heating of tumor tissue and the time allowed between radiation and the hyperthermia treatment are just two of them. The extent of heat-radiosensitization is related to the heat dose as well as to the time span between the two treatments. The results reported in this thesis show that certain drugs influence heat-induced nuclear protein binding and, as such, affect heat-radiosensitization. Especially the time factor (time-span in between treatments) may be modified by drugs and previous heat treatments (thermotolerance). This type of insight in underlying mechanisms allows for favorable manipulation of clinical protocols.

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SAMENVATTING

WARMTE-GEINDUCEERDE VERANDERINGEN IN DE CELKERN
Relatie met hypertherme celdood en stralingssensibilisatie

Hyperthermie (een warmtebehandeling van cellen boven hun normale groeitemperatuur) kan leiden tot het verlies van het reproductive vermogen van eukaryote cellen. Tevens kan het de stralingsgevoeligheid van deze cellen verhogen. Zowel binnen de biologie alswel binnen de geneeskunde is er toenemende belangstelling voor hyperthermie. Binnen de biologie kan het worden gebruikt als middel om de reacties van cellen onder uitwendige "stress" situaties te onderzoeken. Kennis van de regulatie en functie(s) van stress-geinduceerde eiwitten ("heat shock proteins": HSPs) kan mogelijk leiden tot een beter begrip van processen als genexpressie, celproliferatie en aanpassing van cellen aan een veranderde omgeving. Klinisch gezien is hyperthermie interessant omdat het kan worden aangewend als een adjuvans in de radiotherapeutische behandeling van kanker. Meer kennis van de processen die leiden tot hypertherme celdood en -stralingssensibilisatie is nodig voor optimaal klinisch gebruik van deze combinatietherapie.
Met behulp van in vitro experimenten met menselijke (HeLa S3) cellen en cellen van de muis (Ehrlich Ascites Tumor cellen and LM fibroblasten) is onderzoek gedaan naar zowel hypertherme celdood als hypertherme stralingssensibilisatie.

Het celdodende karakter van hyperthermie (40-47^{o}C) neemt toe met de hoogte van de temperatuur en met de tijd van blootstelling aan deze temperatuur. De effecten van warmte werden vooral onderzocht op het niveau van de eukaryote celkern. Een verhoogde binding van eiwitten aan structuren in deze celkern werd waargenomen ten gevolge van de hypertherme behandeling van cellen (hoofdstuk 2,7). De hoeveelheid extra gebonden kerneiwit én de duur van deze verhoogde binding bleken gerelateerd te zijn aan de mate van hypertherme celdood. Deze correlatie bleef bestaan (hoofdstuk 2,3) onder condities waar de warmtegevoeligheid van de cellen was verhoogd (met agentia als ethanol en procaine) of verlaagd (met het agens glycerol of via de inductie van thermotolerantie door een voorbehandeling met warmte).
De verhoogde eiwitbinding in de kern bleek voornamelijk op te treden aan het kern(eiwit)skelet, de zogenaamde kernmatrix (hoofdstuk 4,5,11).
Metname de regulatoire (topoisomerase II) plaatsen ter hoogte van de aanhechting van de DNA lussen aan de kernmatrix bleken te zijn veranderd mogelijk ten gevolge van deze verhoogde eiwitbinding (hoofdstuk 6). Niet het aantal (hoofdstuk 4) maar de aard (hoofdstuk 5) van deze aanhechtingsplaatsen bleek te verschillen. Gezien het feit dat de kernmatrix als een dynamische structuur wordt beschouwd, waar verscheidene DNA-gebonden processen zich lijken af te spelen (zie 1.3), wordt gesuggereerd dat de verhoogde eiwitbinding aan de matrix deze processen aantast, hetgeen dan

warmtedood tot gevolg heeft. Als mogelijk mechanismen worden voorgesteld (hoofdstuk 12): een verminderde plooibaarheid van de kernmatrix, een verminderde mogelijkheid tot regulatie van de DNA supercoiling, een verminderde bereikbaarheid van aan de matrix gelegen regulatoire DNA sequenties en "late" DNA schade. De eiwitten die na verwarming in verhoogde mate kerngebonden blijven zijn niet-histon-eiwitten en deze zijn niet van cytoskeletaire origine. Een verhoogde activiteit van de DNA polymerasen α en β werd gevonden in kernen geisoleerd uit verwarmde cellen (hoofdstuk 7). Met behulp van gel electroforese (SDS-PAGE) werd gevonden dat er een grote verscheidenheid aan polypeptiden in geisoleerde kernstructuren van verwarmde cellen gebonden bleef, die niet of nauwelijks aanwezig waren in dezelfde geisoleerde structuren van onverwarmde cellen (hoofdstuk 5,11). Met behulp van immunoblotting werd aangetoond dat één van deze eiwitten behoort tot de groep van de HSP70 eiwitten. De gevonden abnormale eiwitbinding in de kern wordt verondersteld de aanleiding te zijn voor HSP synthese. Met de uit de literatuur bekende eigenschappen van HSP70, wijzen onze gegevens op een mogelijke rol van HSP70 bij het herstel van de warmte-geinduceerde verandering in eiwitbinding aan het kernskelet. Meer HSP70 ten tijde van cellulaire verwarming (bv. in thermotolerante cellen) versnelt het herstel van de verhoogde eiwitbinding aan kernstructuren na de verwarming van cellen, waardoor tijdelijke "thermoresistentie" ontstaat (hoofstuk 2,3).

Celdood na ioniserende straling is hoogstwaarschijnlijk het gevolg van niet- of fout-gerepareerde DNA schade (zie: 1.3, 12.2). De toename in stralingsgevoeligheid van cellen na verwarming werd dan ook onderzocht op het niveau van DNA schade inductie en reparatie. Hyperthermie heeft vooral effect op dit laatste. Reparatie van schade gedetecteerd met de alkalische ontwindings techniek (hoofstuk 9) en de fluorescente halo techniek (hoofdstuk 11) bleek te zijn geremd. De verhoging van de stralingsgevoeligheid van cellen door hyperthermie kan echter niet volledig verklaard worden door remming van de DNA reparatie (12.2). De uiteindelijke effectiviteit van DNA reparatie is hoogstwaarschijnlijk afhankelijk van alsnog onbekende factoren die bijdragen tot hypertherme stralingssensibilisatie.

Aanvankelijk werd er een goede relatie gevonden tussen het verlies van cellulaire activiteit van de reparatie enzymen DNA polymerase α en β en de mate van stralingssensibilisatie door warmte (hoofdstuk 8,12). Het effect van thermotolerantie op beide parameters was vergelijkbaar voor de situatie waarin warmte direct gevolgd werd door de bestraling (hoofdstuk 10). Wanneer echter warmte en straling gescheiden werden middels een tijdsinterval hield de correlatie niet langer stand. Hypertherme stralingssensibilisatie verdween sneller in tolerante dan in niét-tolerante cellen. Het herstel van DNA polymerase activiteit verliep echter op gelijke wijze in de tolerante en niet-tolerante cellen (hoofdstuk 10). Experimenten waarin het gecombineerde effect van hyperthermie en aphidicoline (een DNA polymerase α remmer) op DNA reparatie werd onderzocht, toonde eveneens aan dat warmte-geinduceerd verlies van cellulaire DNA polymerase α activiteit geen doorslaggeven-

de factor is bij door hyperthermie geinduceerde remming van DNA reparatie (hoofdstuk 9). Ook de analyse van literatuur gegevens (zie 12.2) laat grote twijfel bestaan over een functionele relatie tussen het verlies van cellulaire DNA polymerase activiteit en stralingssensibilisatie. Veranderde beschikbaarheid van de DNA polymerases voor het reparatie proces door de verhoogde en gerealloceerde binding aan kernstructuren na verwarming van cellen, is nog een nader te onderzoeken mogelijkheid.
Duidelijke resultaten werden verkregen met betrekking tot een correlatie tussen de totale toename van eiwitbinding in de kern op het moment van straling en de remming van de DNA reparatie (gemeten met de halo-assay: hoofdstuk 11) en tussen de toename van kerngebonden eiwit en de mate van stralingssensibilisatie (overleving: hoodstuk 10) door hyperthermie. Deze correlatie bleef tevens bestaan onder condities waarin cellen thermotolerant waren gemaakt. Ook het versnelde verlies van hypertherme stralingssensibilisatie in tolerante cellen was terug te vinden op het niveau van verhoogde kerneiwit binding (hoofdstuk 10). Mogelijke mechanismen via welke een verhoogde kerneiwit binding tot een verhoging van de stralingsgevoeligheid zou kunnen leiden zijn:

1. verandering in de distributie en binding van reparatie enzymen (functionele activiteit) zoals waargenomen voor DNA polymerasen (hoofdstuk 7)
2. reductie in the bereikbaarheid van het beschadigde DNA voor de reparatie enzymen. Dit wordt afgeleid uit de verlaagde detectie van schade gemeten met de halo-assay (hoofdstuk 11), de verlaagde bereikbaarheid van de topoisomerase II plaatsen in het DNA (hoofdstuk 6) en gegevens uit de literatuur.

Concluderend kan worden gesteld dat hyperthermie veranderingen in de celkern teweeg brengt die belangrijk lijken voor zowel hypertherme celdood als hypertherme stralingssensibilisatie. De parameter "verhoogde eiwit binding in de kern" is gecorreleerd aan de hypertherme celdood als hierbij zowel met de mate als de tijdsduur van deze binding rekening wordt gehouden. Voor de verhoogde stralingsgevoeligheid kunnen veranderingen in de eiwit binding in de kern een bepalende rol spelen via veranderde interacties tussen beschadigd DNA en de reparatie enzymen, leidend tot een minder adequate reparatie.

SUMMARY

HEAT-INDUCED ALTERATIONS IN THE CELL NUCLEUS
Relation to hyperthermic cell killing and radiosensitization

Hyperthermia (exposure of cells to temperatures above their normal growth temperature) may kill eukaryotic cells and may also enhance the radiosensitivity of those cells that survived the heat treatment. Studies on the action of hyperthermia are of biological as well as of clinical importance.
Biologically, hyperthermia serves as a tool to investigate cellular responses to environmental stress. Knowledge of regulation and function(s) of stress induced proteins ("heat shock proteins": HSPs) are helpful in elucidating the mechanisms of gene control and, cell proliferation, and understanding adaptation or protection of cells to an altered environment. Clinically, the possible use of hyperthermia as an adjuvant in the radiotherapeutic treatment of cancer needs the understanding of mechanisms that underlay heat-induced cell death and radiosensitization. By in vitro heating of established human (HeLa S3) and rodent (Ehrlich Ascites Tumor and LM fibroblast) cell lines, both heat killing and radiosensitization were investigated.

Eukaryotic cells are progressively killed by hyperthermia (40-46^{o}C) with increasing time and temperature. The effects of such heat treatments were investigated at the level of the cell nucleus. Upon exposure of cells to hyperthermic temperatures, changes in the tightness of protein binding to nuclear structures were observed (Chapters 2 and 7). The amount and duration of such binding was found to be related to ensuing the extent of heat killing; this correlation held under conditions that both enhanced (using heat sensitizers like procaine or ethanol) and reduced (using the heat protector glycerol or via the induction of thermotolerance) thermal killing (Chapters 2 and 3).
The enhanced binding of proteins appeared to occur specifically at the nuclear matrix (Chapters 4,5 and 11) and at least in part to regulatory (topoisomerase II) sites present at the basis of DNA loops attached to the nuclear matrix (Chapter 6), thereby affecting the nature (Chapter 5) but not the number (Chapter 4) of DNA-matrix attachment sites. Since the nuclear matrix is a highly dynamic structure, involved in the regulation of various DNA-associated processes (see 1.3), it is suggested that the enhanced binding of proteins to this structure may affect these functions and result in thermal cytotoxicity. A less malleable matrix, an inhibition of DNA supercoiling ability, the restriction of matrix-attached regulatory sequences, and "late" DNA damage, may cause this enhanced nuclear protein binding may to become cytotoxic (Chapter 12). The proteins involved in the enhanced binding to the nuclear structure appear to be non-histone proteins and are not of cytoskeletal origin. More DNA polymerase α and β activity was found to be retained in nuclei isolated from heated cells (Chapter 7). Polyacryl-

amide gel electrophoretic analysis (SDS-PAGE) revealed an abundance of polypeptides that remained bound to nuclear structures after heating of cells while they were only present to a minor extent or even absent in similar structures from unheated cells (Chapters 5 and 11). One of these proteins was characterized by immunoblotting as belonging to the group of HSP70s. The aberrant protein-protein binding in the nucleus directly after heating may serve as a trigger for HSP synthesis. Combined with data from the literature about the properties of the HSP70s, our results point to a role of HSP70 in the restoration of the heat-induced alterations in nuclear protein binding. Hence, the presence of an increased amount of HSP70s in the nucleus at the time of heating of cells (e.g., in thermotolerant cells) will lead to an enhanced rate of restoration of normal nuclear architecture after the heat treatment, leading to protection against thermal cell death (Chapter 2 and 3).

Radiation-induced cell killing is probably caused by non- or misrepaired damage to the DNA (see 1.3; 12.2). The observed increased radiation sensitivity upon exposure of cells to heat was investigated with respect to DNA damage induction and repair. Hyperthermia mainly affects the latter. Repair of radiation-induced damage as measured using the alkaline unwinding technique (Chapter 9) and the fluorescent halo-assay (Chapter 11) was inhibited. The effect of hyperthermia on DNA repair rates as such is not sufficient to fully explain radiosensitization (Chapter 12). The efficiency of DNA repair is dependent on several, yet unknown factors which may contribute to the extent of thermal radiosensitization.

Initially a good correlation was found between the loss of cellular activity of the repair enzymes DNA polymerase α and β and the extent of heat radiosensitization (Chapters 8 and 12). The effect of thermotolerance on heat-induced loss of polymerase activities resembled the extent of radiosensitization when heat treatment was immediately followed by radiation (Chapter 10). However, as the time interval between heat and radiation was increased, this correlation did not hold. Heat radiosensitization disappeared more rapidly in tolerant than in nontolerant cells. The recovery of cellular polymerase activities, however, occurred with similar kinetics in both tolerant and nontolerant cells (chapter 10). Combined application of heat and aphidicolin (a DNA polymerase α inhibitor) on DNA repair also revealed that heat-inactivation of cellular DNA polymerase α activity cannot be a (major) determinant in hyperthermic inhibition of repair (Chapter 9). Analysis of data from the literature (see 12.2) also sheds doubt on a functional relation between heat-induced loss of cellular DNA polymerase activity and radiosensitization. Changes in availability of DNA polymerases for the damaged DNA in the cells, due to enhanced binding of these enzymes to the nuclear matrix and to intracellular reallocation after heating, may be important factors and should be taken into consideration.

Data were obtained showing that the overall observed increase in binding of proteins to the nuclear matrix at the moment of radiation was related to the inhibition of DNA repair (using the halo-assay: Chapter 11) and the extent

of radiosensitization (survival: Chapter 10) after hyperthermia. This correlation held under conditions of thermotolerance; also the more rapid recovery from radiosensitization in tolerant as compared to nontolerant cells was reflected at the level of nuclear protein binding (Chapter 10). Enhanced nuclear protein binding at the time of irradiation may lead to radiosensitization through:

1. a change in distribution (functional activity) of repair enzymes as suggested for the DNA polymerases (Chapter 7).
2. a reduction in the accessibility of the damaged DNA for the repair enzymes as suggested by the reduced detectability of damage using the halo-assay (Chapter 11), the reduced accessibility of the topoisomerase II sites in the DNA (Chapter 6), and data from the literature.

In conclusion, the results show that hyperthermia causes changes at the level of the cell nucleus that may be important for both thermal cell death as well as for thermal radiosensitization. The parameter "enhanced nuclear protein binding" correlates with hyperthermic killing when both the extent and duration of this binding are taken into account. For the enhanced radiosensitivity, the heat-induced alterations in intranuclear protein binding may play a determining role, altering the normal interactions between damaged DNA and repair enzymes, leading to less adequate repair.

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H.H. Kampinga, G. v.d. Kruk, A.W.T. Konings, Reduced DNA break formation and cytotoxicity of the topoisomerase II drug 4'-(9'-acridinylamino) methanesulfon-m-anisidide when combined with hyperthermia in human and rodent cell lines. Cancer Research (1989) in press

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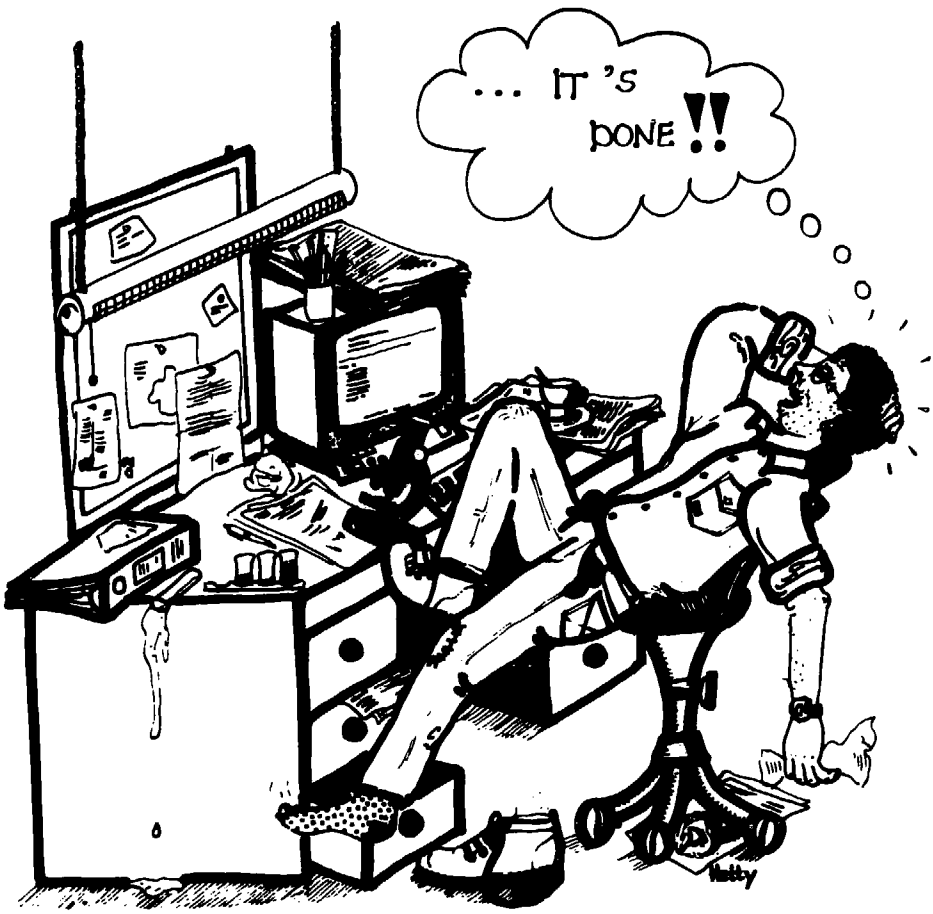
... IT 'S
DONE !!

STELLINGEN

behorend bij het proefschrift

HEAT-INDUCED ALTERATIONS IN THE CELL NUCLEUS
Relation to hyperthermic cell killing and radiosensitization

van

Harm H. Kampinga

Groningen 26 mei 1989

1.

De waargenomen toename van het eiwitgehalte van de geïsoleerde celkern ten gevolge van een hyperthermie behandeling van cellen kan beter worden omschreven als verhoogde kerneiwitbinding dan als kerneiwitaccumulatie.
dit proefschrift

2.

De conclusie van Warters dat hyperthermie DNA-afhankelijke processen remt op het niveau van de kernmatrix, wordt niet voldoende ondersteund door zijn experimentele gegevens, en is derhalve prematuur.
Warters R.L. Radiation Res. 115 (1988) 258

3.

Radford's veronderstelling, dat slechts de initiële produktie en niet de mate van reparatie van "dubbelstrengsbreuken" relevant is om de lethale respons van zoogdiercellen na bestraling te begrijpen, is niet alleen in tegenspraak met zijn eigen "critical-target-misrepair" model, maar ook niet conform zijn eigen experimentele gegevens en die van (vele) anderen.
Radford I.R., Hodgson G.S. and Matthews J.P. Int. J. Radiat. Biol. 54 (1988) 63

4.

Daar trypaanblauwopname en morfologische veranderingen geen accurate maat zijn voor hypertherme celdood, is de conclusie van Riabowol et al., dat microinjectie met HSP 70-antibodies de toxiciteit van een hypertherme behandeling verhoogt, (vooralsnog) onjuist.
Riabowol K.T., Mizzen L.A. and Welch W.J. Science 242 (1988) 433

5.

Hypertherme behandeling van autoloog beenmerg transplantaat verdient op grond van de experimenten van Moriyama et al. meer aandacht.
Moriyama Y., Narita M., Sato K., Urushiyama M., Koyama S., Hirosawa H., Kishi K., Takahashi M., Takai K. and Shibata A. Blood 67 (1986) 802

6.

Hoewel het gebruik van hyperthermie in de behandeling van AIDS -in principe- tot de mogelijkheden behoort, is de door Yatvin gegeven onderbouwing hiervan volstrekt onvoldoende en schept deze onterechte verwachtingen.
*Weatherburn H. Br. J. Radiology **61** (1988) 862; Yatvin M.B. Medical Hypothesis 27 (1988) 163*

7.

Als de recente berichten over het "broeikas effect" bij wetenschap, politiek en samenleving in onvoldoende effect resulteren, is de kans groot dat bij ons nageslacht meer dan alleen de gemoederen zullen worden verhit.
*Schneider S.H. Science **243** (1989) 771*

8.

Het feit dat velen een enorme vliegangst hebben, geeft aan dat men niet alleen in de techniek, maar vooral in de statistiek geen vertrouwen heeft.

9.

Dat rokers, naast een poging tot zelfdoding, bovendien willens en wetens ook bij anderen de kans op longkanker verhogen, wijst op de waarschijnlijkheid dat roken, naast de longen, wellicht ook de hersenen aantast.

10.

De hitparades van tegenwoordig zijn een dreun in je gezicht.

11.

Werkeloosheid is een overtreding van de grondwet door de meerderheid van het volk.

12.

Proefschriften kunnen bij de R.U.G. klaarblijkelijk nog tot stand komen ondanks de "goedkoop-duurkoop" politiek bij het aanschaffen van copieermachines.

13.

Evolutie o.k., maar door een groots Creator.

14.

Want wie nadenkt over God, valt van de ene verbazing in de andere en komt voor vraag na vraag te staan.
Stellinga P. In: Langs de rand (Jeugdburo N.B.J.B.: Drachten)