ORGANIZATION OF DNA IN AND AROUND THE REPLICATION POINT IN EUKARYOTIC CELLS

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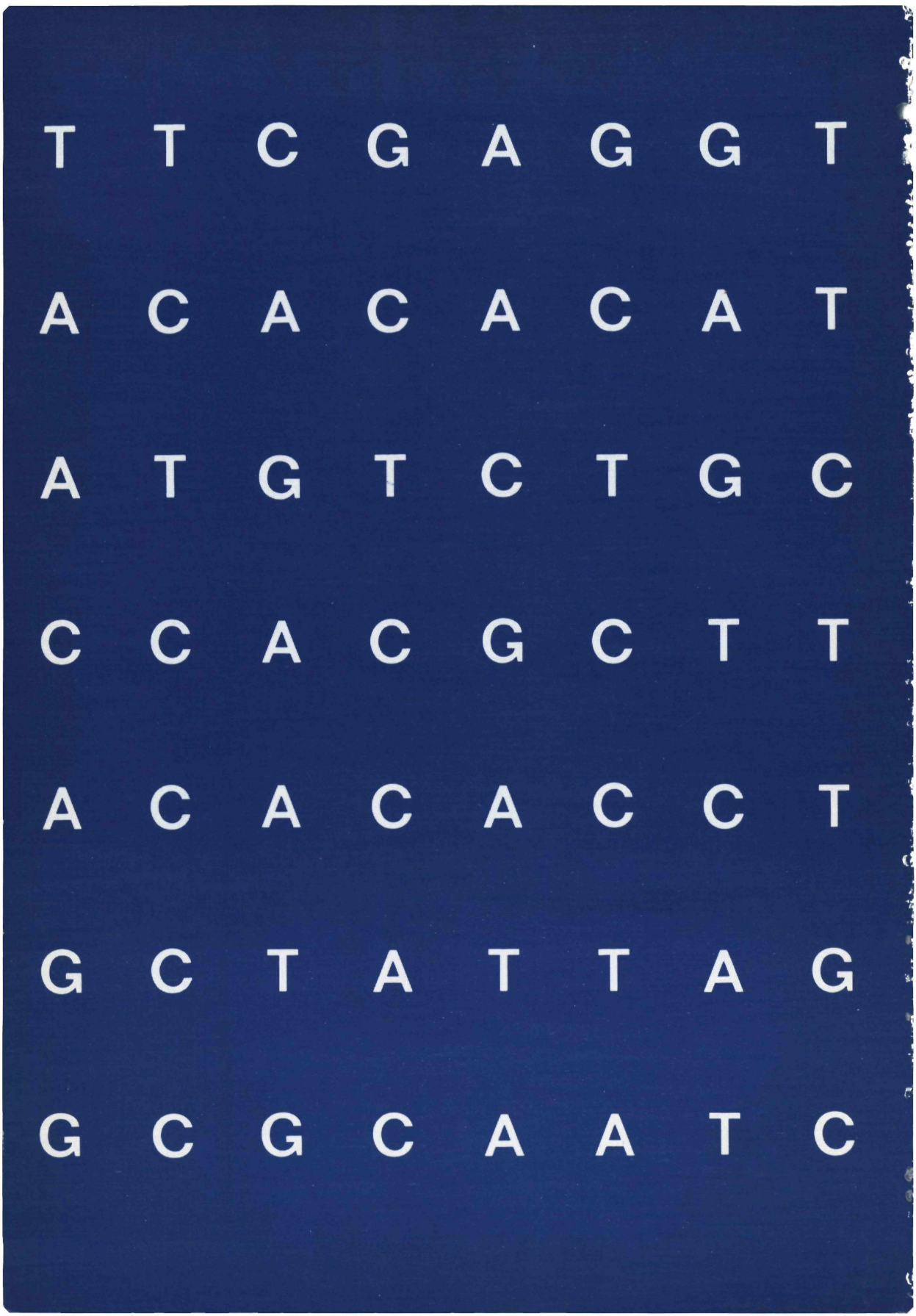
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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN
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PROF. DR. P.G.A.B. WIJDEVELD
VOLGENS HET BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN OP
DONDERDAG 12 FEBRUARI 1981
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GEBOREN TE HILVERSUM

Druk: Stichting Studentenpers
Nijmegen
1981

Aan allen, die op hun
wijze deel hebben gehad
in de totstandkoming
van dit proefschrift.

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Chapter I

GENERAL INTRODUCTION

I. GENERAL INTRODUCTION

I.1. *Introduction*

In each organism duplication of the genetic material is one of the most fundamental processes. For the eukaryotic cell this is a formidable task since in a time-lapse of a few hours only, a genome with a length of up to several meters, has to be copied. This genome, subdivided into several chromosomes, consists of chromatin, i.e. DNA intimately associated with a number of classes of proteins, of which the class of the histones in one of the most prominent. The replication process, therefore, requires coordinately a correct duplication of DNA, the synthesis of the right amounts and types of proteins and the assembly of these components to form two daughter chromatin strands that are identical to the parental one. Moreover, the mechanism has to provide for that in each cell cycle duplication takes place only once. It will be clear that, besides temporal regulatory processes, functional organization of the genome for replication during interphase has received considerable attention in recent years.

I.2. *Chromatin Structure*

The basic structural unit of chromatin, whether in interphase or during mitosis, is the nucleosome (1-5). It consists of a stretch of DNA of approximately 200 base-pairs, complexed to a histone octamer (2,6,7) consisting of 2 molecules of H_{2A}, H_{2B}, H_3 and H_4 each. This structural repeating unit can be further subdivided into a "core particle", consisting of 140 base-pairs (8-11) wound around the outside of the histone octamer (12-16), and a "linker", i.e. a stretch of DNA of 40-60 base-pairs on the average, varying from species to species (10,11,14,17) and even within a species (18). Core-particles can be isolated from chromatin by a limit digestion with Staphylococcal nuclease, since the linker-regions are more susceptible to the action of the enzyme (8-11,19). Further analysis has revealed that DNA is wound

around the cylindrical histone octamers in 1,75 turns (15,20,21), so inducing one negative supercoil for each nucleosome (22). Ultrastructurally, chromatin appears in a beads-on-a-string conformation (1,2,23,29), in which the positioning of the nuclosomes along the DNA strand is a-specific with respect to base-sequence (24,25). In vivo, and partly due to histone H_1 which interacts with the linker-region (26,28), the beaded structure most likely exists in a more compact, solenoidal form (29,30). It is generally thought that the metaphase chromosome consists of a super-structure of the solenoid, in the modelling of which non-histone chromosomal proteins may play an important rôle (31,32).

Analysis of chromatin structure with DNase I showed that core-DNA is accessible to the nuclease, which cleaves DNA at intervals of 10 base-pairs. This could indicate that DNA is not smoothly bent around the histone-core (33), but instead exists in a periodically kinked form with a repeat length of 10 base-pairs (32,34).

With respect to DNA-replication, as well as to transcription, this basic organization of chromatin has important consequences. For instance, it provides one of the levels of regulation of the above-mentioned processes.

I.3. *Chromatin Replication*

The eukaryotic genome appears to be replicated in a temporally and spatially ordered pattern. It has been shown that specific parts of the genome replicated at defined periods within the S-phase (35-37), which suggests the existence of multiple units of replication. These units were shown to exist in subsequent years and were called "replicons" (38,39). Replicons appear to be clustered, i.e. duplication of sets of adjacent replicons takes place simultaneously (38-41). In each replicon, synthesis of daughter strands was found to start at a specific "origin" (42). From each origin two replication forks emerge, indicating that replication proceeds bi-directionally (38,43-45). As a consequence, "replication-

loops" are generated, which have been demonstrated to exist ultra-structurally (46). Termination seems to take place irrespective of the base-sequence, where two replication loops fuse (47). The size of replicons has been shown to vary from species to species (48) and, within a species, has been shown to depend on the stage of development (46). The observed values averaged 20-60 µm (48).

I.4. *Intermediates of Replication*

Since all known DNA-polymerases can only synthesize DNA in the 5'-3' direction (49), DNA-replication at first was thought to proceed in semidiscontinuous way. However, with the probable exception of small DNA-virusses (50-52), DNA-replication is now assumed to occur discontinuously on both parental strands (53-55).

Primary products of this process are the so-called "Okazaki fragments", replicative intermediates sedimenting at approximately 4S (51-54,56,57). *In vitro*, Okazaki fragments were shown to be primed by RNA-fragments of approximately 10 base-pairs (58,59). Studies using intact eukaryotic cells, however, have provided contradictory evidence on this subject (54,60-62). RNA-priming would be consistent with the fact that none of the known DNA-polymerases is able to initiate new DNA-chains (49).

The striking similarity of the length of the Okazaki fragments and the repeat length of nucleosomal DNA suggested that initiation of Okazaki fragments might be directed by the periodicity of spacing of the nucleosomes along the DNA-strand (63,64).

Okazaki fragments have been postulated to be initiated between the nuclosomes in the linker region (63,64), possibly in a pre-fork mode of DNA-synthesis (63,65). The fact that, *in vitro*, the fragments are initiated at random with respect to template base sequence (58,59), as would be expected from the positioning of the nucleosomes, is in agreement with this postulate.

In the next step of the replication process, Okazaki fragments

which seem to be incorporated into nucleosomes prior to ligation (66,67), are ligated to ultimately from intermediates of replicon size (53,54,56,57,68,69). Whether this elongation mechanism proceeds by the way of classes of intermediates of discrete lengths is still a matter of controversy, though their occurrence has been reported (53,69,70). In the final step the replicon-sized intermediates are joined to form mature chromosomal DNA (53,68,69).

I.5. *Inhibitors of DNA-synthesis*

Evidence confirming the phased mechanism of DNA-replication has been provided in abundance by studies that employ inhibitors affecting DNA-synthesis. Putative targets of such inhibitors are initiation (both of S-phase and of individual replicons), elongation of nascent chains and ligation of primary and secondary replicative intermediates. Inhibitors affecting initiation of S-phase generally do not act directly on the replication process, but instead, mostly seem to influence one or several steps in G_1-phase. This class of inhibitors, more or less falling outside the scope of this thesis, comprises several inhibitors of protein and RNA-synthesis and certain categories of mitogens (71,72). Initiation of replicons can be inhibited by damaging the DNA by means of irradiation. Doses introducing 1 hit, presumably a single-strand break for every 10^9 dalton DNA (the size of a replicon-cluster), are sufficient to produce this effect (73-75). Since this target size also corresponds to the size of the supercoiled subunits of the chromosome (76), it is generally assumed that, because of the relaxation of the supercoil in the replicon-cluster, initiation of every single replicon of that cluster is prevented (74,75). Inhibitors, such as actinomycin D, a known inhibitor of replicon initiation (77), acridines and anthracyclines, which by intercalating into DNA reduce its amount of supercoiling (78), might have an effect similar to that of X-rays. Alternatively, their helix-stabilizing properties (79-81) could also be the reason for their effect on replicon initiation, for which analogous to initiation of transciption

(82) a local denaturation at the origin is considered to be an essential step (63,64).
However, also inhibitors with a completely different mode of action affect initiation of replicons. Ara-C. a competitor with dC for DNA-polymerase (83,84), for instance, was shown to prevent the onset of replicon duplication (85). No clear reason for this effect is known at this moment but a cause might be an enrichment of origins with G-C-sequences (86).
Finally, it is necessary to mention inhibitors of protein synthesis, although their effect on replicon initiation only seems to be an indirect one (87).
A reduction of the rate of chain elongation seems to be the most manifest effect of inhibitors of protein synthesis (87,88). It was, therefore, concluded that DNA-replication was highly dependent on ongoing protein synthesis,possibly of histones(89). The nature of this coupling, however, is not yet quite clear (87,90,91).
Other classes of inhibitors have also been shown to retard fork progression. Amongst them is the class of the nucleoside analogues, which exert their action either by competitive inhibition of DNA polymerase (83,84 92) or by altering the properties of the 3'-ends of the nascent chains as a consequence of their incorporation into the DNA (93-95). A similar phenomenon is observed, when substances affecting nucleotide metabolism, such as hydroxyurea and FdUrd, are used (96,97).
Whether the inhibitors reducing the rate of fork progression, simultaneously inhibit ligation of primary replicative intermediates, is hard to determine, for as a consequence of inhibition of ligation, there will also be a relative accumulation of Okazaki fragments in pulse-labelling experiments. On the other hand, ligation of replicon-sized intermediates to form mature chromosomal DNA has been observed to be affected by an inhibitor of protein synthesis, cycloheximide (98). That ongoing protein synthesis is required for this process, might also be indicated by the observation that *in vitro* DNA-synthesizing systems, such as isolated nuclei, which lack protein synthesis, usually are defective in this respect (99-101).

I.6. *Replication and Intranuclear Structures*

In the bacteria the cell-envelope has been shown to be involved in the process of duplication of the genome (102,103). Analogously, for eukaryotic cells, the nuclear envelope has been proposed to play a comparable rôle. This seemed to be confirmed by the observation that replicating DNA was preferentially found in the 'M"-band (104,105), which consists of a complex of Mg-sarcosinate and membrane-material (106). Autoradiographic studies, however, revealed that synthesis of DNA occurs throughout the nucleus and is not confined to regions in the immediate proximity of the nuclear membrane (107-109). This apparent contradiction was solved when by means of extraction of interphase nuclei with high concentrations of salt, the existence of a fibruous network within the nucleus was demonstrated (110-113).
Comparable observations were done on isolated and dehistonised metaphase chromosomes (114,115). The nuclear protein structure is generally believed to consist of a pore complex-lamina, which apparently can be isolated separately (116,117), residual nucleoli and an internal network or matrix (110, 112). In conformity with the autoradiographic observations, biochemical analysis revealed that the replicating DNA is associated with the nuclear protein structure preferentially (118-120).

The aim of the study, presented in this thesis, was to gain further insight in the relationship between replicating chromatin and the intranuclear protein structure. Accordingly, both the mechanism of the replication of chromatin and the mode of attachment of replicating DNA to the nuclear structure were studied.
Replication was analysed mainly by interfering in the process by means of two different inhibitors of DNA-synthesis, i.e. arabinosylcytosine, to which chapter 2 is devoted, and daunomycin, the mode of action of which is described in chapter 3. In chapter 4 the attachment of dehistonised, replicating DNA to the nuclear protein structure is examined

in detail, using several different nucleases as probes. In chapter 5 it is investigated whether replicating DNA is attached to the nuclear protein structure in one site only. Finally, in chapter 6, the efforts and the results thereof, to gain insight in chromatin structure of newly replicated DNA are described.

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Chapter II

ENHANCED RELEASE OF NASCENT SINGLE STRANDS FROM DNA SYNTHESIZED IN THE PRESENCE OF ARABINOSYLCYTOSINE

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ENHANCED RELEASE OF NASCENT SINGLE STRANDS FROM DNA SYNTHESIZED IN THE PRESENCE OF ARABINOSYLCYTOSINE

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Summary

Arabinosylcytosine at a $1 \cdot 10^{-4}$ molar concentration inhibited thymidine incorporation into DNA by more than 95% In sucrose gradients the labelled dThd was predominantly found in short DNA chains Labelled arabinosylcytosine (aC) was incorporated into DNA, as was labelled dThd, indicating that it causes a preferential inhibition of the chain polymerization rather than termination of nascent chains This was confirmed by the observation of the conversion of short chains to normal size DNA molecules in chase experiments.

In the absence of NaCl and at neutral pH a release of more than 50% of the nascent label as single strands was repeatedly observed upon sucrose gradient centrifugation This release could be significantly reduced by 1 M NaCl, indicating that in the presence of aC the in vivo structure was preserved This was further confirmed by the fact that aC did not cause detachment of DNA from a rapidly sedimenting nuclear structure

Based on these results, a replication model slightly different from the one suggested by Okazaki, is proposed, in which initiations of new nascent chains can occur at certain distances ahead of the replication fork.

Introduction

In recent years inhibitors which interfere in a specific way with the replication process have been used succesfully in investigations on DNA synthesis and its regulation The overall replication comprises a number of steps, of which the initiation of replicons, the initiation of new DNA chains, the polymerization of the nascent chains and the joining of Okazaki fragments by polynucleotide ligase are putative targets of such inhibitors.

Abbreviations SDS sodium dodecyl sulphate aC arabinosylcytosine dThd, deoxythymidine

The antitumor agent arabinosylcytosine (aC) [1—3] has been shown to be a powerful inhibitor of DNA synthesis in eukaryotic organisms but the mode of its action is not yet entirely clear. In the cell the nucleoside is converted to aCTP [4] and incorporated into replicating DNA [5—7]. DNA synthesis, in vitro, is apparently terminated after aCMP has been attached to the 3'-end of the growing chain [8], however, in vivo experiments show the nucleotide to be incorporated into internal positions [5—7,9]. DNA-polymerase activity in vitro is apparently inhibited by competition of aCTP with dCTP (10—14], while there is some evidence that, in vivo, the initiation of new replicons is inhibited preferentially [15]. Other mechanisms of action of the inhibitor have also been suggested [16,17]. The main aim of this investigation was to establish the effect of aC on the initiation and elongation of Okazaki fragments. Particular attention was paid to the release of nascent single strands.

Materials and Methods

Source of materials used. 1-β-D-Arabinosylcytosine was purchased from Sigma and used without further purification. [*Me*-^{3}H]dThd (20 Ci/mmol), [5-^{3}H]aC (15 Ci/mmol) and [2-^{14}C]dThd (52.8 Ci/mol) were products of N.E.N. Pronase (nuclease-free) was obtained from Calbiochem. Calf thymus DNA was from Boehringer and hydroxyapatite (DNA-grade) from Biorad. All other chemicals were of analytical grade.

Cell culture and labelling procedures. Calf liver cells were grown in monolayer culture as described previously [18], except that a serum concentration of 10% was used. All labelling and inhibition experiments were performed at 37°C using pre-warmed solutions. DNA was prelabelled by addition of 0.04 μCi/ml [^{14}C]dThd for at least 36 h. After growing in label-free medium for another 2 h cells were pulse-labelled with 5 μCi/ml [^{3}H]dThd for 10 min or 1 h in the presence or absence of aC, or with 100 μCi/ml [^{3}H]dThd for 1 min without inhibitor. When aC was used, cells were routinely preincubated with the inhibitor for 30 min. For experiments on inhibition kinetics the cells were grown and prelabelled on cover slips. These were transferred to the medium of choice for appropriate times. The cells were then washed in 0.9% NaCl and dissolved in 0.5% SDS and appropriate volumes of the solution were used for the determination of the radioactivities [19].

Sedimentation analysis. Cell lysates were prepared in 0.5% SDS as described previously [19,20] and gently homogenized by passsage through a 20 cm long capillary with a diameter of 1 mm (10 times at a pressure of 0.5 atm.). 4—6 ml of the lysates were layered on the top of either 20—65% neutral (10 mM Tris · HCl, pH 7.5) or 5—25% alkaline (0.1 M NaOH containing 10 mM EDTA) sucrose gradients. The latter were prepared on a cushion of 4 ml 65% sucrose. The centrifugations were performed in a SW 27.2 rotor for 17 h at 20°C and at 24 000 or 20 000 rev./min, respectively. Fractions were collected starting from the bottom of the tube. For determinations of the sedimentation values the low molecular weight fractions of neutral sucrose gradients were combined, calf thymus DNA was added as a carrier and the DNA was precipitated by mixing with 2.5 vols. of 96% ethanol containing 1% potasssium acetate. After storing for 16 h at −15°C the precipitate was collected by centrifugation and redis-

solved in a 0.1 M NaOH solution containing 0.5% SDS and 10 mM EDTA. A sample of 1.0 ml was layered on top of a 5—20% alkaline sucrose gradient and centrifuged at 36 000 rev./min in a SW 40 rotor for 17 h at 20° C. S-values were estimated according to McEwen [21].

Chromatography on hydroxyapatite. The procedure described previously [22] was used with minor modifications. Combined peak fractions of the neutral sucrose gradients were digested with 50 μg/ml pronase for 30 min at 37° C. The samples were then diluted with an equal volume of 0.04 M phosphate buffer (pH 7.0). Hydroxyapatite, washed twice with 0.02 M phosphate buffer, was added and adsorption was allowed to proceed for 20 min. The suspension was poured onto a column and DNA was eluted by a 0.02—0.5 M phosphate gradient [23]. Fractions of 2 ml were collected for the determination of the radioactivity.

Determination of the radioactivitiy. DNA containing samples of appropriate size were processed [20] and the radioactivities were counted in a Philips Liquid Scintillation Analyser using a toluene-base scintillation fluid.

Results

1. Accumulation of replication intermediates

In the standard dose-response experiments it was found that the [^{3}H]dThd incorporation into DNA was inhibited by more than 90% at aC concentrations of 1 μM and higher. Therefore, experiments were routinely carried out at aC concentrations of $1 \cdot 10^{-4}$ M, ensuring an inhibition of more than 95%.

It was then investigated whether this inhibition was due to a reduced rate of initiations of new chains or whether other steps of the replication process such as chain elongation or joining of Okazaki fragments were preferentially affected. This was done by comparing sedimentation patterns of newly synthesized DNA in neutral and alkaline sucrose gradients.

[^{14}C]dThd prelabelled cells were labelled for 1 h with [^{3}H]dThd in the presence and absence of aC. The sedimentation patterns of ^{3}H- and ^{14}C-labelled DNA of the uninhibited cells coincided fairly well (Figs. 1A and C). However, the ^{3}H counts of the pulse-labelled DNA, in neutral gradients, exhibit one peak coinciding with the prelabelled DNA, and in addition, a slowly sedimenting peak. In alkaline gradients the two peaks were also predominant, though all other size classes were present (Figs. 1B and D). Increased amounts of pulse-labelled DNA which sedimented slowly in alkaline sucrose gradients were also obtained by use of lower aC concentrations [15], but so far this material has not been further analysed.

It might be argued that the slowly sedimenting DNA is degraded DNA and the labelled dThd-nucleotides are reincorporated into replicating DNA. However, chase experiments in the presence of aC showed no change of chain length of the pulse-labelled low molecular weight DNA and only a partial decrease in the amount of this material after 120 min (Fig. 2, column A).

In contrast, in the absence of aC, all slowly sedimenting DNA was shifted to the peak of ^{14}C-prelabelled DNA in about 120 min (Fig. 2, column B) indicating that this material represents a regular intermediate of the replication process.

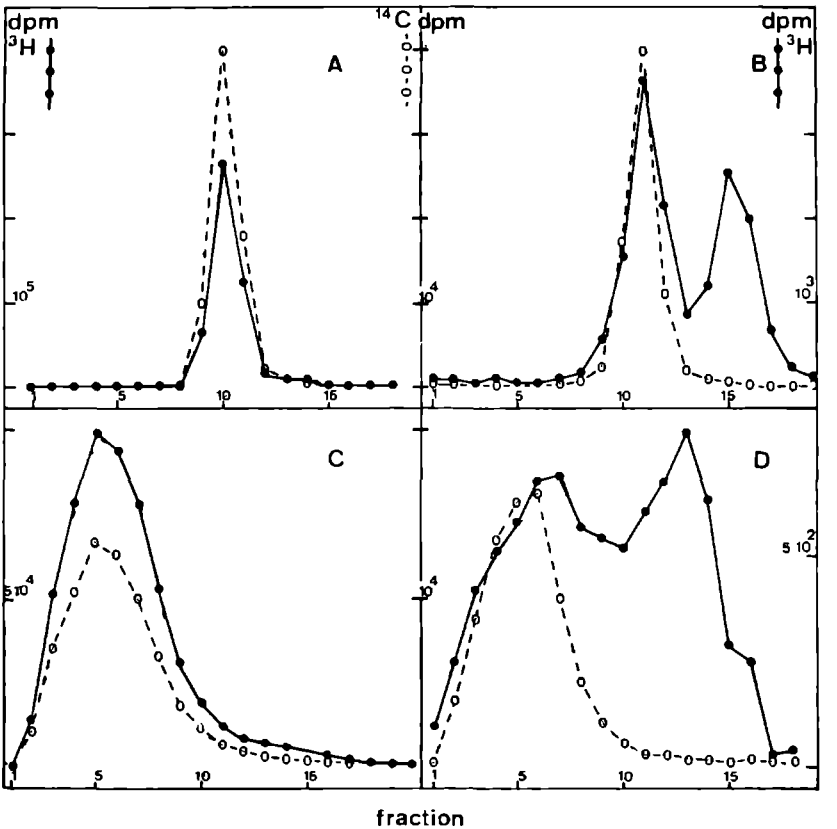

Fig 1. Sucrose gradient sedimentation patterns of labelled DNA from SDS-lysed cells. Cells, prelabelled with [^{14}C]dThd, were pulse-labelled for 1 h with [^{3}H]dThd in the absence (A and C) and in the presence (B and D) of $1 \cdot 10^{-4}$ M aC. Neutral gradients A and B, alkaline gradients· C and D.

[^{3}H]aC was incorporated into DNA in essentially the same way as was [^{3}H] dThd. Sedimentation analysis, on neutral sucrose gradients, of DNA labelled at both low and high concentrations of the inhibitor (Figs. 3A and B) also demonstrated the occurence of a fraction of 3 S-DNA. This DNA could be chased into high molecular weight DNA (Fig. 3C), and no loss of label was observed even when the chase was prolonged to 4 h.

2. Analysis and origin of the slowly sedimenting DNA

The DNA synthesized in the presence of aC was further analysed by chromatography on hydroxyapatite. Almost all of the label of the rapidly sedimenting fraction was eluted at the position of DNA duplexes, while the slowly sedimenting pulse-labelled DNA was recovered in the single-stranded DNA fractions (Fig. 4). Essentially the same results were obtained with DNA of control cells which were labelled for 1 min in the absence of aC (results not shown). Others have also found similar results for the majority of the fractions of slowly sedimenting nascent DNA, isolated under non-denaturing conditions [20,22, 24–27].

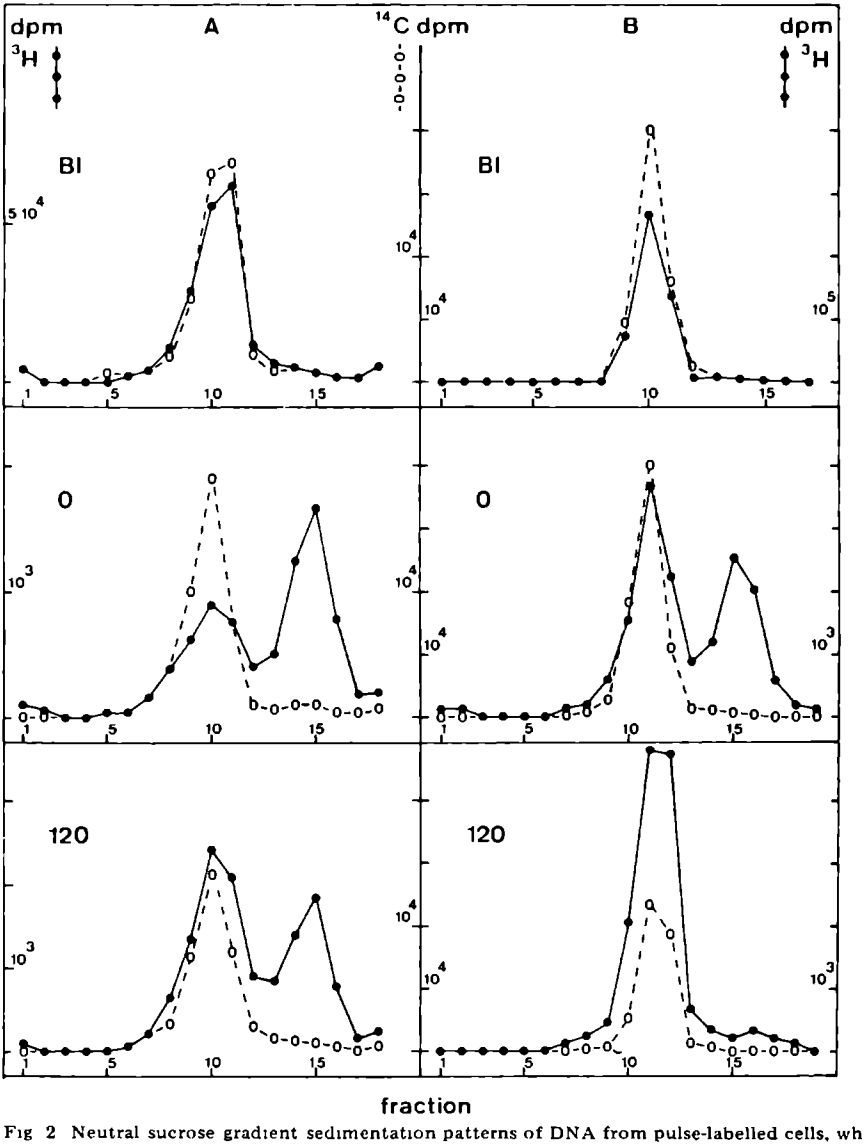

Fig 2 Neutral sucrose gradient sedimentation patterns of DNA from pulse-labelled cells, which were subsequently incubated in aC-containing and aC-free medium Cells, prelabelled with [^{14}C]dThd, were pulse-labelled for 1 h with [^{3}H]dThd in the presence of 1 10^{-4} M aC (except for the control) The cells were then transferred to label-free medium containing either 1 · 10^{-4} M aC (column A), or without inhibitor (column B). Cells were incubated for 2 h and were then lysed in SDS. B1. control, 1 h pulse without aC 0, 1 h pulse in the presence of aC; 120, 1 h pulse in the presence of aC followed by 2 h chase.

There is strong evidence that the release of nascent single strands from replicating DNA occurs by branch migration. This branch migration is facilitated by low stability of the double helix in the isolation media usually employed [22]. The release can be considerably suppressed when lysates are prepared at high NaCl concentrations which stabilize the secondary structure of the DNA molecule. A greatly reduced release was also observed when nuclear lysates of cells labelled in the presence of aC were prepared in 1 M NaCl/pronase [22]

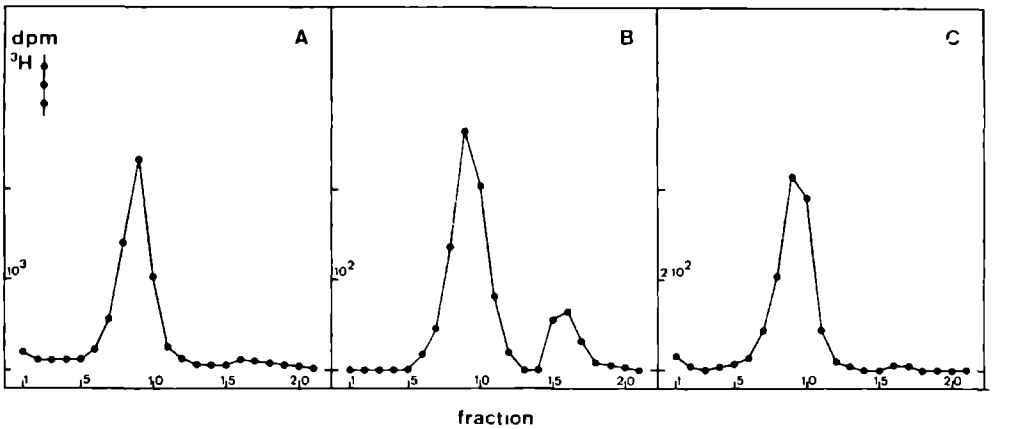

Fig. 3. [^{3}H]aC incorporation into DNA. Cells were pulse-labelled for 1 h with 5 μCi/ml [^{3}H]aC of different specific activities and the SDS-lysates were analyzed on neutral sucrose gradients. Sedimentation patterns of DNA from cells treated with (A), 3 10^{-7} M aC (B) 1 10^{-6} M aC, and (C) 4 10^{-6} M aC followed by an incubation of 1 h in aC-free medium

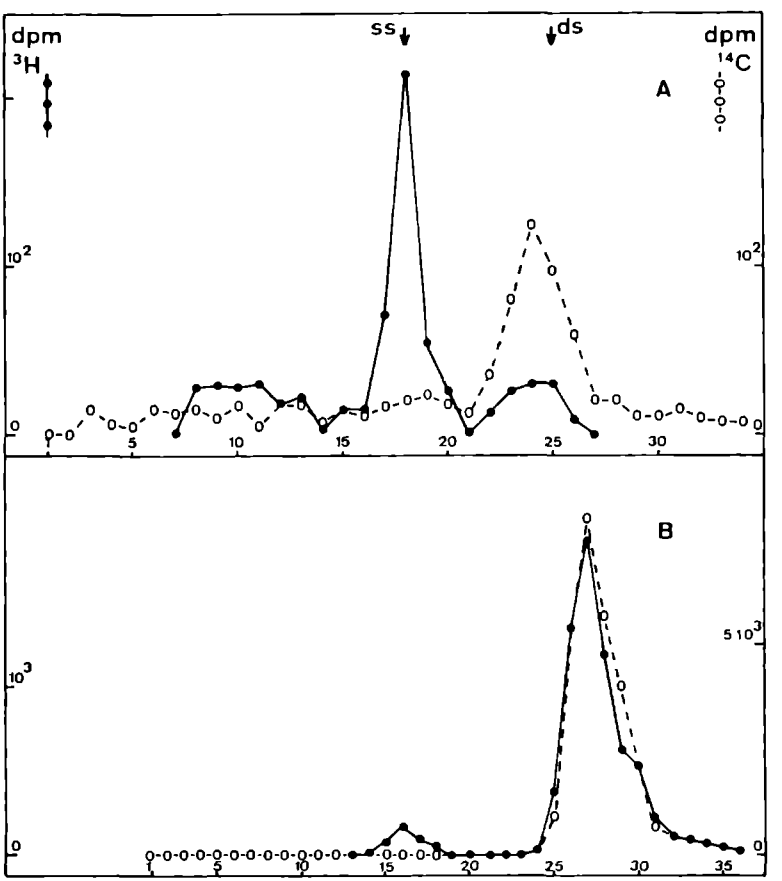

Fig. 4 Chromatography on hydroxyapatite [^{14}C]dThd-prelabelled cells were pulse labelled for 1 h with [^{3}H]dThd in the presence of $1 \cdot 10^{-4}$ M aC and then lysed in SDS. Slowly sedimenting and bulk DNA were isolated from neutral sucrose gradients, then adsorbed on hydroxyapatite and eluted with an increasing concentration phosphate buffer Elution patterns of (A), slowly sedimenting, and (B), bulk DNA.

(Fig. 5A). Centrifugation in neutral sucrose gradients resulted in a significant proportion of the ^{3}H-labelled DNA sedimenting slightly ahead of the peak of the parental DNA. When this fraction was isolated and redissolved in 0.5% SDS the 3 S nascent chains were again released in alkaline as well as neutral sucrose gradients (Figs. 5B and C).

Recently, Wanka et al. [28] have shown that replicating DNA remains

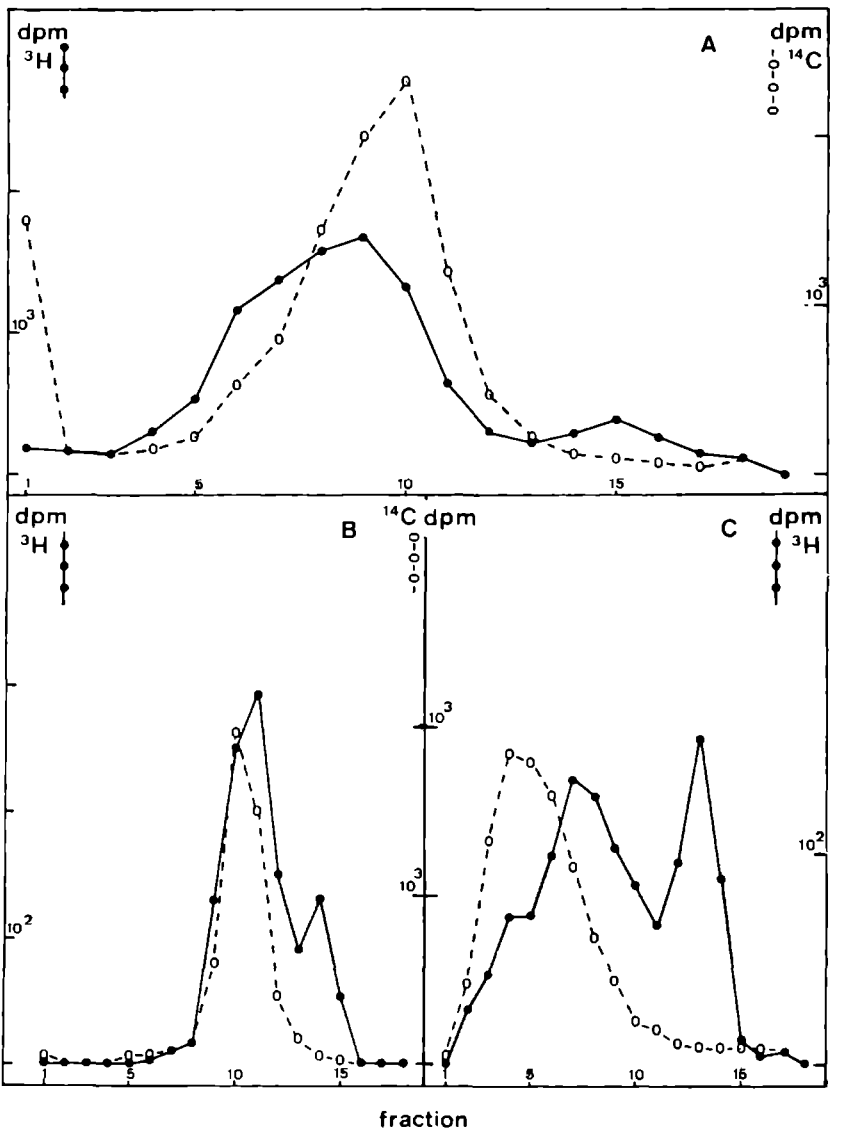

Fig. 5. Suppression of release of 3 S-chains by 1 M NaCl. [^{14}C]dThd prelabelled cells were pulse-labelled for 1 h with [^{3}H]dThd in the presence of 1 10^{-4} M aC. Nuclei were isolated and dissolved in 1 M NaCl/pronase. (A) Sedimentation pattern of the nuclear lysate on a neutral 1 M NaCl/sucrose gradient. DNA from fractions 4—7 was isolated, resuspended in SDS and analysed on neutral (B) and alkaline (C) sucrose gradients

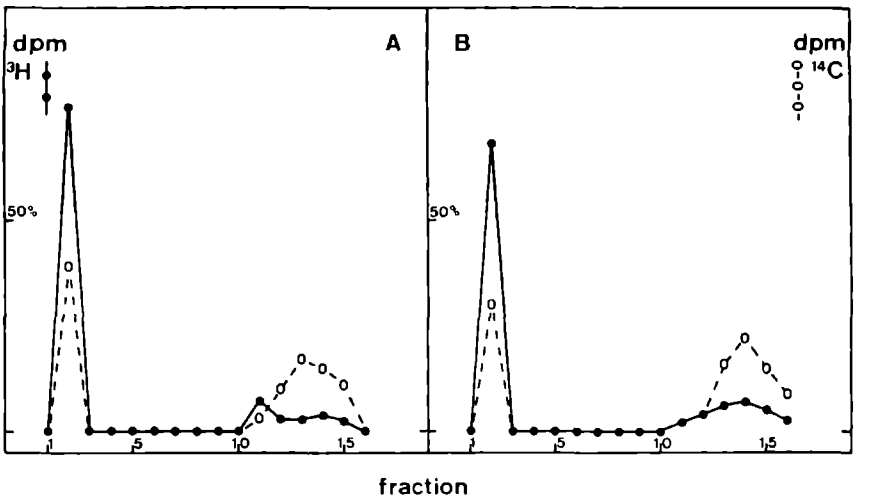

Fig. 6. Association with the rapidly sedimenting nuclear structure. [^{14}C]dThd prelabelled cells were pulse-labelled for 1 h with [^{3}H]dThd in the presence of $1 \cdot 10^{-4}$ M aC or for 1 min in the absence of the inhibitor. Nuclei were isolated and dissolved in 1 M NaCl. Sedimentation patterns on neutral sucrose gradients [28] of DNA from aC treated cells (A) and 1-min pulsed cells (B). Total incorporation: (A), ^{14}C. 12 400 dpm, ^{3}H: 25 200 dpm, (B), ^{14}C 14 400 dpm; ^{3}H: 7000 dpm.

associated with a rapidly sedimenting structure in nuclear lysates prepared in 1 M NaCl. Such a structure could play an important role in the ordered replication of the nuclear DNA molecules [29]. Therefore, to examine whether the inhibition by aC might be accompanied by a dissociation of the replicating DNA from this nuclear structure, a NaCl lysate was prepared from nuclei isolated from cells which were grown in the presence of both label and inhibitor. Fig. 6A shows that the newly synthesized DNA remained preferentially associated with the rapidly sedimenting structure. Obviously, the inhibition by aC has no effect on the binding of the replicating DNA.

Discussion

Our labelling experiments show a relative accumulation of short nascent DNA chains in the presence of aC. The sedimentation values vary up to 5 S with a maximum at 3 S (results not shown), while Okazaki fragments in eukaryotes are about 4 S [30,31]. The most likely cause for a preferential accumulation of growing Okazaki fragments is a deceleration of the polymerization process, at an unchanged, or slightly decreased rate of initiation of new chains. This is consistent with the observation, in vitro, that the DNA polymerase activity is inhibited by aC in a competitive way [11—14].

This conclusion was reached previously by Wist et al. [14]. They showed with an in vitro system, that aCTP reduced the length of Okazaki fragments produced during a 60-s pulse by 50%. Further, contrary to our observations, these nascent chains could be chased, at rates near to those in inhibited nuclei, into high molecular weight DNA.

We have shown that the labelling patterns are not, to any significant extent, caused by aC-induced repair processes, degradation of newly synthesized DNA,

or detachment of replicating DNA from the rapidly sedimenting nuclear protein structure. The low molecular weight DNA increases to normal size DNA chains during chases in the absence of aC, indicating that it represents regular intermediates of the DNA replication.

Our results indicate that, as in other systems [5,7,9], aC is incorporated into DNA in internal positions. One may assume that this is the normal case, at least in mammalian cells, and that the chain termination, as found in vitro [8], is not representative for the in vivo situation.

The incorporation of label into DNA chains which are multiples of Okazaki fragments indicates that joining of these fragments continues in the presence of aC. Fridland [15] has found an increase in the average size of longer DNA chains during inhibition by aC. From the shift of the sedimentation peak from 38 to 70 S he concluded that the initiation of new replicons is inhibited. Initiation of Okazaki fragments and initiation of new replicons may therefore be considered to be different types of processes. It is obvious that the interference of aC with the initiation of new replicons must be stronger than that with the formation of Okazaki chains. Nevertheless, the mode of action might be the same if one assumes that the origins of replicons are enriched with clusters of cytosine sequences and, therefore, would be particularly subject to the action of aC. Future experiments will have to show whether the initiation of new replicons is inhibited by aC in this way or whether a basically different mechanism is involved.

Release by branch migration of nascent single strands from duplexes with parental strands requires that the complementary regions in the opposite parental strands are fully unpaired [22]. According to the conventional Okazaki model of replication this above mentioned release can only apply for the chains which are polymerized in the direction of the fork movement [32], i.e. only the strands which, in principle, could be synthesized continuously. Thus release of nascent single strands from unhibited [22] and aC inhibited cells is proof of a two-strand discontinuous replication. Such a mechanism has also been inferred from results obtained with HeLa cells [33,34], bacteriophages [35], and mammalian viruses [36].

Release of single stranded DNA by brach migration would only apply for the leading Okazaki pieces in the replication fork. These leading Okazaki pieces would only account for a small fraction of the pulse-label incorporation into nascent DNA. Our data show, however, that up to 50%, occasionally even more, of the pulse-label was released as single-stranded 3 S material. A simple interpretation of these data is possible on the basis of a modified version of the concept of pre-fork replication [37] and the microbubble hypothesis [38] shown in Fig. 7.

According to this model new Okazaki fragments are initiated at a certain distance ahead of the two forks of the active replicon. Duplexes of nascent and template strands are formed in such a way that the complementary regions of the parental chains remain unpaired. Consequently branch migration will release the nascent chains from both template strands and therefore is not limited to a maximum of 50% of the pulse-labelled chains. In particular, the proportion will increase as the number of small pre-fork eye loops piles up under conditions of inhibition of the chain polymerization at an unchanged rate

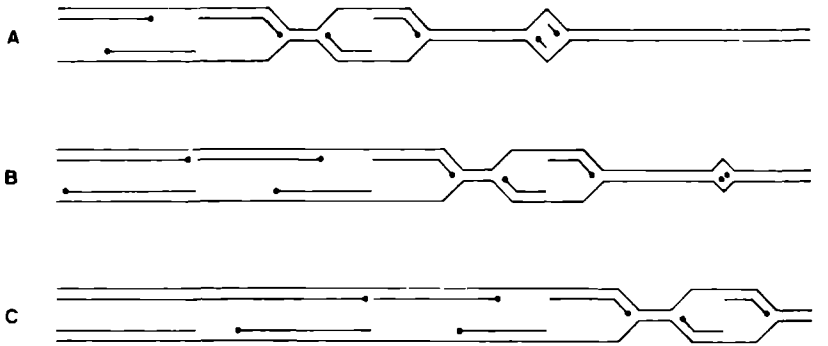

Fig. 7 Diagram of the involvement of pre-fork initiation in the DNA replication A, B and C represent successive steps involving formation and growth of small pre-fork eye loops by initiation and polymerization of Okazaki fragments The main fork moves by alteration of the polymerization of nascent chains and by the fusion of the pre-fork eye loops with the main loop of the active replicon Several modifications of the basic model can be envisaged varying numbers of pre-fork eye loops, varying distances between the initiation points, varying rates of polymerization of the nascent chains, etc

of initiation as found in the presence of aC. The model also accounts for the presence of a greater number of uncompleted Okazaki fragments than that required for the original Okazaki model [30]. One might also consider a slight modification of the model, in which the regions between the main fork and the pre-fork eye loops have become single stranded by the action of certain classes of proteins. These are able to melt out the double-stranded parental DNA and are supposed to keep the strands separated until the complementary nascent strands are synthesized [39].

Acknowledgements

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Chapter III

INHIBITION OF DNA SYNTHESIS IN MAMMALIAN CELLS BY DAUNOMYCIN

Preferential Inhibition of Replicon Initiation at Low Concentrations

Eur J Biochem 102, 409–416 (1979)

Inhibition of DNA Synthesis in Mammalian Cells by Daunomycin

Preferential Inhibition of Replicon Initiation at Low Concentrations

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The effect of the intercalating agent daunomycin on DNA synthesis was studied in cultured bovine liver cells. At low daunomycin concentrations (1 and 2 μM) the rate of [^{3}H]thymidine incorporation decreased progressively with the duration of exposure to the inhibitor. This was accompanied by a shift of nascent DNA intermediates of replicon size to higher sedimentation values on sucrose gradients, indicating that daunomycin preferentially affects the initiation of replicating units, both in asynchronous and synchronized cells.

At high daunomycin concentrations (12 μM) the rate of chain growth was also markedly reduced. This was indicated by a rapid and nearly complete cessation of the [^{3}H]thymidine incorporation and an accumulation of nascent DNA intermediates of low molecular weight. These observations are discussed in relation to a pre-fork mode of DNA synthesis.

Daunomycin, a potent anticancer agent [1,2] of the anthracycline group, has been shown to be a strong inhibitor of DNA and RNA synthesis [3–6]. The compound exerts its inhibitory effect by binding to the DNA template [7–11] by which process both the charged amino group of the daunosamine sugar residue and the chromophore are involved.

Daunomycin is taken up into intact cells rather rapidly [4, 7, 12, 13] and accumulation in the cell was shown to be closely correlated to the degree of inhibition of nucleic acid sythesis [4, 13]. While *in vivo* DNA and RNA synthesis are inhibited to about the same extent [4, 13, 14], *in vitro* the DNA polymerase seems to be slightly more sensitive to the drug than RNA polymerase [6]. This was recently confirmed for the closely related anthracycline compound adriamycin [15].

Several authors [16, 17] have presented evidence for the requirement of a short stretch of RNA as a primer for DNA synthesis. Recently, Barthelemy-Clavey et al. [18] demonstrated that *in vitro* daunomycin inhibits initiation of RNA synthesis probably by interfering with the strand-opening step, which transforms the RNA-polymerase-DNA complex to a specific and stable form [19]. Elongation of the RNA strands did not seem to be affected significantly, indicating that once initiation has occurred, dissociation of daunomycin from the opened helix is fast enough not to be rate-limiting.

On the basis of these considerations it was investigated whether daunomycin might also preferentially inhibit the initiation step of the replication process. A preliminary indication that one type of initiation, i.e. the initiation of new replicons, is affected is the observation that *in vivo* the degree of inhibition of DNA synthesis increases upon prolonged incubation of cells with low concentrations of the inhibitor [4].

MATERIALS AND METHODS

Cell Culture and Labelling Procedures

Bovine liver cells were grown in monolayer cultures, either on coverslips or in Carrel-flasks, as described previously [20], except that a serum concentration of 10% was used.

All labelling and inhibition experiments were performed at 37°C using pre-warmed solutions. DNA was pre-labelled by addition of 0.04 μCi/ml [2-^{14}C]-thymidine (52.8 Ci/mol) for at least 36 h. After growing in label-free medium for another 2 h, cells were pulse-labelled with [Me-^{3}H]thymidine (20 Ci/mmol) in the presence or absence of daunomycin (Boehringer).

For experiments on the kinetic aspects of the inhibition by daunomycin, the cells were grown and labelled on coverslips. These were transferred to the medium of choice for the appropriate time. Pulses were terminated by rinsing the cells with ice-cold 0.9% NaCl in 50 mM Tris-HCl pH 8.0.

Synchronization Procedure

To cells, grown in monolayers in Roux bottles, thymidine was added to a final concentration of 2 mM. After 18 h of exposure, the thymidine-containing medium was replaced by fresh medium. [^{14}C]thymidine was added to a final concentration of 0.02 μCi/ml and the cells were allowed to grow for 15 h. Subsequently the cells were blocked for a second time by removing the radioactive medium and adding medium containing 2 mM thymidine. The block was reversed after 2.2 h. Experiments were performed during the period of maximal DNA-synthetic activity, i.e. 8 h after the termination of the second block.

Sedimentation Analysis

Cell lysates were prepared in 0.5% sodium dodecyl sulfate as described previously [21]. Appropriate samples were layered on the top of either 20–65%, neutral (10 mM Tris-HCl pH 8.0) or 5–25% alkaline (0.1 M NaOH containing 10 mM EDTA) sucrose gradients. The latter were prepared on 65% sucrose cushions containing 0.4 g CsCl/ml. To the samples to be analysed on alkaline gradients NaOH and EDTA were added to final concentrations of 0.1 M and 10 mM respectively. Centrifugation conditions will be given with the figures.

For the determination of the effect of daunomycin on the initiation of replicons, nuclei were first isolated from the cells in 0.1% Triton X-100 in 50 mM Tris-HCl containing 10 mM EDTA, pH 8.0 [22] and purified by rigorously forcing the suspension twice through a hypodermic needle with a diameter of 0.7 mm. The nuclei were then collected by centrifugation and resuspended in an appropriate volume of Triton-Tris-EDTA (0.2 ml for each coverslip). Subsequently 0.4 ml of the suspension was layered on top of 5–25% alkaline sucrose gradients prepared on 65% sucrose cushions containing 0.4 g CsCl/ml between two dodecylsulfate layers (0.6 ml each; final concentrations 0.5% sodium dodecylsulfate, 0.1 M NaOH, 10 mM EDTA) and lysis was allowed to proceed for 2 h in the dark at room temperature. Centrifugation was performed for 17 h at 14000 rev./min and at 20 °C in a SW-27 rotor.

Fractions were collected starting from the bottom of the tube and processed as described elsewhere [21]. The radioactivities were counted in a Philips LSA using a toluene-based scintillation fluid.

Sedimentation coefficients were estimated according to Meiwen [23].

RESULTS

To quantify the inhibitory effect of daunomycin on the process of DNA replication, dose-response experiments were performed. It was found that concentrations of daunomycin of 10 μM and higher inhibited [^{3}H]thymidine incorporation into DNA by more than 90% in cells pre-incubated with the inhibitor for 45 min (Fig. 1A).

From the time courses of inhibition one can conclude that, at the higher concentrations tested, maximal inhibitory effect was reached within 30–45 min. At the lowest concentration, however, inhibition was not complete even after 90 min. From these observations some tentative conclusions can be drawn concerning the mode of action of daunomycin. At the lowest concentration (1 μM), initiation of new replicons could be inhibited preferentially, while replication of replicons already initiated may be less affected. As a consequence, the number of active replicons and therefore the [^{3}H]thymidine incorporation will decrease with increasing duration of exposure to the inhibitor. At higher concentrations elongation of the nascent chains within the still-active replicons will also become inhibited, thus causing a more immediate reduction of the [^{3}H]thymidine incorporation.

To investigate further which step of the replication process is affected by daunomycin, the sedimentation patterns of both continuously-labelled and pulse-labelled DNA were analysed on alkaline sucrose gradients. For this purpose [^{14}C]thymidine pre-labelled cells were pulse-labelled for 1 h with [^{3}H]-thymidine in the presence of two different concentrations of daunomycin. As a control, pre-labelled cells were also pulsed for 1 h in the absence of the inhibitor. Fig. 2A shows that the sedimentation patterns of both pre-labelled and pulse-labelled DNA from uninhibited cells coincide fairly well. Essentially the same was found when cells had been pulse-labelled in the presence of 1 μM daunomycin (Fig. 2B). Interestingly, it was found that the pulse-labelled DNA obtained from cells treated with 12 μM daunomycin could be resolved into a class of relatively slowly sedimenting DNA and a class with a sedimentation value close to that of the pre-labelled DNA (Fig. 2C). The former class does not seem to consist of nascent chains of one definite length, but shows a broad distribution over a range from 8 S to 25 S and is similar to the distribution after short pulses in the absence of the inhibitor. This relative accumulation of low-molecular-weight intermediates indicates that in the presence of 12 μM daunomycin chain elongation and possibly ligation are inhibited markedly.

Chase experiments were then done to see whether the slowly sedimenting class of DNA represents regular intermediates of the replication process. [^{14}C]-Thymidine pre-labelled cells were pulse-labelled with [^{3}H]thymidine for 1 h in the presence of 12 μM daunomycin. The cells were then immediately transferred to label-free and inhibitor-free medium and

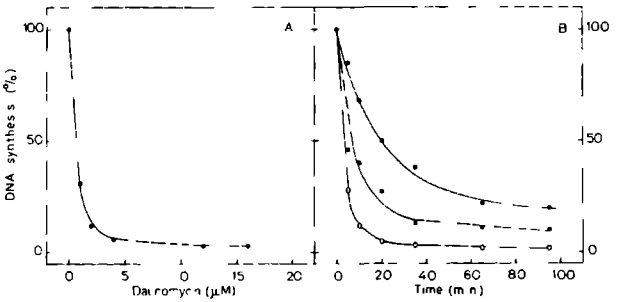

Fig. 1. *Effect of A the daunomycin concentration and B the pre-incubation time on the [^{3}H]thymidine incorporation.* Cells pre-labelled with [^{14}C]thymidine were pre-incubated (A) with various concentrations of daunomycin for 45 min or (B) for various times with three different concentrations of daunomycin. Subsequently they were pulse-labelled with 10 μCi/ml [^{3}H]thymidine for 10 min (A) or 10 min (B) in the presence of the inhibitor. Incorporation of the control without daunomycin was set at 100. Daunomycin concentration in (B): (●) 1.0 μM, (■) 2.0 μM, (○) 1.2 μM.

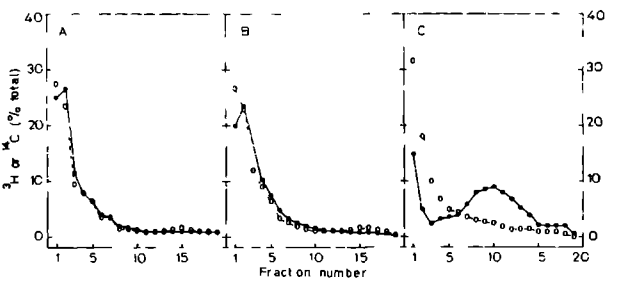

Fig. 2. *Alkaline sucrose gradient sedimentation patterns of labelled DNA from dodecylsulfate-lysed cells.* Cells pre-labelled with [^{14}C]thymidine were pulse-labelled for 1 h with 5 μCi/ml [^{3}H]thymidine in the absence (A) and in the presence of 1 μM (B) and 12 μM (C) daunomycin respectively. The lysates were centrifuged for 16 h at 23000 rev./min in a SW27 rotor. Direction of sedimentation is to the left. Total ^{3}H (●) was (A) 1088000, (B) 525400, (C) 18200 dis./min; total ^{14}C (○) was (A) 52900, (B) 52600, (C) 65600 dis./min. Unless indicated otherwise, cells were pre-incubated with the indicated daunomycin concentration for 45 min.

then incubated for up to 3 h. Fig. 3 shows that within these 3 h the greater part of the nascent label can be shifted to the position of the peak of the pre-labelled DNA. This indicates that the slowly sedimenting DNA chains are regular replication intermediates.

From the observed shift one can conclude that inhibition by daunomycin is not easily reversible. To obtain more definite data the following experiment was performed. Cells were firstly treated with 12 μM daunomycin for 45 min after which they were transferred to inhibitor-free medium and incubated for various periods of time. Subsequently samples of the cells were pulse-labelled with [^{3}H]thymidine for 1 h after which the level of inhibition compared to cells not treated with daunomycin and the DNA distribution on alkaline sucrose gradients were determined.

After both 1 h and 2 h of incubation the rate of incorporation was still inhibited by 97 %, and the major part of the pulse label was found in the low-molecular-weight intermediates indicating that the rate of chain growth is still reduced markedly (Fig. 4B and C). However, 20 h after removal of the inhibitor the pulse label was incorporated into high-molecular-weight DNA (Fig. 4D) but the rate of incorporation in this experiment amounted to only 55% of the control rate. These observations indicate that inhibition of DNA replication by high concentrations of daunomycin is only partly reversible.

As pointed out above, the process of initiation seems to be the most likely target for the action of daunomycin. Two types of initiation can be discerned: initiation of replicons and initiation of Okazaki fragments. The time course of inhibition at 1 μM suggested that the former is preferentially affected by daunomycin. Since Fridland recently showed that the cytostatic agent arabinosyl-cytosine inhibits initiation of new replicons preferentially [24], a similar approach was chosen to establish more precisely the mode of action of daunomycin.

Briefly, cells were preincubated with 1 μM daunomycin for various periods of time after which they were labelled with [^{3}H]thymidine for 12 min in the

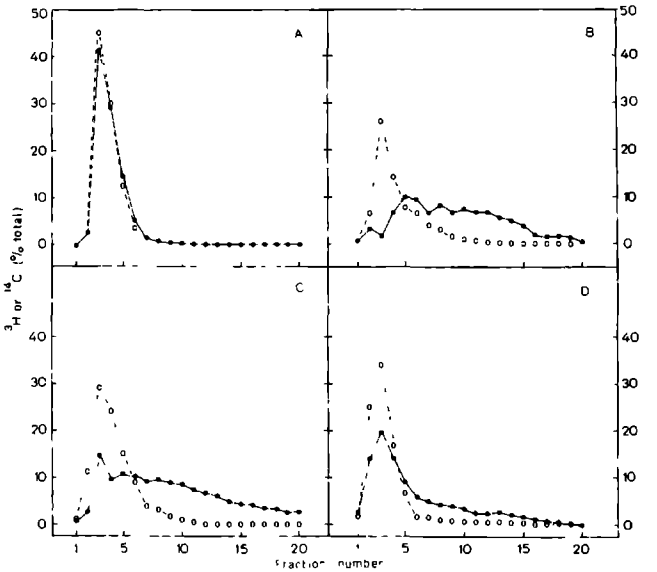

Fig 3 *Alkaline sucrose gradient sedimentation patterns of DNA from pulse labelled cells which were subsequently incubated in daunomycin-free medium* Cells pre-labelled with [^{14}C]thymidine were pulse labelled with 5 μCi ml^{-1} [^{3}H]thymidine for 1 h in the absence (A) and in the presence of 12 μM daunomycin The cells pulse labelled in the presence of the inhibitor were subsequently incubated in inhibitor free medium for 0 (B) 1 (C) and 3 (D) h For further details see under Fig 2 Total ^{3}H (●) was (A) 2723000 (B) 31400, (C) 45300 (D) 29400 dis min ^{14}C (○) was (A) 66900 (B) 78400 (C) 64400 (D) 57000 dis min

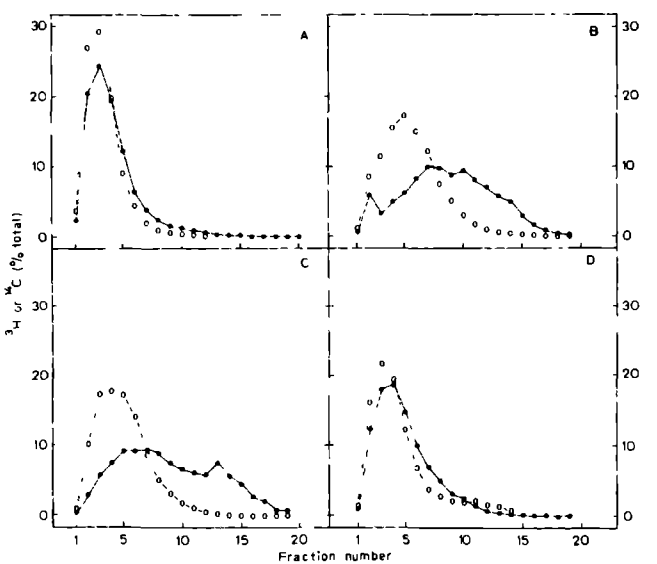

Fig 4 *Alkaline sucrose gradient sedimentation patterns of DNA from pulse-labelled cells previously treated with daunomycin followed by an inhibitor free incubation* Cells which were first treated with 12 μM daunomycin for 45 min were subsequently incubated in inhibitor-free medium for 0 h (B) 1 h (C) and 20 h (D) and then pulse-labelled with 5 μCi ml^{-1} [^{3}H]thymidine for 1 h Control cells (A) were only pulse-labelled with [^{3}H]thymidine for 1 h For further details see under Fig 2 Total ^{3}H (●) was (A) 1630000 (B) 47700 (C) 53200 (D) 1036000 dis min total ^{14}C (○) was (A) 59500 (B) 79400 (C) 64000 (D) 69300 dis min

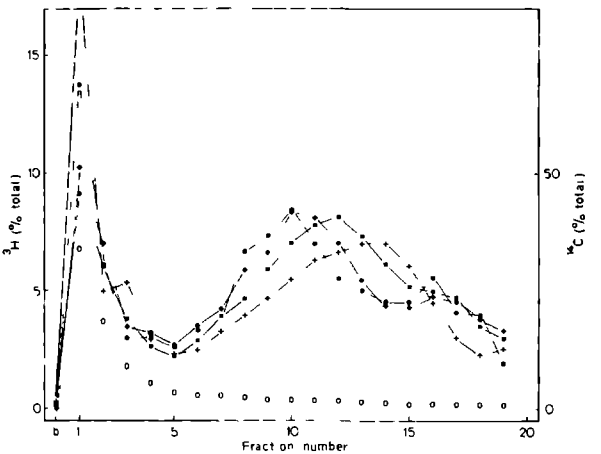

Fig 5 *Effect of pre-incubation with 1 μM daunomycin on the sedimentation pattern in alkaline sucrose gradients* Cells pre-labelled with [^{14}C]thymidine (○ ○) were pulse-labelled with 10 μCi ml^{-1} [^{3}H]thymidine for 12 min in the absence (+ +) and in the presence of 1 μM daunomycin after various pre-incubation times 30 min (■ ■) 60 min (◆ ◆) and 90 (● ●) min Nuclei were isolated and lysed in sodium dodecylsulfate on top of the gradient Centrifugation was performed in a SW27I rotor for 17 h at 14000 rev min Total ^{3}H was (□) 305000 (■) 87300 (◆) 33800 (●) 28500 dis min total ^{14}C (○) was on average 38000 dis min In the pattern of the control (+) fraction 1 contained 20 of the total ^{3}H

presence of the inhibitor The sedimentation patterns of the labelled DNA obtained from these lysates are shown in Fig 5

It can be seen from Fig 5 that without pre-incubation the ^{3}H-labelled DNA is resolved into two classes One class represents DNA of extremely high molecular weight (over 100 S) and most probably consists of multiples of replicons or replicons ligated to fully duplicated DNA The other class showing a broad distribution between 10 S and 80 S with a peak at 30 S is though to consist of nascent and partially replicated replicons Upon increasing duration of pre-incubation it can be seen that the peak of this distribution shifts to higher sedimentation values This can be taken to indicate that replicons once initiated continue to be elongated but that the inhibitor reduces the rate at which new replicons are initiated which results in a reduction of the number of the smaller intermediates Since a blocking of cells in the G_1 phase by daunomycin has been reported [25] it had to be shown that the observed shift was not caused by this effect of daunomycin Therefore we performed an experiment similar to that described above using S-phase cells obtained after a double-thymidine block As shown in Fig 6A maximal DNA synthesis occurred at approximately 8 h after release of the cells from the second block Consequently experiments were performed starting at the beginning of the ninth hour As can be seen in Fig 6B at 1 μM daunomycin, essentially the same shift to higher sedimentation values of replicon-sized intermediates was observed as found in asynchronous cells This shift is not the consequence of a proportion of the cells leaving the S phase and entering the G_2 phase during the pre-incubation time since control cells pulsed either at 8 or 9 h after release from the thymidine block showed a similar distribution pattern of their replicon-sized DNA intermediates

Finally we examined whether daunomycin affected the kinetics of initiation and elongation of Okazaki fragments Cells preincubated with 1 μM or 12 μM daunomycin for 45 min were briefly pulse-labelled with [^{3}H]thymidine Subsequent analysis on alkaline sucrose gradients showed that at both concentrations of daunomycin the length of the Okazaki fragments was equal to that of the Okazaki fragments obtained from uninhibited cells (Fig 7A and B 1 μM experiment not shown) At both concentrations the absolute amount of label in the short chains was reduced but only at 12 μM was a significant relative increase of label in the Okazaki fragments observed suggesting a markedly reduced rate of chain elongation This was confirmed by the finding that in the presence of 12 μM of the inhibitor the Okazaki fragments were incorporated into high-molecular-weight DNA at considerably lower rates than in control cells (Fig 7C and D)

DISCUSSION

Our results confirm the observation that daunomycin is a potent inhibitor of eukaryotic DNA

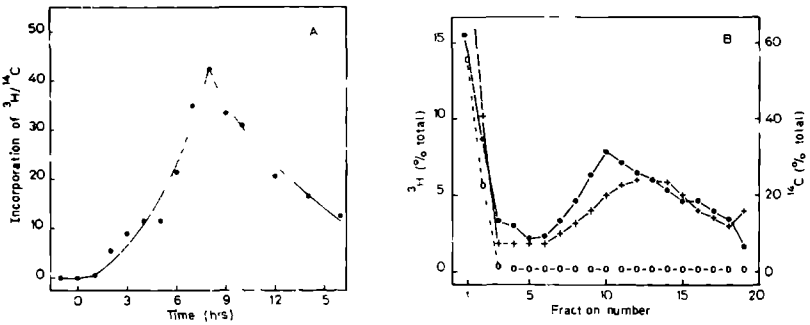

Fig 6 *A Time course of [^{3}H]thymidine incorporation in synchronized cells. B Effect of pre-incubation with 1 μM daunomycin on the sedimentation pattern in alkaline sucrose gradients.* (A) Cells grown on coverslips were synchronized by a double-thymidine block. Between the two successive blocks the cells were labelled with [^{14}C]thymidine. At $t = 0$ the medium containing thymidine was removed and at the indicated times samples of two coverslips each were pulse-labelled with 16 μCi/ml [^{3}H]thymidine for 10 min. Incorporation is given as the amount of ^{3}H incorporated/^{14}C. The sample $t = -1$ h was pulse-labelled in the presence of 2 mM thymidine 1 h before termination of the block. (B) Cells pre-labelled with [^{14}C]thymidine (○—○) synchronized by a double thymidine block were pulse-labelled with 10 μCi/ml [^{3}H]thymidine for 12 min both 8 and 9 h after release in the absence of daunomycin (+—+) (patterns coincident) 9 h after release in the presence of daunomycin (●—●) the pulse being preceded by 60 min of preincubation with the inhibitor. For further details see Fig 5. Total ^{3}H in the control was 702 100 dis/min 223 500 dis/min in the presence of daunomycin; total ^{14}C in the control was 22 300 dis/min 20 900 dis/min in the presence of daunomycin

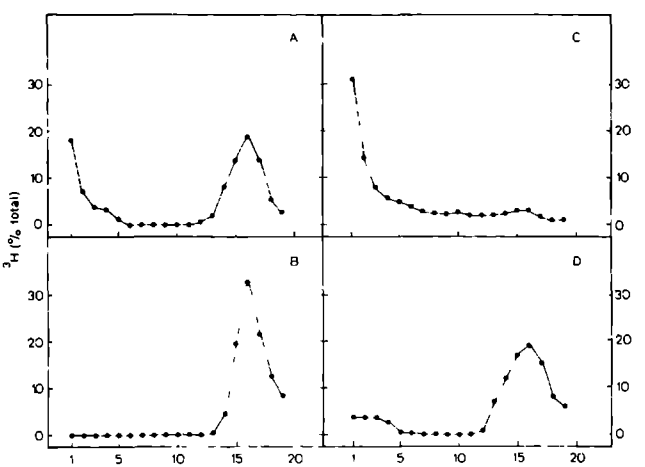

Fig 7 *Effect of high daunomycin concentrations on the alkaline sedimentation patterns of briefly pulsed DNA.* Cells were either pulse-labelled with 50 μCi/ml [^{3}H]thymidine for 1 min (A) and 5 min (C) in the absence of the inhibitor or pulse-labelled for 1 min (B) and 5 min (D) in the presence of 12 μM daunomycin after a 45 min pre-incubation. The dodecylsulfate lysates were analysed on alkaline sucrose gradients. Centrifugation was performed on a SW27H rotor for 17 h at 26000 rev/min. Total ^{3}H was (A) 1910 (B) 800 (C) 19650 (D) 1960 dis/min

replication. The concentration dependence of inhibition was fairly similar to that found by Rusconi et al [4]. However, most probably due to the fact that in our experiments the cells were pre-incubated with the inhibitor, the level of inhibition observed was somewhat higher. Essentially the same results were obtained by Bachur et al [13].

As shown before [4, 2, 13], daunomycin is taken up by cells rather rapidly. Release of the inhibitor from the DNA has been shown to occur at a very low rate [12]. This most probably is the reason for the persistent inhibition of the [^{3}H]thymidine incorporation after removal of daunomycin from the medium. It might also explain the altered distribution pattern of pulse-labelled DNA, even after 3 h of incubation in daunomycin-free medium.

Obvious short-term detrimental effects of daunomycin were not observed in the course of this study.

No loss of ^{14}C-labelled DNA could be observed during incubations following daunomycin administration confirming the fact that hardly any detached cells were detected. Upon prolonged incubation in inhibitor-free medium following a 1-h exposure to 1 μM daunomycin between 25% and 50% of the cells were found to be markedly enlarged (at the 36th hour) while their shape resembled that of fibroblasts. The remainder of the cells appeared to be rather normal. Detached cells were hardly found even after 96 h of incubation. However, when cells had been treated with 12 μM daunomycin for 1 h a significant amount of detached cells could be observed after 36 h while after 96 h hardly any attached cells were detected.

Moreover, upon sucrose gradient centrifugation in experiments on the effect of daunomycin on the initiation of replicons the peak of ^{14}C-labelled DNA did not show a significant broadening upon prolonged incubation with 1 μM inhibitor. This indicates that strand scission or formation of alkali-labile bonds as a consequence of daunomycin intercalation did not occur on a detectable scale. This is apparently contrary to the findings of Schwartz [26] but in our experiments only DNA strands below 100 S were analyzed, while Schwartz studied only very-high molecular-weight DNA (over 200 S). The latter system therefore was much more sensitive to the daunomycin effects mentioned above.

From the time courses of inhibition some tentative conclusions concerning the mechanism of inhibition by daunomycin can be drawn. At 12 μM [^3H]thymidine incorporation ceases rather abruptly. At 1 μM however, the incorporation only decreases slowly, maximal inhibition being reached much later than at the higher concentration. One possible explanation for this observation could be that after addition of daunomycin only replicons already initiated are completed while no (or hardly any) new replicons start to be duplicated. The [^3H]thymidine incorporation should then cease after a period of time needed to complete the largest replicon, provided that the inhibition of replicon initiation were complete. The observation that a fairly constant rate of incorporation of 20% is attained after 60 min of exposure suggests that the initiation is not totally blocked. This effect might also be contributed to by an apparent increase in inter-origin distance of replicons due to the blocking of a proportion of the origins.

The assumption of a preferential inhibition of the initiation step is further supported by an analysis of the distribution of intermediate-length DNA chains. Both in asynchronous and in synchronized cell cultures a shift of replicon-sized intermediates to higher sedimentation values was observed upon prolonged incubation with 1 μM daunomycin. This can be explained by a decrease of the number of short growing chains by the decreased rate of replicon initiation. In addition, due to the larger distances between operative origins in active replicon clusters, the number of chains longer than replicon length will also increase.

Tentatively, the preferential inhibition of the replicon initiation by daunomycin could be explained in the way suggested for the initiation of transcription [18]. Both processes require local strand separation in the DNA double helix. This opening would be counteracted by the known double-helix-stabilizing effect of daunomycin and would be in agreement with results obtained with another intercalating agent, actinomycin D [27]. This interpretation may be over-simplified however in view of our recent evidence suggesting that initiation of Okazaki fragments also requires local strand separation in pre-fork loops [28], possibly between successive nucleosomes [29]. The data presented in this study do not provide evidence for a preferential inhibition of the initiation of Okazaki fragments in a way comparable to the inhibition of replicon initiation. The suggestion that the selective inhibition of replicon initiation at low daunomycin concentrations is due to the double-helix stabilizing effect of daunomycin therefore remains uncertain. This is even more so since a similar preferential inhibition has been observed by low concentrations of arabinosylcytosine [24] which has no effect on the double helix stability.

In brief, these results suggest that in spite of possible similarities the two types of initiation are distinctly different. One can think of various explanations for the differential sensitivities of the two types of initiation. For instance, origins of replicons could differ from the remainder of the DNA in base composition or sequence and might therefore be more sensitive towards daunomycin (and arabinosylcytosine). Alternatively, the origins of replicons could be complexed to proteins differently from the rest of the chromatin supposedly because they are attached to a nuclear matrix [30]. Another possibility is that the change of the DNA supercoil structure caused by intercalating agents [31] might affect initiation of clusters of replicons [32, 33]. The results further show an additional effect of daunomycin at 12 μM. From the accumulation of small replicative intermediates and the reduced rate of incorporation of Okazaki fragments into high-molecular-weight DNA it can be concluded that chain elongation is also inhibited to a significant extent. This effect is probably comparable to the effect of the inhibition on DNA synthesis in cell-free systems *in vitro* [6, 9, 15]. Due to the high level of inhibition the primary daunomycin effect (i.e. blocking of the initiation of replicons) becomes obscured. The reduction of the elongation rate can be explained by assuming that, even at the low concentration, daunomycin, intercalated into DNA, interferes with the translocation of the polymerase molecule along the DNA strands. Though this is not a significant effect

at 1 μM higher concentrations (i e increasing amounts of intercalated daunomycin molecules) will cause an increasing reduction of the rate of chain elongation This effect might also be contributed to by daunomycin bound to DNA only electrostatically for instance in a stacked form

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Chapter IV

ANALYSIS OF THE ATTACHMENT OF REPLICATING DNA TO A NUCLEAR MATRIX IN MAMMALIAN INTERPHASE NUCLEI

Analysis of the attachment of replicating DNA to a nuclear matrix in mammalian interphase nuclei

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ABSTRACT

The attachment of replicating DNA to a rapidly sedimenting nuclear structure was investigated by digestion with various nucleases. When DNA was gradually removed by DNase I, pulse label incorporated during either 1 min or during 1 hour in the presence of arabinosylcytosine, remained preferentially attached to the nuclear structure. Single strand specific digestion by nuclease S_1 or staphylococcal nuclease at low concentrations caused a release of about 30% of the pulse label, without significantly affecting the attachment of randomly labelled DNA. The released material had a low sedimentation coefficient and contained most of the Okazaki fragments. The remaining pulse label was less accessible to further digestion by double strand specific nuclease activity than the bulk DNA. The results suggest that an attachment of the replication fork to the nuclear structure occurs at the point where it is about to branch out.

INTRODUCTION

Chromosomal DNA of mammalian cells was found to be attached to a nuclear structure which is resistant to 2 M NaCl (1). From the results of enzymatic digestion experiments it appeared that proteins are the main components involved in the stabilization of the structure. Biochemical and ultrastructural studies (2) revealed that the protein structure related to what has been described respectively as nuclear protein matrix (3) or core complex-lamina (4).

Replicating DNA was found to be attached to the nuclear protein component by replication at the location of the replication fork (1). The experiments reported here were carried out in order to obtain a more detailed insight into the mode of binding of the replicating DNA molecule. Lysates of nuclei labelled *in vivo* under various conditions were digested with different DNA degrading enzymes and the degradation kinetics of pulse labelled and continuously labelled DNA were compared.

MATERIALS AND METHODS

Cell culture and labelling procedures. Monolayers of bovine liver cells were grown in Carrel-flasks as described elsewhere (5), except that a serum concentration of 10% was used. DNA was pre-labelled by addition of 0.04 μCi/ml 2-^{14}C-dThd (spec. activity 52,8 μCi/mmole, NEN) for at least 30 hours. After growing cells in label-free medium for another 2 hours, me-^{3}H-dThd (spec. activity 20 Ci/mmole, NEN) was added. The cells were pulse labelled for 1 or 20 minutes with 50 μCi/ml and 5 μCi/ml respectively. When ara-C (Sigma) was used, the cells were preincubated with the inhibitor for 30 minutes after which they were pulse labelled with 10 μCi/ml ^{3}H-dThd for 1 hour in the presence of ara-C. The incorporation of label was stopped by rinsing the cells with ice-cold 0.1% Triton, 50 mM Tris-HCl buffer pH 8.0.

Preparation of nuclear lysates and sucrose gradient centrifugation. Nuclear lysates were prepared as described elsewhere (1). Briefly, nuclei, isolated in 0.1% Triton, 5 mM Tris-HCl, pH 8.0, were suspended in 50 mM Tris-HCl pH 8.0 and an equal volume of 2 M NaCl was added. The lysate was gently homogenized and layered on a neutral, 1 M NaCl containing 15%-40% sucrose gradient, prepared on a 65% sucrose cushion containing 0.4 g/ml CsCl. Centrifugation was performed in a Spinco SW 27-2 rotor for 1 hour at 20 000 rpm and at 20°C. To analyze the size distribution of pulse labelled nascent DNA chains, nuclei were dissolved in 0.5% SDS, 0.1 M NaOH, 10 mM EDTA. The lysate was layered on a 5%-20% alkaline sucrose gradient prepared on a 65% sucrose cushion. Centrifugation was performed in a Spinco SW 27-1 rotor for 17 hours at 24 000 rpm and at 20°C. The gradients were fractioned starting from the bottom of the tube. The fractions were processed and the radioactivity was determined as described previously (6).

Enzymatic DNA digestions. Nuclear lysates in 1 M NaCl were subjected to one of the following nuclease treatments:

a. DNase I. Lysates were incubated with various concentrations of DNase I (Sigma, electrophoretically purified) in the presence of 7.5 mM $MgCl_2$ for 30 minutes at 37°C.

b. Nuclease S_1. For incubations with S_1 (Sigma) the pH of the nuclear lysates was lowered to 4.5 by adding 0.5 M sodium acetate buffer (pH 4.5) to a final concentration of 0.05 M. Incubations with the enzyme were carried out in the presence of 1 mM $ZnSO_4$ for 30 minutes at 37°C.

c. Staphylococcus nuclease. Lysates were incubated with various concentrations of Staphylococcus nuclease (Worthington) in the presence of 1 mM $CaCl_2$ for 10 minutes at 37°C.

d. When successive incubations with nuclease S_1 and DNase I were carried out, the pH was firstly lowered to 4.5 as described above. Nuclease S_1 was added to a final concentration of 3 µg/ml, and the samples incubated as described above. The incubation was terminated by addition of 0.5 M Tris-HCl pH 8.0 and 0.25 M EDTA to final concentrations of 100 mM and 2 mM respectively. Then DNase I was added and the samples were incubated in the presence of 10 mM $MgCl_2$ for 30 minutes at 37°C.

In all cases described, the incubations were terminated by addition of 0.25 M EDTA to a final concentration of 30 mM.

RESULTS

1. Release of DNA by DNase I digestion. Nuclear DNA attached to the protein component can be isolated as a rapidly sedimenting complex from nuclear NaCl lysates by ultracentrifugation (1). A preferential association of newly replicated DNA with the protein component was assured in view of the relative enrichment of pulse label, in particular after partial release of DNA by shearing or limited DNase treatment.

For a more precise determination of the DNA regions attached to the protein structure, nuclear lysates of cells pulse labelled for 1 min and 20 min respectively, were digested with varying concentrations of DNase I. Fig. 1 shows that a DNase treatment which removed 95% of the total (pre-labelled) DNA, still left 40% of the 1 min pulse labelled DNA attached to the nuclear protein component. Identical results were obtained with lysates prepared in 2 M NaCl. The $^3H/^{14}C$ ratio of the DNA present in the rapidly sedimenting complex increased continuously, as the release of DNA by the enzyme progressed (fig. 1E). An increase of the ratio was also observed when lysates obtained after pulse labelling for 20 min were treated with DNase I, or were exposed to various degrees of shearing. However, the relative $^3H/^{14}C$ ratios after a 20 min pulse were significantly lower than those obtained after pulse labelling for 1 min (fig. 1E). This suggests that the most newly synthesized DNA must be located very close to the attachment sites, or, in other words, the region of attachment must be next to the replication fork.

This finding was confirmed by another experiment carried out after labelling DNA for 1 hour in the presence of 400 µM arabinocytosine. This analogue has been shown to cause a strong reduction of the rate of DNA chain growth (7). Thus, in the presence of arabinocytosine only a limited region adjacent to the replication fork becomes labelled during one hour incorporation of radioactive thymidine. The results of the DNase digestion of the

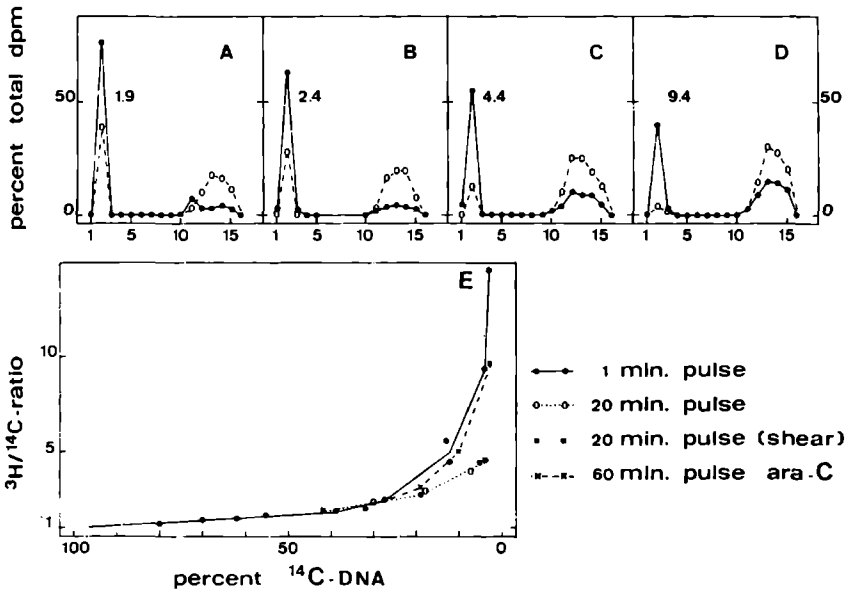

Figure 1. Effect of DNase I digestion on the label distribution in nuclear lysates.
A nuclear lysate was prepared from cells pulse labelled for 1 min with ^3H-dThi. The lysate was divided into 4 portions and DNase I was added to final concentrations of 0 (A), 0.3 (B), 0.6 (C) and 1 (D) µg/ml. After incubation the samples were analysed by sucrose gradient centrifugation. Direction of centrifugation was from right to left. The numbers in each panel represent the ratios of the percentages ^3H and ^{14}C dpm present in the rapidly sedimenting material. The average total dpm per gradient were 19 000 for ^3H and 11 000 for ^{14}C. Fig. E shows the $^3H/^{14}C$-ratios of the rapidly sedimenting complex as a function of the proportion of total DNA remaining. Results of experiments with different labelling programs are shown. For each separate curve one nuclear lysate was used. In one experiment (shear) a graded release of DNA from the complex was obtained by shearing, i.e. samples were forced various times through a 0.5 mm glass capillary at a pressure of 1 atm.

nuclear lysates from such cells were very similar to those obtained with a 1 min pulse experiment without inhibitor (fig. 1E).

2. Release of DNA by nuclease S_1. The data reported so far indicate that, in contrast to prokaryotes (e.g. 8), discontinuous synthesis of both daughter strands is the general mode of replication in eucaryotic organisms (7, 9, 10). This implies that short unpaired regions occur temporarily in the parental strands behind the branch point. In order to find out whether these regions are accessible to single strand specific nucleases while the DNA is bound to the nuclear structure, we have digested nuclear lysates with

nuclease S_1. Fig. 2A-D shows that after a 1 min pulse about 30% of the pulse label was removed by enzyme concentrations which did ot significantly affect the amount of bound pre-labelled DNA. The almost exclusive release of pulse label at low enzyme concentrations is also indicated by the abrupt decrease of the $^3H/^{14}C$ ratio of the complex (fig. 2E). Similar results were obtained using cells pulse labelled for 1 hour in the presence of arabinosylcytosine. The decrease in the ratio was only slightly greater when high enzyme concentrations were used which also released marked amounts of pre-labelled DNA. Apparently, only a limited portion of the most recently synthesized DNA is removed from the complex by the action of the single strand specific enzyme activity. The pulse label released after a 20 min pulse was only 10% or even less (fig. 2E).

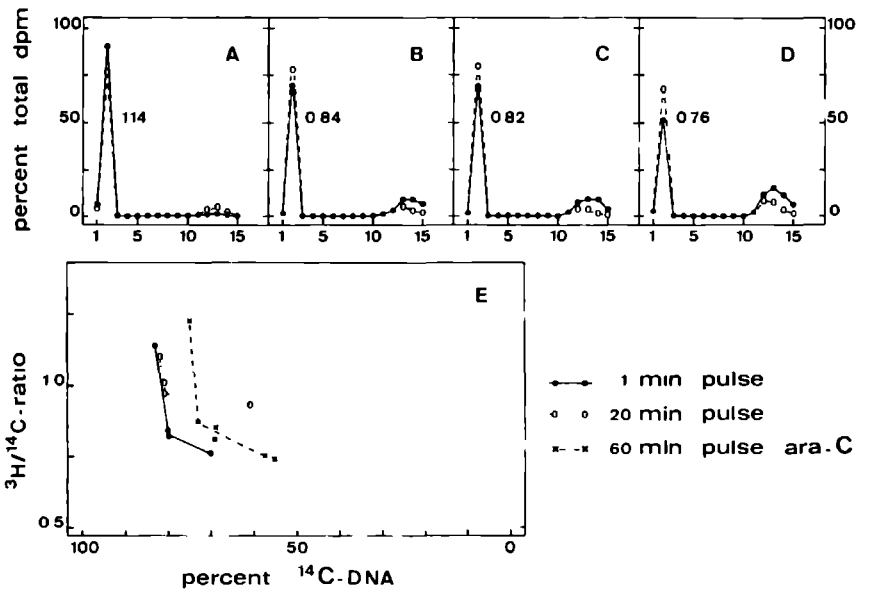

Figure 2. Effect of nuclease S_1 digestion on the label distribution in nuclear lysates.
Portions of a nuclear lysate prepared as described in fig. 1, were digested with 0 (A), 1 (B), 3 (C) and 10 (D) µg/ml nuclease S_1. For further details see fig. 1. The average total dpm per gradient were 12 000 for ^3H and 13 000 for ^{14}C. Fig. E shows the $^3H/^{14}C$ ratios of the rapidly sedimenting complex as a function of the proportion of total DNA remaining. Results from experiments with different labelling programs are shown. For each separate curve one nuclear lysate was used. In each curve the point at the highest ^{14}C DNA percentage represents the non-incubated control.

It is a reasonable assumption that the released material mainly consists of Okazaki fragments hydrogen bonded to small species of the complementary parental chains cut out by the nuclease. This assumption was compatible with the results of chromatography on hydroxyapatite (data not shown) and by the sedimentation analysis presented in fig. 3. On prolonged centrifugation the pulse labelled material sediments at a much lower rate than the small amount of pre-labelled DNA which is generally released from the complex due to unavoidable shearing (1). It can be roughly estimated that the amount of the parental fragments bound to the Okazaki pieces is of the order of 0.01% of the total nuclear DNA. This is compatible with the negligible proportion of pre-label associated with the slowly sedimenting peak of pulse labelled DNA (fig. 2).

Finally it was found that the amount of pulse label released from the complex by nuclease S_1 was of the same order as the amount of pulse label found in Okazaki pieces under the same labelling conditions. These are represented by the material sedimenting at a rate of less than 10 S in alkaline sucrose gradients (fig. 4).

The possibility had to be ruled out that the accessibility to nuclease S_1 might be caused by a modification of the complex at pH 4.5, the pH optimum for the nuclease (11). We have therefore carried out DNase I digestions at pH 4.5, using higher enzyme concentrations. The results, not reported here, were the same as those at pH 8.0 presented in the preceding section.

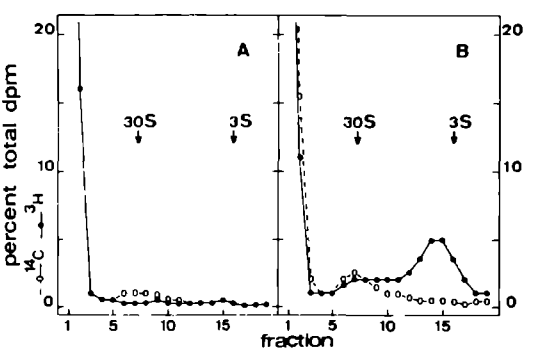

Figure 3. Sedimentation analysis of the pulse labelled DNA fragments released by nuclease S_1 digestion.
A nuclear lysate was prepared as described and a portion of it was incubated with 3 µg/ml nuclease S_1. The sample (B) and an undigested control (A) were centrifuged for 17 hours at 24 000 rpm. The total dpm per gradient were:
A. ^{3}H: 7 050 dpm (78% in fraction 1); ^{14}C: 5 200 (73% in fraction 1).
B. ^{3}H: 3 050 dpm (32% in fraction 1); ^{14}C: 5 600 (67% in fraction 1).

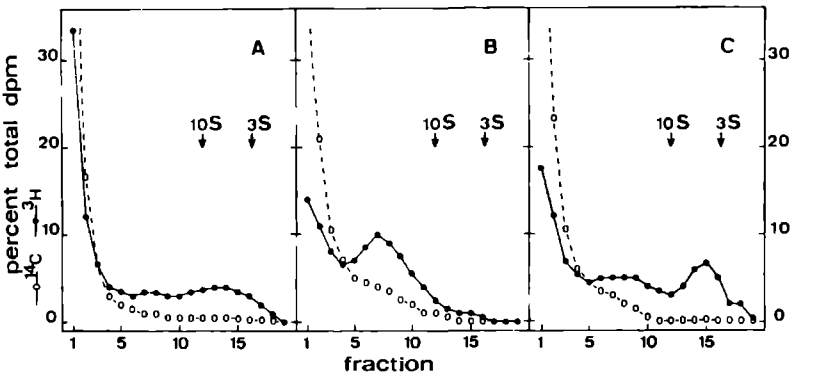

Figure 4. Analysis of the size distribution of pulse labelled DNA, present in the various nuclear lysates.
Nuclei, isolated from cells, pulse labelled for 1 or 20 minutes, or for 60 minutes in the presence of ara-C, were lysed in alkaline SDS. The SDS lysates were analysed in alkaline sucrose gradients. (A) 1 minute pulse, (B) 20 minutes pulse, (C) 60 minutes pulse in the presence of ara-C. The total dpm in gradients A-C were as follows: A: ^3H: 26 250 dpm; ^{14}C: 20 150 dpm (64% in fraction 1). B: ^3H: 2 075 800 dpm; ^{14}C: 14 350 dpm (36% in fraction 1). C: ^3H: 116 500 dpm; ^{14}C: 8 000 dpm (41% in fraction 1).

Digestion with RNase was found to have no effect (7).

From these data one can conclude that the attachment of the replicating DNA, although close to the replication fork, does not include those parts of the parental strands to which Okazaki fragments are hydrogen bonded.

3. Release of DNA by Staphylococcus nuclease. In order to avoid the uncertainties arising from the incubation with nuclease S_1 at low pH, we digested nuclear lysates at neutral pH with staphylococcal nuclease, which has a preferential single strand specificity (12, 13). It was further expected that due to the ability of the enzyme to degrade double stranded DNA as well, the combined activities at higher enzyme concentrations might lead to new insights about the attachment sites as discussed below.

Fig. 5 shows that, in spite of the slight double strand nucleolytic activity of the enzyme, the digestion at low enzyme concentrations of nuclear lysates prepared after labelling for 1 min showed a similar decrease of the $^3H/^{14}C$ ratio in the bound DNA as observed by digestion with nuclease S_1. This indicates again the preferential release of nascent DNA by breaks in the single stranded regions. As could be expected there was an increasing release of randomly labelled DNA at higher enzyme concentrations. Simultaneously, the $^3H/^{14}C$ ratio of the DNA, still associated with the complex, increased. An essentially similar increase of the ratio was found when a nuclear lysate was

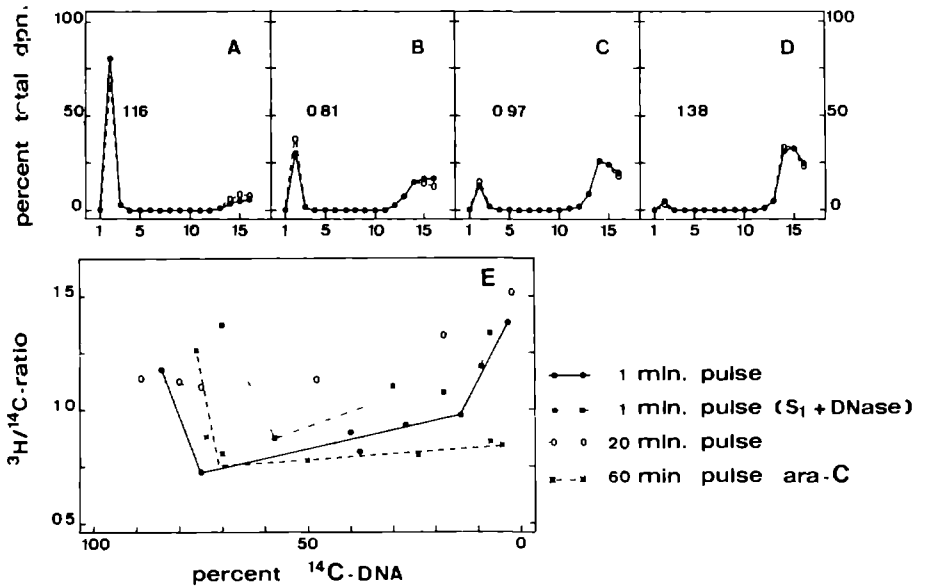

Figure 5. Effect of Staphylococcus nuclease digestion on the label distribution in nuclear lysates.
Portions of a nuclear lysate prepared as described in fig. 1 were digested with Staphylococcus nuclease at the following concentrations: 0 (A), 0.5 (B), 1.0 (C)and 2 µg/ml (D). For further details see fig. 1. Average dpm were 71 500 dpm for ^3H and 11 500 for ^{14}C respectively. Fig. E shows the $^3H/^{14}C$ ratios of the rapidly sedimenting complex as a function of DNA remaining. Results from experiments with different labelling programs are shown. For each separate curve one nuclear lysate was used. In each curve the point at the highest ^{14}C DNA percentage represents the non-incubated control. In one experiment a nuclear lysate, prepared from cells pulse labelled for 1 minute was incubated with nuclease S_1 and subsequently the various samples were digested by DNase I at concentrations of 0, 0.3, 0.6, 1.0 and 1.5 µg/ml respectively.

digested by DNase I after the nascent DNA regions had been removed by nuclease S_1 (fig. 5E). Apparently, a marked proportion of the pulse labelled DNA which is not removed by single strand specific nucleolytic activity, is situated at or close to the DNA region attached to the protein structure. For obvious reasons the changes of the label ratios were much less pronounced in the complex obtained after a 20 min pulse label experiment.

When a similar digestion by staphylococcus nuclease was carried out with a nuclear lysate prepared after labelling cells for 1 hour in the presence of arabinosylcytosine, a marked decrease of the $^3H/^{14}C$ ratio at low enzyme concentrations was again observed. In contrast to the 1 min and 20 min pulse ex-

periment, however, the ratio did not show the increase observed during the release of most of the randomly labelled DNA at high enzyme concentrations (fig. 5E). This difference is most likely to be due to the mode of inhibition of the DNA replication by arabinosylcytosine as will be discussed below.

DISCUSSION

Models on the spatial and temporal organization of the DNA replication during the S phase and segregation of the daughter molecules in mitosis imply the existence of a scaffold-like structure to which the DNA is attached. Dingman (14) suggested that such a structure - at that time believed to be represented by the nuclear envelope - would provide binding sites for the origins of the tandemly arranged replicons (see fig. 6).

Recent ultrastructural studies on histone-depleted metaphase chromosomes have revealed a central scaffold to which the DNA molecule is anchored at intervals comparable to known replicon lengths (15, 16). Essentially, the structure shows a number of features inherent to the model just mentioned. Indirect evidence of the binding of chromosomal DNA to a scaffold-like nuclear protein structure, resistant to 2 M NaCl, has also been obtained with interphase nuclei (1). The results suggested that, apart from this binding which possibly occurs at the origins of replicons, replicative DNA was attached by additional binding sites located close to the replication forks. This finding is important, because it gives experimental evidence for another postulate of the Dingman model, which is the attachment of the replication point.

A more precise location of the bound DNA region was obtained by studying the release of pulse labelled DNA from the complex by producing random breaks in the DNA molecule by DNase I or shearing. The probability of a DNA fragment being released from a DNA loop (fig. 6) by such breaks will decrease the closer it is situated to a bound region. The results presented above show that the release of pulse label is particularly low after very short pulses, indicating that the attachment must occur close to the most recently synthesized DNA segment, i.e. to the replication fork. A similar conclusion can be drawn from the results of DNase I digestion after a more selective labelling of the replication fork in the presence of arabinosylcytosine.

If the binding of the fork would occur just slightly ahead of the branch point as shown in fig. 6B, the removal of the pulse labelled part immediately adjacent to the branch point by for example nuclease S_1 would result in a disconnection to the DNA-molecule. Consequently the remaining pulse label

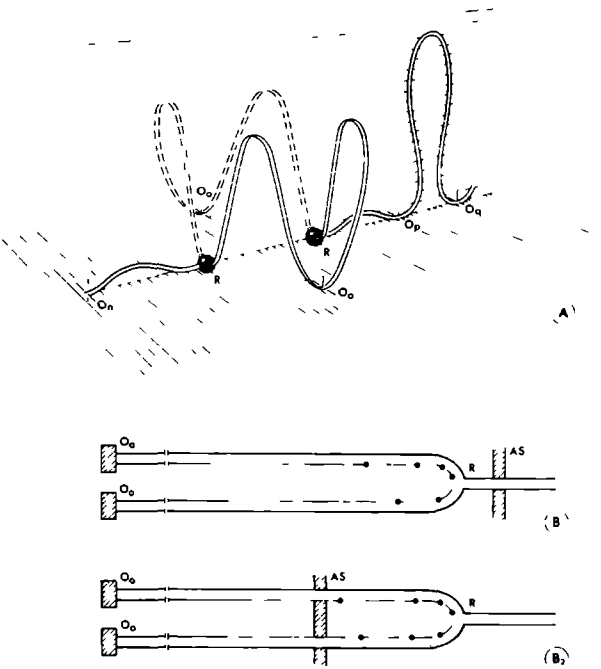

Figure 6. A: Model of the spatial organization of the DNA in the nucleus (according to Dingman). DNA is attached to the matrix at the origins of replication (O_n; O_o''; O_p and O_q). O_o' and O_o'' represent replicated origins. Additional binding to the matrix takes place at the replication forks (R).

B: B_1 and B_2 represent two possible modes of attachment of the replication forks to the matrix. Our results are compatible with the occurrence of an attachment site (AS) behind the branch point.

would then be situated at a free end. Random breaks by, for example, subsequent digestion with double strand specific nuclease should then result in a preferential release of the remaining pulse labelled region. Our results, however, show that the subsequent release of pulse label by DNase I or high concentrations of Staphylococcal nuclease occurs at a slower rate than the release of the randomly labelled DNA. Obviously there must be an attachment of the replication fork behind the region which is cut out by the single strand nuclease. This mode is presented in fig. $6B_2$. This finding does not rule out the possibility of additional binding sites just ahead of the branch point or within the single strand gaps.

A different result was obtained after pulse labelling in the presence of arabinosylcytosine, in that the $^3H/^{14}C$ ratio of the bound DNA was not signi-

ficantly altered by high Staphylococcus nuclease concentrations as compared to low concentrations. This is for the following reason. It is inherent to the Dingman model that origins of replicons are attached to a nuclear scaffold. In an asynchronous cell culture these origins will be pulse labelled at random just like any other part of the nuclear DNA. The average $^{3}H/^{14}C$ ratio of the origins will thus be the same as the ratio of the total nuclear DNA, and double strand nuclease activity would not cause an alteration of the latter except for the contribution which is due to the attachment of the pulse labelled replication points. Only few origins of replicons will become labelled in the presence of arabinosylcytosine which has been shown to be a strong inhibitor of the initiation of new replicons (17). Release of DNA fragments by double strand breaks will then result in a decrease of the $^{3}H/^{14}C$ ratio of the complex, which more or less compensates the increase owing to the attachment of parts of the pulse labelled replication forks.

We would like to point out that our interpretation would possibly need some modification if, owing to erroneous incorporation of incorrect deoxyribonucleotides (for instance dUMP) as observed under certain conditions in bacterial systems (18, 19) post replication repair would occur. This might lead to resynthesis of more or less extensive DNA-"patches". Pulse label incorporated into such patches could be resistent to nuclease S_1, and if attached to the protein structure, also less accessible to DNase I. If repair would take place rapidly, the thereby induced single strand gaps would be located near to the branch point. Such a mechanism, which could make a semi-discontinuous mode of DNA synthesis appear to be totally discontinuous, would not affect our interpretations. However, these phenomena have not been observed in mammalian cells so far. In addition we have found that the apparent labelling patterns of replicative intermediates in our cell strain are not affected significantly by any kind of repair replication (10).

The exact location of the attachment site is of particular interest for the function of the nuclear structure in the replication model proposed by Dingman (14). A most important point of the model is, that it provides a mechanism for the complete unwinding of the parental DNA molecule, which is required for the separation of the two daughter molecules during the subsequent mitosis. This is explained by the particular spatial fixation of the origin as well as the replication fork to the scaffold (see fig. 6). The unwinding occurs as a consequence of the translocation of the DNA molecule along the replication binding sites. Obviously complete unwinding can be ensured if the binding occurs at, or at least extends into, the 2 branches

of the fork. Such a mode of binding is strongly supported by our results.

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Chapter V

THE STRUCTURE OF THE ATTACHMENT SITE

V. THE STRUCTURE OF THE ATTACHMENT SITE

V.1. *Introduction*

In chapter 4 it has been shown that in the replication points DNA is attached to the nuclear matrix behind the branch point. The existence of an attachment site *in front of* the branch point could neither be demonstrated nor ruled out by our results. The reason for this is the aspecific labelling of parental DNA with ^{14}C-dThd, as will be pointed out below.

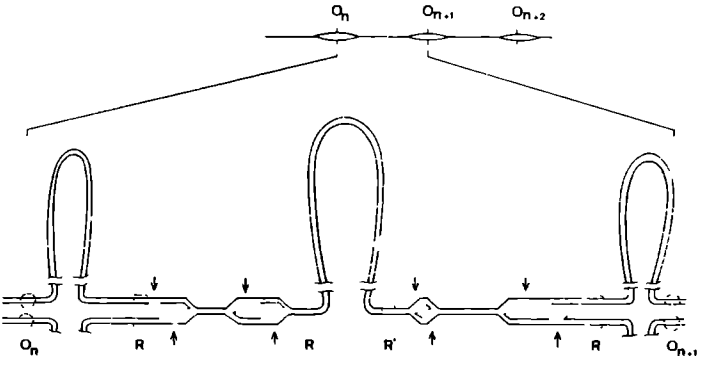

Fig. 1. *Model of the spatial organization of replicating DNA.* DNA is attached to the nuclear scaffold at the replication origins of the individual replicons (0_n, 0_{n+1}, 0_{n+2}). Additional attachment sites (R) were shown to exist behind the branch points. In case of absence of attachment sites (R') in front of the replicating regions, digestion by single-strand-specific nucleases (arrows) of the single-strand gaps opposite to growing Okazaki fragments should cause detachment of the unreplicated loop from the nuclear matrix.

From figure 1 it is obvious that detachment of the Okazaki fragments from the nuclear matrix by single-strand-specific nuclease will be accompanied by release of part of the parental DNA when an attachment site in front of the branch point does not exist. The DNA detached consists of the unreplicated loops between pairs of approaching replication points, originating from successive origins within clusters of replicons. However, since approximately 10^5 replicons (1-3) have to be duplicated in 12 hours (the length of S-phase in our cell-line (4)), and since the average duplication time

for a replicon is assumed to be 30 minutes (5,6), at each moment in S-phase approximately 4% of the replicons are actively engaged in the process of replication. As these replicons on the average will have been duplicated for 50%, one can expect an upper limit of 2% of the randomly labelled DNA to be susceptible to the action of single-strand-specific nucleases. The actual amount that will be detached, is most likely to be even somewhat smaller. This is due to the fact that part of the DNA of the terminal replicons of each cluster will remain bound to the nuclear matrix after digestion of the single-strand regions because of its attachment to an inactive terminal replicon of an adjacent cluster. Therefore, one has to expect a release of 1-2% of the randomly labelled DNA from the nuclear matrix, obtained from asynchronuous cells, by the action of single-strand nucleases. As this proportion is within the experimental error of the method employed, a different approach had to be adopted.

During S-phase DNA does not seem to be replicated randomly. A temporal order seems to exist in which DNA sequences that are duplicated in a particular period in one S-phase, are duplicated in the corresponding period in each following S-phase (7-12). Consequently, if in synchronized cells only the, for instance, early replicating clusters of replicons would be labelled in one S-phase and S1-digestion would be carried out early in the next S-phase, one would expect a considerable part of the label to be detached from the nuclear matrix in case of the absence of an attachment site in front of the branch point. A second advantageous effect of synchronization is that because of the increase of the proportion of cells in S-phase, the absolute amount of replicating DNA and consequently the amount of label susceptible to nuclease S1, will also increase. Therefore, an experimental scheme was designed in which cells were synchronized at the beginning of S-phase by two successive blocks of excess deoxynucleotides. Between the blocks DNA was pulse-labelled and after the second block the Nuclease S1-digestion was carried out.

V.2. *Materials and Methods*

V.2.1. *Cell synchronization and labelling procedures.*

To asynchronuous cells, grown in monolayers as described elsewhere (4), 2mM dAdo was added for 16 hours. The dAdo-containing medium was then removed, the cells were washed with fresh medium and medium containing 50 uM dGua was added. This nucleotide was present in the medium to shorten recovery time since due to excess dAdo, the dGua-pool is depleted most (13). Subsequently replicating DNA was labelled with 0.5 uCi/ml ^{14}C-dThd (spec. activity 52.8 mCi/mmol; NEN) for 30 minutes starting 20 minutes after release from the dAdo-block. Then the dGua- and ^{14}C-dThd-containing medium was replaced by fresh medium, in which the cells were allowed to grow for another 10 hours to complete S-phase. 2mM dAdo was again added for 16 hours, after which the block was lifted as described before. Thereupon ^{3}H-dThd (20 uCi/ml; spec.activity 20 Ci/mmol; NEN) pulses of 5 minutes were given at the times indicated, after which the nuclei were isolated.
When dThd was used to synchronize cells, a protocol as described in chapter 3 was followed. Between the blocks replicating DNA was labelled with ^{14}C-dThd at the time of maximal DNA-synthetic activity, i.e. 6-9 hours after release from the block.

V.2.2. *Analysis of the nuclear lysates*.

Nuclei were lysed in 2M NaCl and the lysates were treated with nuclease S1 as described in chapter 4. The lysates were then analysed on 15-40% neutral sucrose gradients, which were spun at 20,000 rpm in SW27II-rotors for 1 hour. Alternatively, the lysates were analysed for the amount of ^{14}C-dThd-labelled DNA detached by Nuclease S1 by spinning down the rapidly sedimenting material in a Sorvall HB-4 rotor for 20 minutes at 8,000 rpm and determining the amount of label in the supernatant.

V.2.3. *Density labelling and isopycnic centrifugation.*

To cells, synchronized by a double-block and labelled with ^{14}C-dThd for 1 hour between the blocks, 10 uM ^{3}H-BrdUrd (2,5 uCi/ml; NEN) was added at an appropriate time after the second block. Samples of cells were taken at the times indicated. The cells were then lysed in 0,5% SDS in 50 mM Tris-HCl pH 8.0 and the lysates were digested successively with 50 ug/ml RNase A (Sigma) and 50 ug/ml pronase (nuclease-free; Sigma) for 30 minutes each at 37^{o}C. DNA was subsequently isolated and analysed on CsCl-gradients as described before (14).

V.3. *Results*

In the first approach, the analysis of whether or not attachment to the nuclear matrix occurs in front of the branch point, dAdo was used as the blocking agent. After the first block ^{14}C-dThd was added to label early replicating clusters of replicons. Cells were then allowed to complete S-phase after which they were again blocked in medium that contained dAdo. After reversal of the second block, cells were pulse-labelled for 5 minutes with ^{3}H-dThd at various times, in order to label nascent DNA, which served as an internal control for the Nuclease S1 digestion. Nuclear lysates were then prepared, treated with Nuclease S1 and analysed on sucrose gradients. In Figure 2 both the experimental scheme and the results of 3 seperate experiments are given. Compared to the results presented in chapter 4, it is obvious that digestion by Nuclease S1 detaches considerably more ^{14}C-labelled DNA from the rapidly sedimenting complex obtained from selectively labelled cells than from the complex obtained from continuously-labelled asynchronuous cells. Moreover, though the proportion of ^{14}C-labelled DNA detachable by Nuclease S1 immediately after release from the second block seems to be rather variable, the amount released nevertheless tends to decrease in time, as one would expect to occur.
However, as synchronization by excess dAdo might cause lesions

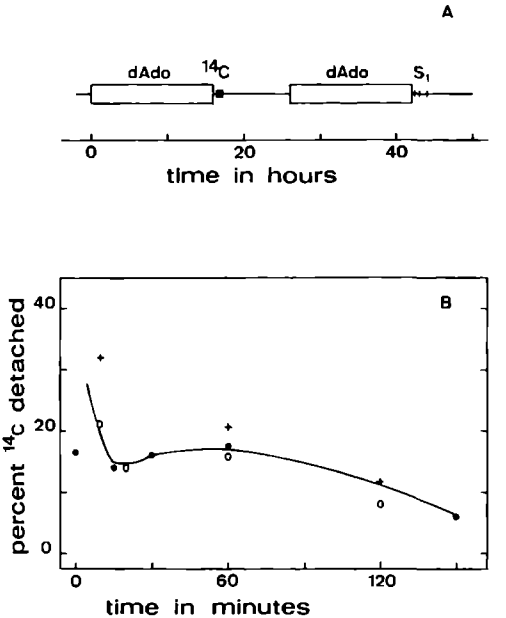

Fig. 2.a. *Protocol of the experiments to analyse the Nuclease S1-sensitivity of selectively labelled DNA.* Cells were synchronized by successive dAdo-blocks. ^{14}C-dThd was present for 30 minutes between the two blocks. Samples, taken at different times after release of the cells from the second block, were digested by Nuclease S1 and the amount of label released from the nuclear matrix was determined.

2.b. *Time-course of detachment by Nuclease S1 of selectively labelled DNA from the nuclear matrix obtained from cells synchronized by dAdo.*

(15,16) control experiments had to be performed to see whether the synchronized cells showed the normal replicative type of DNA-synthesis. Therefore, cells were labelled with ^{14}C-dThd for 1 hour between the two dAdo-blocks. Subsequently, following the second block, ^{3}H-BrdUrd was added and samples were taken at different times. DNA was isolated, purified and analysed on CsCl-density gradients. Surprisingly, even after 24 hours of labelling with BrdUrd, a shift of the ^{14}C-labelled DNA to intermediate density could not be observed (Figure 3). This indicates in all probability that dAdo had blocked replicative DNA-synthesis irreversibly and

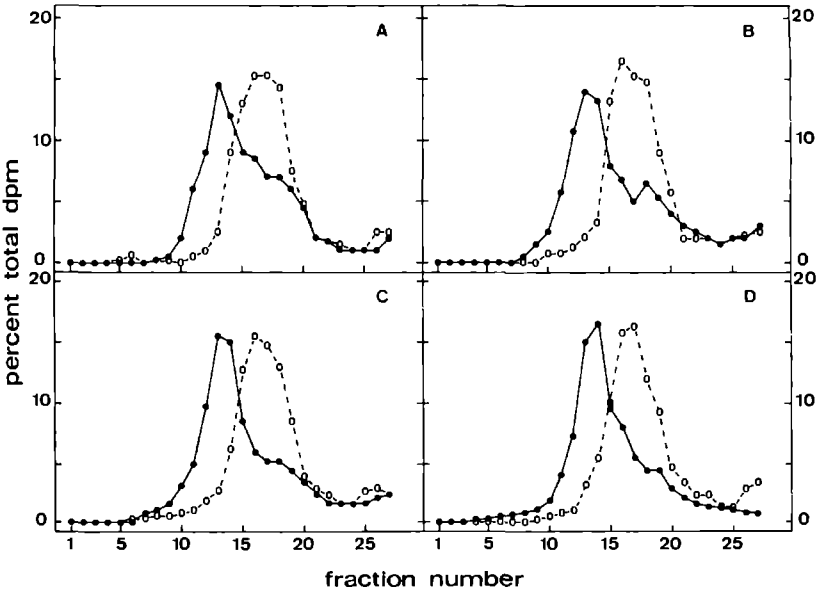

Fig. 3. *Isopycnic analysis of DNA labelled in successive S-phase in dAdo-synchronized cells.* Cells were synchronized by a double dAdo-block. 3 hours after the first block, DNA was labelled with ^{14}C-dThd for 1 hour. Immediately after the second block, ^{3}H-BrdUrd was added and the shift of the ^{14}C-labelled DNA to intermediate density was followed on CsCl-gradients. Samples were taken at 3 (A), 4.5 (B), 6.5 (C) and 24.5 (D) hours after the time of addition of ^{3}H-BrdUrd to the cells.
Total dpm per gradient were:

	A:	B:	C:	D:
^{3}H:	4,734	14,260	23,800	126,000
^{14}H:	1,810	1,810	1,650	1,200

has induced some type of unscheduled DNA-synthesis. Moreover, a marked proportion of the cells was found to stop proliferating and ultimately died. Since the observed effect of Nuclease S1 is obviously not related to replication and since for this type of experiments an undisturbed replication is an absolute prerequisite, the use of dAdo for synchronization procedures had to be abandoned. As an alternative, dThd was used in all further experiments.

Employing dThd as the synchronizing agent it was firstly investigated whether the cells could enter S-phase after the double-block. Therefore, a density-shift experiment, similar to the one described above, was done. Figure 4 shows that DNA, which had been labelled for 1 hour with ^{14}C-dThd 7 hours

after the lifting of the first block, had completed its shift to intermediate density between 14 and 18 hours after release of the cells from the second block. It can, therefore, be concluded that DNA labelled between the blocks, is replicated in the next S-phase but that, possibly due to the synchronization procedure, the temporal order of replication is not very strict.

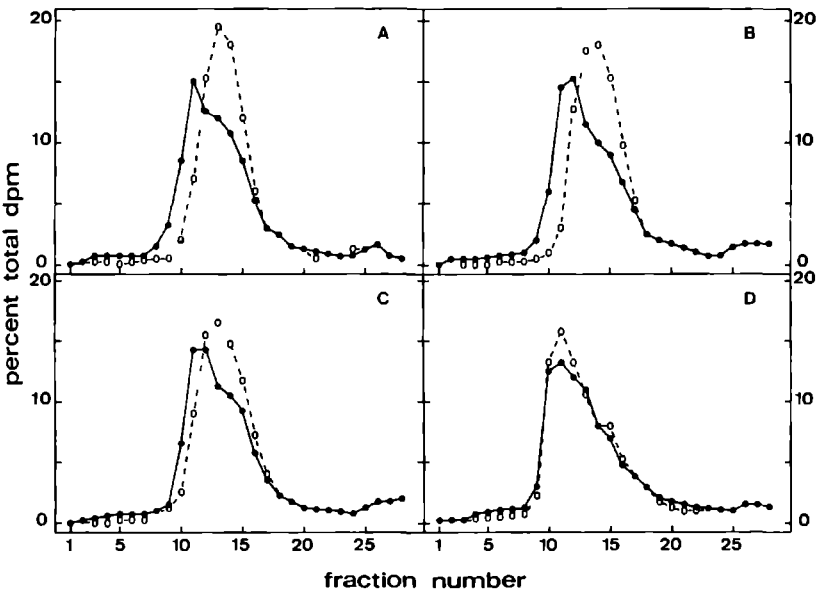

Fig. 4. *Isopycnic analysis of DNA labelled in successive S-phases in dThd-synchronized cells.* Cells were synchronized by a double dThd-block. 7 hours after release of the cells form the first block, DNA was labelled with ^{14}C-dThd for 1 hour. 6 hours after the second block, ^{3}H-BrdUrd was added and the shift of the ^{14}C-labelled DNA was followed on CsCl-gradients. Samples were taken at 1.0 (A), 2.5 (B), 8.0 (C), and 23.5 (D) hours after addition of the ^{3}H-BrdUrd label.
Total dpm per gradient were:

	A	B	C	D
^{3}H:	61,670	171,960	355,000	510,600
^{14}C:	2,850	3,550	4,030	5,110

Since replicative DNA-synthesis seems to proceed in this system, the Nuclease S1-sensitivity of the ^{14}C-labelled DNA could be analysed next. To that purpose cells were labelled with ^{14}C-dThd for 1 hour between the successive dThd-blocks. After release from the second block samples were taken at the times indicated and nuclear lysates were prepared, which were

treated with Nuclease S1. Figure 5.a. shows that the proportion of ^{14}C-labelled DNA, detached by Nuclease S1 in the S-phase following the second block, attains a maximum at a time corresponding to the period of labelling in the previous S-phase. After several hours the proportion detached decreases to values still significantly higher than those in unsynchronized cells. The time-course seems to indicate that an attachment site in front of the fork might be absent, though evidence is still rather inconclusive. This could be due to loss of phase synchrony as a result of the synchronization procedure (17), as also is indicated by the broad maximum. Since the density-shift experiments do not exclude excess dThd-induced repair synthesis, which occurs simultaneously with replication, it had to be ruled out that the S1-susceptibility of the ^{14}C-labelled DNA was to some extent the consequence of the single-strand gaps and nicks, which are known to be generated temporarily by certain types of repair (18).

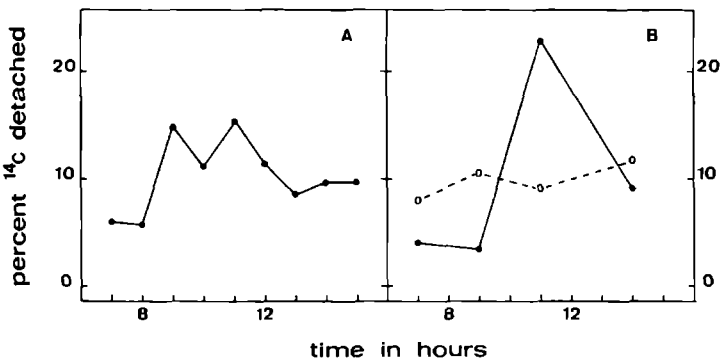

Fig. 5. *Time-courses of detachment of ^{14}C-labelled DNA from the nuclear matrix by Nuclease S1.*
Fig. 5.a. Cells were labelled with ^{14}C-dThd for 1 hour, starting 9 hours after release of the cells from the first dThd-block. After the second block, samples were taken at the times indicated (t=0 corresponding to the time of release of the cells from the second block) and treated with Nuclease S1.
5.b. As under a., except that between the blocks cells were both continuously-labelled with ^{14}C-dThd and pulse-labelled with ^{3}H-dThd for 1 hour starting 9 hours after release from the first block.
●——●——●: pulse-labelled DNA.
0- -0- -0: continuously-labelled DNA.

To examine the contribution of repair, a slightly different experiment than the one described above was performed. Immediately following the first dThd-block the cell population was divided into two. Between the blocks one of the samples was labelled with ^{14}C-dThd continuously while the other was only labelled for 1 hour at the time of maximal DNA-synthetic activity. Figure 5.b. shows that the ^{14}C-pulse-labelled DNA was most sensitive to Nuclease S1 immediately after the time corresponding to the period, in which in the previous S-phase the label had been present. The continuously-labelled DNA showed a relatively constant, though rather high, Nuclease S1-susceptibility. This observation confirms the results presented before, though the rather small difference between the proportions of DNA detached from the nuclear lysates, obtained from ^{14}C-pulse-labelled and ^{14}C-continously-labelled cells respectively, only allows cautious conclusions. Further experiments will be needed for corroboration.

V.4. *Discussion*

The existence of a stringently fixed temporal order for the replication of DNA in eukaryotic cells is still under investigation. One of the main difficulties in precisely demonstrating this temporal order lies in the absence of natural synchrony in higher eukaryotes. This is contrary, for instance, to the high degree of synchrony of replication occurring in macroplasmodia of Physarum polycephalum, in which organism DNA-duplication was shown to follow a fixed order (7). In yeast too, this was demonstrated (10).
In higher eukaryotes the presented evidence indicates that replication does not proceed completely at random throughout S-phase. In synchronized cells certain DNA-classes were found to be duplicated in ordered time-sequences (12). This temporal order, however, does not seems to be very strict (17,19) and synchronization by a number of drugs was found to have a disturbing effect on the sequence programming (17,18). Nevertheless, the occurrence, to some extent, of sequence programming has to be considered a prerequisite for this

study which is aimed at demonstrating the existence or absence of an attachment site in front of the replication fork. Evidence presented on the Nuclease S1-sensitivity of the selectively labelled DNA of cells synchronized by excess dThd, suggests that at times somewhat later than the time of labelling in the previous S-phase, an increased amount of DNA could be detached from the nuclear matrix. As this amount is higher than the background level, i.e. the proportion of detached DNA continuously labelled between the successive dThd-blocks, this observation favors the model in which the unreplicated loops between two approaching replication points are not attached to the nuclear matrix. The amount of selectively labelled DNA, detached by Nuclease S1, however, is relatively low. Most probably this is a consequence of a rather low degree of sequence programming, as is indicated by the density-shift experiments. DNA, pulse-labelled between the successive dThd-blocks, was found to be duplicated throughout the whole of the next S-phase. In case of the existence of a stringently fixed temporal order of replication an abrupt shift would have been found. Excess dAdo disturbed the replication process even more, blocking DNA-synthesis and reducing cell-viability.

In view of the detrimental effects of the synchronization procedures which have been used in this study, the results presented cannot be considered to be more than tentative. Confirmation will have to be obtained from experiments with cells synchronized by mitotic selection or, assuming the mechanism of DNA-replication in lower and higher eukaryotes to proceed esentially similar regarding spatial organization, with naturally synchronous systems such as Physarum polycephalum.

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Chapter VI

CHROMATIN ORGANIZATION IN THE REPLICATION POINTS

VI. CHROMATIN ORGANIZATION IN THE REPLICATION POINTS

VI.1. *Introduction*

The major part of nuclear chromatin has been shown to be organized in nucleosomes (1). These structural repeating units consist of a histone octamer around which a 140 base-pair segment of DNA is wound. Along the DNA string, successive nucleosomes are separated by linkers of an average length of 40 to 60 base-pairs, to which histone H1 is bound. The linker regions are readily accessible to nucleases, while the DNA-segment associated with the histone octamer is strongly shielded (1,2). Whether or not this structure is retained in the immediate vicinity of the replication point is not yet clear. Ultrastructural data have been presented indicating that chromatin structure on both sides of the replication fork is identical (3).

Most biochemical evidence, however, shows that newly replicated chromatin, *in vivo*, is more susceptible to endonucleolytic degradation than is the remainder of the chromatin (4-7). One of the hypotheses presented to account for this difference is an asymmetric segregation of the parental nucleosomes during replication, resulting in a transient absence of nucleosomes from one of the two double helices behind the replication fork (4). Recent results seem to confirm this assumption (8).

Further evidence in support of this view has been obtained from studies on replication in the absence of protein synthesis, i.e. *in vitro* replication (9,10) or replication in the presence of cycloheximide (11). Aditionally, it was observed that the nucleosomes immediately behind the replication fork have an altered conformation (9,10,12).

It has been suggested that the unshielded internucleosomal regions give access to transcriptional and replication factors (13,14). In particular Okazaki fragments might be initiated at these sites. This is compatible with the finding that the size of Okazaki fragments roughly matches the average length of the DNA segment contained by the nucleosome (15). Initiation of Okazaki fragments in the internucleosomal regions could

imply the occurrence of pre-fork replication loops (13), for which we have recently provided experimental evidence (16). Furthermore we have shown that replicating DNA is intimately associated with a nuclear protein skeleton, which can be isolated from nuclei that have been lysed in 2M NaCl (17). After removal of the histones by the high salt concentration and owing to the single-strand gaps between the growing nascent strands, a substantial part of the nascent DNA can be cut out by single-strand-specific nucleases and can be separated from the skeleton by centrifugation (18). In this study we investigate whether the single-strand gaps in the DNA of replicating chromatin are shielded by nucleosomes.

VI.2. *Materials and Methods*

VI.2.1. *Cell culture and labelling procedures.*

Bovine liver cells were grown in monolayers in Carrel-flasks as described elsewhere (19), except that a serum concentration of 10% was used. DNA was pre-labelled by addition of 0.02 uCi/ml 2-^{14}C-dThd (specific activity 52.8 mCi/mmol; NEN) for at least 30 hours. After growing the cells in label-free medium for another 2 hours, the cells were pulse-labelled for 1 minute with 50 uCi/ml me-^{3}H-dThd (specific activity 20 Ci/mmol; NEN). Incorporation was stopped by rinsing the cells with ice-cold 0.1% Triton X-100 in 50 mM Tris-HCl, pH 8.0. Nuclei were then isolated as described before (17) and resuspended in 50 mM Tris-HCl pH 8.0.

VI.2.2. *Enzymatic digestions and preparation of the nuclear lysates.*

For digestions by Nuclease S1 the pH of the nuclear suspension was adjusted to 4.5 by adding 0.5 M acetate-buffer (final concentration 50 mM). Incubations were carried out for 30 minutes at 37^{o}C in the presence of 1 mM $ZnSO_4$.
Incubations with the single-strand-specific nuclease from Physarum polycephalum, isolated as described elsewhere (20), were carried out in 50 mM Tris-HCl pH 8.0 for 30 minutes at 37^{o}C.

All incubations were stopped by addition of EDTA to a final concentration of 30 mM. Subsequently nuclear lysates were prepared as described before (18), except that a final concentration of 2 M NaCl was used. Alternatively, lysates of which the pH was adjusted to 4.5 *after* lysis of the nuclei, and lysates obtained from nuclei, which had been incubated in medium of pH 4.5 for 30 or 60 minutes, were subjected to digestion by Nuclease S1.
Furthermore, lysates were incubated with the Physarum enzyme after 10-fold dilution with 50 mM Tris-HCl pH 8.0. To avoid reassociation of histones with the DNA attached to the nuclear skeleton, heparin was added to a final concentration of 20 µg/ml (21).

VI.2.3. *Sedimentation analysis.*

The lysates were analysed on neutral, 2M NaCl containing 15-40% sucrose gradients, prepared on 65% sucrose cushions containing 0.4 g/ml CsCl. Centrifugation was performed in a Spinco SW27II rotor for 1 hour at 20,000 rpm and at 20°C. Fractions of 2.5 ml were collected starting from the bottom of the tube and the radioactivity was determined as described before (22).

VI.3. *Results*

We have shown recently (18) that incubation of the rapidly sedimenting DNA-protein complex, obtained from nuclei lysed in 2M NaCl, with the single-strand-specific enzyme Nuclease S1 results in detachment of a substantial part of the nascent DNA without concomitant loss of detectable amounts of parental DNA. The nascent DNA detached was shown to consist almost exclusively of Okazaki fragments hydrogen bonded to parental segments.
In order to investigate whether the single-strand regions in replicating DNA are protected by nucleosomes from the action of single-strand nucleases, pre-labelled cells were pulse-labelled with ^{3}H-dThd for 1 minute and the nuclei were isolated.

Subsequently, part of the nuclei was incubated with Nuclease S1 and then lysed in 2M NaCl. The other part was first lysed in 2M NaCl, after which the lysate was treated with the single-strand nuclease.
Sedimentation analysis of the untreated NaCl-lysates in sucrose gradients revealed that almost 100% of the DNA remained attached to the nuclear skeleton (Figure 1.a.). Incubation

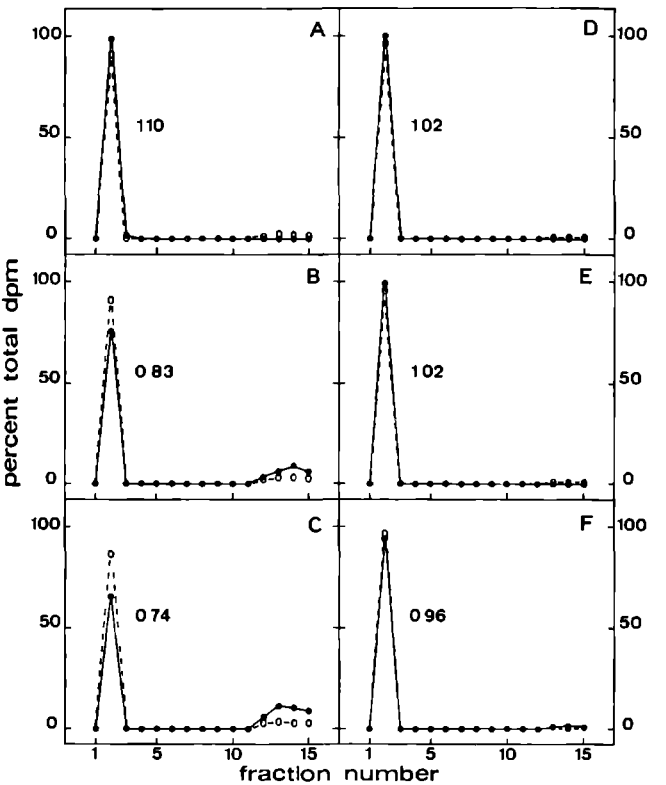

Fig. 1. *Effect of Nuclease S1 digestion on nuclear lysates and nuclei.* Nuclei were isolated from ^{14}C-dThd-labelled cells, which were pulse-labelled with ^{3}H-dThd for 1 minute. Half of the nuclei was lysed in 2M NaCl and the lysate was divided into 3 portions. Nuclease S1 was added to final concentrations of 0 (A), 100 (B) and 1000 (C) units/ml. The other half of the nuclei was also divided into 3 portions and Nuclease S1 was added to final concentrations of 0 (D), 100 (E) and 1000 (F) units/ml. Subsequently the nuclei were lysed by addition of NaCl to a final concentration of 2M. All lysates were analysed by sucrose gradient centrifugation. Sedimentation is from right to left. The numbers in each panel represent the ratios of the percentages of ^{3}H and ^{14}C present in the rapidly sedimenting material.
Average total dpm per gradient were 2,500 for ^{3}H and 8,000 for ^{14}C.

of the nuclei at pH 4.5 prior to lysis had no effect on the sedimentation pattern (Figure 1.d.). In accordance with our previous results (18), digestion of the NaCl-lysates with Nuclease S1 resulted in a 30% release of the pulse-labelled DNA without significant loss of pre-labelled DNA (Figures 1.b. and 1.c.). Treatment of nuclear suspensions with the enzyme caused only a very small and variable release of nascent DNA (Figures 1.d. and 1.e.).

Routinely, between 60 and 90% of the pulse-label released by digestion of nuclear lysates remained associated with the rapidly sedimenting complex when whole nuclei were digested. The most plausible reason for the inaccessibility of newly replicated DNA in chromatin is a partial shielding of the single-strand regions by nucleosomes. It is not likely that H1-histones are involved, because digestion of nuclei in the presence of 0.4M NaCl gave the same results as incubations performed without NaCl (results not shown).

A particular disadvantage of the use of Nuclease S1 is its requirement for a low pH. Microscopical examination showed that at pH 4.5 the nuclei had shrunk to about half their original diameter. This implies that an artificial reorganization of the nuclear proteins, and maybe also some displacement of nucleosomes along the DNA-strands, cannot be excluded. Moreover, it had to be made sure that the relative insensitivity of newly replicated chromatin towards Nuclease S1 is not due to a collapse of a very labile structure of replicating chromatin in a medium of low pH and low ionic strength. Therefore, nuclei were first incubated at pH 4.5, then lysed by addition of 2M NaCl and digested as described before. The pre-treatment had no significant effect on the release of nascent DNA from the nuclear skeleton (Figure 2). Moreover, preliminary electrophoretic data indicate that the poly-peptide composition of the rapidly sedimenting complex, obtained from nuclei incubated at pH 4.5 for up to 60 minutes, did not differ significantly from the poly-peptide composition of nuclear skeletons isolated at pH 8.0 (Schellinx, unpublished results). Whether these observations imply that chromatin structure in

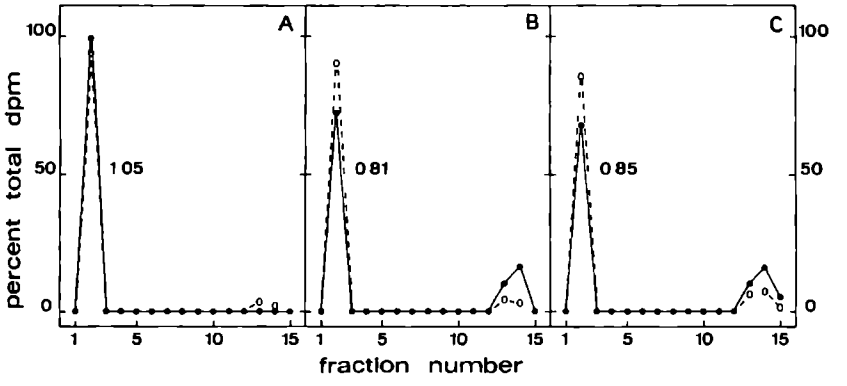

Fig.2. *Effect of the incubation of nuclei at pH 4.5 on digestion of nuclear lysates by Nuclease S1.* Nuclei were isolated from ^{14}C-dThd-labelled cells, which were pulse-labelled with ^{3}H-dThd for 1 minute. Nuclei were lysed in 2M NaCl immediately (A and B) or after incubation in medium of pH 4.5 for 60 minutes (C). Nuclease S1 was then added to final concentrations of 0 (A) and 300 (B and C) units/ml. For further details see under figure 1.
Average total dpm per gradient were 2900 for ^{3}H and 2200 for ^{14}C.

medium of pH 4.5 is identical to chromatin structure under physiological conditions, remains to be proven. For this reason we have performed similar experiments with a recently described single-strand-specific endonuclease of Physarum polychephalum (20), which has its maximal activity at pH 8.0. The strong inhibition of this enzyme by high concentrations of salt made it necessary to reduce the NaCl-concentration of the lysate to 0.2 M. The reassociation of histones with the DNA was prevented by addition of heparin. Figure 3 shows some representative results. Like Nuclease S1, the Physarum enzyme specifically detaches nascent DNA (panels A to D). The amount of pulse-label released from the rapidly sedimenting complex (44% in this particular experiment) was always larger than the amount released by Nuclease S1. When nuclear suspensions were digested with the Physarum enzyme, 22% of the pulse-label was detached, while treatment of another portion of the same suspensions with Nuclease S1 only resulted in detachment of 14% of the pulse-label (panels E and F). On the average, the

amount of pulse-label released from the nuclear skeleton by the Physarum endonuclease, added to nuclei, varied from 40 to 50% of the amount released by the enzyme added to nuclear lysates. Approximately the same percentage of nascent DNA was released by the Physarum enzyme, when nuclei had been digested in the presence of 0.4 M NaCl, suggesting that the H1-histones do not protect single-strand regions from the action of the enzyme.

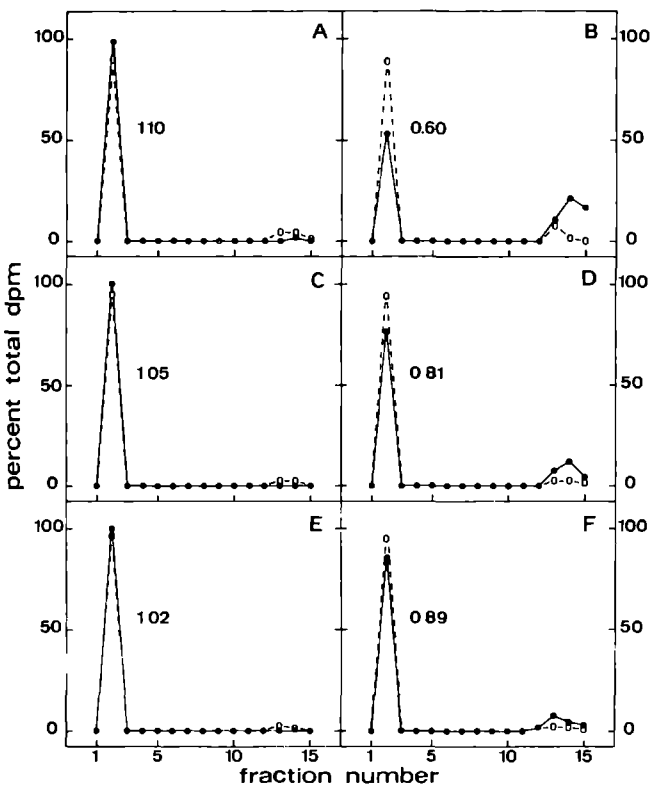

Fig. 3. *Effect of Physarum Zn-endonuclease digestion on nuclear lysates and nuclei.* Nuclei were isolated from ^{14}C-dThd-labelled cells, which were pulse-labelled with ^{3}H-dThd for 1 minute. A portion of the nuclei was lysed in 2M NaCl, the lysate was diluted tenfold and divided into 2 portions. Zn-endonuclease was added to final concentrations of 0 (A) and 300 (B) units/ml. The remainder of the nuclei was divided into 4 portions. Zn-endonuclease was added to 2 portions in final concentrations of 0 (C) and 300 (D) units/ml and, as a control, Nuclease S1 was added (after adjustment of the pH to 4.5) to final concentrations of 0 (E) and 300 (F) units/ml. For further details see under figure 1. Average total dpm per gradient were 1,000 for ^{3}H and 600 for ^{14}C in (A) and (B) and 3,600 for ^{3}H and 2,350 for ^{14}C in (C) through (F).

Replicating DNA in eukaryotic cells is attached to a high-salt resistant nuclear protein skeleton (18,23,24). The attachment site has been shown to be located closely behind the branch point of the replication fork (18), in such a way that the Okazaki pieces, hydrogen-bonded to the parental DNA segments, can be removed specifically by the single-strand-specific Nuclease S1. In the present chapter it is demonstrated that the nascent pieces can be cut out with the same specificity, and even slightly higher efficiency, by a recently isolated and characterized single-strand nuclease of Physarum polychephalum (20). The accessibility of the single-strand regions is strongly reduced in chromatin. The amount of nascent DNA released from intact chromatin in isolated nuclei by the Physarum enzyme is only 40-50% of that released by digestion of histone-depleted DNA. With Nuclease S1 the difference between digestion of chromatin and histone-free DNA is even greater, and, moreover, somewhat less reproducible. This is possibly due to artificial changes of chromatin structure by the low pH, which is required for the enzyme to be active. We will, therefore, base our further discussion solely on the data obtained from the experiments with the endonuclease of Physarum polychephalum.

Our results clearly show that part of the single-strand regions are effectively protected by chromatin proteins. The protection is not significantly altered when the H1-histones are removed by 0.4 M NaCl. Apparently, the internucleosomal linkers are equally accessible to micrococcal nuclease (25) and the single-strand-specific nuclease. We, therefore, suggest that the single-strand regions, not accessible to the Physarum enzyme, are in some way associated with the histone cores of the nucleosomes.

Based on recent results (16-18), Figures 4.a. and 4.b. diagrammaticly show two possible chromatin structures of the replication fork. From diagram 4.a. it can be inferred that release of a piece of nascent DNA can require up to 4 single-strand regions to be digested. For example, for release of the fragments between the initiation points I and II single-

strand breaks have to be introduced at sites 1, 2, 1' and 2' or 3'. The fragments will not be released, if one of the single-strand regions at sites 1,1' or 2 is not accessible to the enzyme. It can be roughly estimated that inaccessibility of only 2 to 3 out of 10 single-strand regions would be sufficient to reduce the release by about 60% as found in our experiments with the Physarum nuclease.

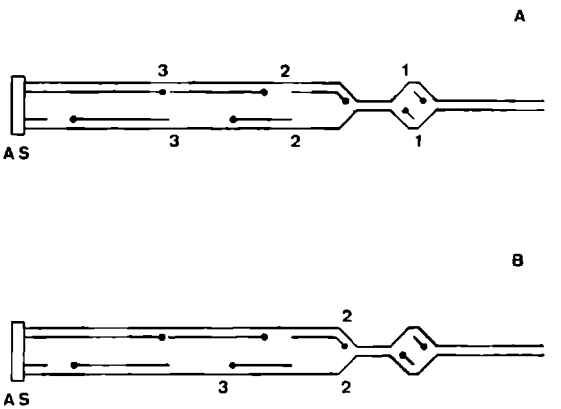

Fig. 4. *Schematic representation of the chromatin structure in the immediate vicinity of the replication fork.*
4.a. Okazaki fragments are initiated between the nucleosomes. Consequently, all single-strand regions (1,2 and 3, 1', 2' and 3') between the growing Okazaki fragments are located in the internucleosomal spaces and, therefore, can be digested by single-strand-specific nucleases.
4.b. Okazaki fragments are initiated within the nucleosomes. Consequently, only a minor amount of the single-strand regions between the Okazaki fragments will be located in the internucleosomal spaces.

If one assumes that initiation of Okazaki fragments occurs near the center of the internucleosomal regions, the amount of nascent DNA removed by digestion of chromatin with a single-strand-specific nuclease should be equal to that removed by digestion of histone-depleted chromatin. This is the consequence of the fact that, in spite of the presence of nucleosomes along the DNA, a stretch of 20 to 30 nucleotides will remain accessible at each single-strand region. However, if the Okazaki fragments are initiated randomly within the

internucleosomal regions, some of those regions might become too short to be accessible to the enzyme, and, therefore, will not be broken.
On the other hand, if synthesis of Okazaki fragments would start within the nucleosomes, single-strand regions in the internucleosomal spaces would also occur, though in a smaller number (Figure 4.b.). Since they appear in one branch only, it can be roughly estimated that no more than 15% of the label, residing in the Okazaki fragments, will be released by the action of single-strand-specific nucleases. The observed release, on the average approximately 40% of the Okazaki fragments is, therefore, more compatible with initiation of the Okazaki fragments occurring in the internucleosomal spaces, as shown in Figure 4.a.
Another factor, which might affect the results, is the possibility of a translocation of the nucleosomes during the experimental procedures. The chance of translocation occurring in media of low ionic strength and at a pH near to neutral, however, seems slight (26) and might even be disregarded. On the other hand, under the conditions required for the Nuclease S1-digestions, the *in vivo* structure might not be retained completely.
Knowledge of the chromatin structure around the replication fork is required for a precise understanding of the duplication of the eukaryotic genome. The experimental approach for an analysis of this structure is severely hampered by its dynamics and instability. Results obtained so far with Staphylococcal nuclease are believed to be related mainly to a region behind the replication fork (4). The use of single-strand-specific nucleases is more specifically directed to the replication fork proper. The tentative conclusion from our results is that Okazaki fragments are initiated at arbitrary positions within the internucleosomal segments of the DNA in chromatin. This is in agreement with the model proposed by Rosenberg (13).

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Chapter VII

SUMMARY/SAMENVATTING

VII. SUMMARY

Recent research in the field of the eukaryotic DNA-replication has led to a more detailed understanding of the molecular mechanism of this process and its temporal regulation. In the first chapter, a no doubt concise survey of this field has been presented.
Contrary to the structure of the cell nucleus, on which recently some attention has been focused, a lot less is known of the spatial organization of nuclear DNA. Moreover, data on the relation between DNA-replication and nuclear structures are almost completely lacking. To this subject, a major part of this thesis has been devoted. Since in nearly all studies of DNA-replication inhibitors can be very useful tools, the mechanism of action of two of those substances was analysed. The results of these analyses are presented in chapters 2 and 3.
Arabinosylcytosine (ara-C), which is described in chapter 2 (in a concentration of 10^{-4}M) was found to reduce the rate of chain elongation of growing DNA-strands markedly. This was indicated by a significant increase of the proportion of label incorporated into Okazaki fragments. These primary intermediates of the replication process were ligated to growing DNA-strands in the presence of the inhibitor, though at a greatly reduced rate compared to controls. This observation suggests that the Okazaki fragments, synthesized in the presence of ara-C, are regular intermediates of DNA-replication. Finally, based on the fact that, after lysis of cells in media of low ionic strength, most of the Okazaki fragments were observed to have single-strand properties, a model was designed in which the primary replication intermediates are initiated in front of the replication fork.
Daunomycin, too, retards fork progression significantly at concentrations of 10^{-5}M, as is shown in chapter 3. This effect, however, obscures another consequence of the addition of daunomycin to the cells, inhibition of the initiation of new replicons. This primary effect of the inhibitor, already occurring at concentrations of 10^{-6}M, results in

a reduction of the number of nascent DNA-chains of replicon length.
In the second part of this thesis, a study of the spatial organization of replicating DNA is presented. Previous work, performed in this laboratory, demonstrated that on sucrose gradients a rapidly sedimenting protein complex can be isolated from nuclei that had been dissolved in 1 or 2 M NaCl. Almost all nuclear DNA was found to be attached to this skeleton. The mode of attachment of DNA to the skeleton was analysed in chapter 4. To that purpose DNA was pulse-labelled for several different periods of time and subsequently the isolated DNA-protein complexes were digested by various DNA-degrading enzymes.
Digestion experiments with DNase I, which introduces nicks into DNA randomly, revealed that continously-labelled DNA was removed from the complex much more efficiently than was pulse-labelled material. Moreover, the susceptibility of pulse-labelled DNA was found to decrease with decreasing pulse lengths. This indicates that DNA synthesized most recently, is most intimately associated with the protein skeleton and that, therefore, an attachment site has to exist in the immediate vicinity of hte replication fork.
Contrary to DNase I, digestion by single-strand-specific nucleases, such as Nuclease S1, results almost exclusively in release of nascent DNA from the skeleton. This is the consequence of the fact that this enzyme introduces gaps and nicks into the single-strand parental DNA between the growing Okazaki fragments. As expected, the material detached was found to exist of the Okazaki fragments, base-paired to short stretches of parental DNA.
Digestion with DNase I of the complex, from which the Okazaki fragments had been removed, demonstrated that the remainder of the pulse-labelled DNA, consisting of mature Okazaki fragments ligated to fully duplicated replicons, still was intimately associated with the nuclear skeleton. A similar conclusion could be drawn from experiments using Staphylococcal Nuclease. This enzyme, which preferentially degrades single-strand DNA, first releases the Okazaki fragments from the

complex before detaching the rest of the DNA.
Taken together, these observations suggest that replicating DNA is not bound to the rapidly sedimenting protein structure in the regions, where Okazaki fragments are being synthesized, The attachment site will rather be situated at some distance *behind* the replication fork. Whether this attachment site is the only location, in which replicating DNA is bound to the nuclear skeleton, remains to be established.
Therefore, in chapter 5, it was investigated whether single-strand-specific nucleases are able to remove additional parental DNA from the rapidly sedimenting complex beside the parental material base-paired to the Okazaki fragments. It is well known that replicons are duplicated in clusters. In case of the absence of an attachment site in front of the replication fork, single-strand-specific nucleases should be able to detach the not yet replicated parental DNA, located between two approaching replication forks. As the potentially detachable material only constitutes a minuscule proportion of the total amount of parental DNA, a method had to be, and was, developed to label this fraction selectively. From the results obtained from experiments performed with the selectively labelled DNA, it can be concluded tentitatively that attachment sites in front of the replication fork are absent.
In the final chapter of this thesis (chapter 6) an analysis of the chromatin structure of replicating DNA is presented. In chapter 4 it was demonstrated that the single-strand-specific enzyme Nuclease S1 removes Okazaki fragments from histone-free replicating DNA. *In vivo*, however, the DNA-chain is wound around cylindrical histone complexes, so adopting the so-called nucleosomal conformation. It appeared reasonable to assume that replicating DNA, associated with histones,would be less susceptible to the action of single-strand-specific nucleases than histone-free DNA actually is. This assumption was verified experimentally with two different single-strand-specific enzymes. Neither of the two nucleases was able to detach from intact chromatin more nascent DNA than 40-50% of the amount which could be removed from histone-depleted chromatin. Based on this observation a model was

proposed suggesting that individual Okazaki fragments are initiated randomly within the internucleosomal spaces.

SAMENVATTING

Het onderzoek dat in de laatste jaren gedaan is naar het verloop van de DNA-replikatie in eukaryotische cellen, heeft een aantal belangrijke inzichten opgeleverd in het mechanisme van die replikatie op molekulair nivo en in de temporele regulatie ervan. In het eerste hoofdstuk wordt een, noodgedwongen beknopt, overzicht gegeven van de stand van zaken op deze onderzoeksterreinen.
Hoewel de struktuur van de celkern recentelijk wel wat meer belangstelling heeft gekregen, is over de ruimtelijke ordening van het DNA in de kern veel minder bekend. Dit geldt evenzeer, en mogelijk zelfs nog in versterkte mate, voor de samenhang tussen het verloop van de DNA-replikatie en strukturen in de kern. Aan dit onderwerp is in dit proefschrift dan ook de nodige aandacht besteed.
Omdat bij vrijwel ieder onderzoek, waarin de DNA-replikatie onder de loep wordt genomen, remstoffen een nuttige rol kunnen spelen werd het werkingsmechanisme van een tweetal van die inhibitoren nagegaan. Het tweede en het derde hoofdstuk zijn hieraan gewijd.
Arabinosylcytosine (ara-C), dat in hoofdstuk 2 aan de orde komt, blijkt in de door ons gebruikte koncentratie van 10^{-4} molair de groei van de DNA-ketens zeer sterk te vertragen. Dit uit zich ondermeer in een duidelijke toename van de relatieve hoeveelheid ingebouwde radioaktiviteit in de Okazaki fragmenten. Deze primaire intermediairen van de DNA-synthese werden ingebouwd in groeiende DNA-ketens, zij het dat dit in aanwezigheid van ara-C slechts zeer langzaam gebeurde. Dit wijst erop dat de Okazaki fragmenten, gevormd in aanwezigheid van de remstof, normale tussenprodukten zijn van de DNA-replikatie. Tenslotte werd, als verklaring voor het feit dat de gesynthetiseerde Okazaki fragmenten ten gevolge van de

lyseprocedure grotendeels enkelstrengs zijn, een model ontworpen dat impliceert dat de Okazaki fragmenten geinitieerd worden vóór de replikatievork.
Ook daunomycine, dat in het derde hoofdstuk beschreven staat, remt bij koncentraties rond 10^{-5} molair de ketengroeisnelheid in sterke mate. Hierdoor wordt echter een ander gevolg van de toevoeging van de inhibitor overschaduwd, te weten remming van de initiatie van nieuwe replikatie-eenheden (de zogenaamde replikons). Dit primaire effekt van daunomycine, dat zich uit in een vermindering van het aantal nieuw gestarte ketens van replikon-lengte, treedt al op bij een koncentratie van 10^{-6} molair.
Het tweede deel van dit proefschrift is volledig gewijd aan de bestudering van de ruimtelijke struktuur van het repliserend DNA. Eerder op dit laboratorium uitgevoerd onderzoek had aangetoond dat uit kernen van kalfslevercellen door lyse in 1 of 2 molair NaCl een op sukrose gradienten snelsedimenterend eiwitkomplex geisoleerd kan worden, waaraan vrijwel al het DNA gebonden is. De wijze van aanhechting van het DNA aan dat skelet is nader onderzocht in hoofdstuk 4. Daartoe werd het DNA gedurende verschillende tijden radioaktief gemerkt en vervolgens met een drietal DNA-afbrekende enzymen behandeld.
Uit de experimenten met DNase I,dat willekeurig verspreide knippen aanbrengt in het DNA,bleek dat kontinu gemerkt DNA veel gemakkelijker uit het komplex werd verwijderd dan DNA dat slechts zeer kort was gemerkt. Verder werd gevonden dat dit puls-gemerkte materiaal resistenter was naarmate de pulsduur korter werd. Dit betekent dat het eiwit-skelet het meest recent gesynthetiseerde DNA het best beschermt, met andere woorden dat er zich in de onmiddellijke nabijheid van de replikatievork een aanhechtingsplaats moet bevinden. In tegenstelling tot DNase I werd vastgesteld dat enkelstrengs specifieke nukleasen als Nuklease S1 vrijwel uitsluitend het nieuw aangemaakte DNA van het skelet losmaken. Reden hiervan is dat dit enzym breuken aanbrengt in het tussen groeiende Okazaki fragementen gelegen parentale DNA,dat dan enkelstrengs is. Zoals te verwachten was, bestond het los-

gemaakte materiaal dan ook grotendeels uit die Okazaki fragmenten,basegepaard met parentaal DNA. Afbraak met DNase I van het komplex,waaruit de Okazaki fragmenten verwijderd waren, liet zien dat de rest van het nieuw gesynthetiseerde DNA nog steeds nauw verbonden was met het eiwitskelet. Dit restant bestaat uit voltooide Okazaki fragmenten, die gekoppeld zijn aan de DNA-ketens van al eerder gedupliceerde replikons. Eenzelfde konklusie kon getrokken worden uit experimenten gedaan met Staphylococcus Nuklease, dat door zijn voorkeur voor enkelstrengs DNA eerst de Okazaki fragmenten van het skelet losmaakt alvorens de rest van het DNA los te knippen.
Deze resultaten doen vermoeden dat replicerend DNA niet aan de snelsedimenterende eiwitstruktuur is gebonden in het gebied, waar de Okazaki fragmenten worden gesynthetiseerd. De bindingsplaats moet veeleer gezocht worden op enige afstand achter de replikatievork. Of deze plaats de enige is, waar replicerend DNA aan het eiweitskelet is aangehecht, is vervolgens onderzocht.
In hoofdstuk 5 is nagegaan of enkelstrengs specifieke nukleasen, behalve het met de Okazaki fragmenten geassocieerde, nog meer parentaal DNA uit het snelsedimenterende komplex verwijderen. Bekend is dat replikons groepsgewijs, in zogenaamde "clusters", gedupliceerd worden. Bij afwezigheid van een aanhechtingsplaats vóór de replikatievork zou het daarom in principe mogelijk moeten zijn met enkelstrengs nukleasen het tussen twee elkaar naderende vorken gelegen nog niet gerepliceerde DNA van het skelet los te maken. Omdat dit deel op het totale parentale DNA slechts een zeer kleine fraktie uitmaakt, werd een methode ontwikkeld om deze fraktie selektief radioaktief te maken. De resultaten van experimenten gedaan met dit selektief gemerkte DNA lijken, voorlopig, te wijzen in de richting van het niet voorkomen van een aanhechtingspunt vóór de replikatievork.
In het laatste hoofdstuk staat een onderzoek beschreven van de chromatinestruktuur van replicerend DNA. Eerder (hoofdstuk 4) was aangetoond dat het enkelstrengs Nuklease S1 Okazaki fragmenten verwijdert uit replicerend DNA, dat histon-vrij

is gemaakt. *In vivo* echter komt het chromatine voor in de zogenaamde nukleosoomstruktuur, cylindrische histonkomplexen waar de DNA-keten omheen is gewikkeld. Naar alle waarschijnlijkheid is het zo met histonen bezette replicerende DNA minder toegankelijk voor het enkelstrengs specifieke enzym dan het histon-vrij gemaakte. Dit werd inderdaad experimenteel bevestigd, en wel met twee verschillende enkelstrengs specifieke nukleasen. Gekonstateerd werd dat deze twee enzymen in staat zijn maximaal de helft van de hoeveelheid Okazaki fragmenten te verwijderen, die uit het histon-vrije materiaal zijn los te maken. Op grond hiervan kon een model worden opgesteld, waarin initiatie van de afzonderlijke Okazaki fragmenten plaatsvindt op plaatsen willekeurig verspreid over de internukleosomale gebieden.

CURRICULUM VITAE

Schrijver dezes werd op 10 mei 1949 geboren in Hilversum. Ondanks een aantal omzwervingen in de tussenliggende jaren werd uiteindelijk niet ver daar vandaan, aan het Baarnsch Lyceum, in 1967 het einddiploma gymnasium β behaald.
In datzelfde jaar werd ook een aanvang gemaakt met de scheikunde studie aan de Rijks Universiteit Utrecht. In december 1970 werd daarop het kandidaatsexamen S2 behaald, in september 1974 gevolgd door het doktoraal. Voor het, wat dat laatste betreft echter zover was waren het bijvak fysiologische chemie (Dr. P.D. Baas en Prof.Dr. H.S. Jansz), het hoofdvak biochemie (Dr. J. de Gier) en tenslotte het bijvak kulturele antropologie (Prof.Dr. J. van Baal) met goed gevolg doorlopen.
De beloning hiervoor van zijde van de Rijks Universiteit Utrecht kwam al snel in de vorm van een tijdelijke aanstelling (augustus t/m december 1974) bij de vakgroep Biochemie van de Mondholte van de Subfaculteit Tandheelkunde, gevolgd door een, eveneens tijdelijke detachering bij de W.W.
Mede dankzij steun van de Stichting Scheikundig Onderzoek in Nederland kwam hieraan op 1 juni 1975 een einde door een aanstelling als wetenschappelijk ambtenaar op het Laboratorium van Chemische Cytologie (Prof.Dr. Ch.M.A. Kuyper) waar onder leiding van Dr. F. Wanka tot 1 november 1979 het onderzoek werd verricht dat tot dit proefschrift heeft geleid.
De laatste hand aan die proeve van bekwaamheid als onderzoeker werd in de, inmiddels al vertrouwde, W.W. gelegd.
Vanaf 1 augustus 1980 is de schrijver verbonden aan het sekretariaat van de Raad van Advies voor het Wetenschapsbeleid.

STELLINGEN

1. Het door Fridland gesuggereerde werkingsmechanisme van arabinosylcytosine levert nauwelijks een verklaring voor de gekonstateerde differentiele effekten van deze remstof op de initiatie respektievelijk de elongatie van nascente replikons.

   Fridland, A. (1977) Biochem. 16, 5308-5312.
   Dit proefschrift hoofdstuk 2.

2. Het verlies in 0,5M NaCl van dubbelstrengs, nascent DNA uit mononukleosomen zou erop kunnen wijzen dat het nukleosoom dicht achter de replikatievork voorkomt in een instabiele replikatieve vorm, die plaats biedt aan beide dochterhelixen.

   Schlaeger, E.J. and Knippers, R. (1979) Nucleic Acids Res. 6, 645-656.
   Dit proefschrift hoofdstuk 6.

3. Dat arabinosylcytosine de replikon-initiatie remt, wordt niet aannemelijker gemaakt door het herhaald en in verschillende tijdschriften presenteren van gelijkluidende resultaten van een beperkt aantal, nauwelijks van elkaar afwijkende experimenten.

   Fridland, A. (1977) Biochem. Biophys. Res. Commun. 74, 72-78.
   Fridland, A. (1977) Biochem. 16, 5308-5312.
   Bell, D.E. and Fridland, A. (1980) Biochim. Biophys. Acta 606, 57-66.

4. De bewering van Vance en de Kruijff dat methylering van fosfolipiden niet van fysiologisch belang kan zijn gezien de geringe grootte van dit effekt, dient, alvorens als serieuze kritiek op het werk van Axelrod en Hirata beschouwd te kunnen worden, experimenteel te worden onderbouwd.

   Vance, D.E. and de Kruijff, B. (1980) Nature 288, 277-278.
   Axelrod, J. and Hirata, F. (1980) Nature 288, 278-279.

5. Uit de konstatering dat mobiele onderzoekers dikwijls de beteren zijn, mag niet zondermeer afgeleid worden dat het veranderen van werkkring de oorzaak is van die hogere kwaliteit.

6. Subsidiëring door SON heeft ook haar schaduwzijden.

7. De skepsis, waarmee in Nederland een onorthodox onderzoeksidee gewoonlijk wordt begroet, kan een niet te onderschatten reden zijn van het, door de Verkenningskommissie Chemisch Onderzoek gekonstateerde, te wensen overlaten van de originaliteit van de Nederlandse chemische research.

   Chemie, nu en straks.
   Een verkenning van het door de overheid gefinancierde chemisch onderzoek in Nederland. (1979)

8. Dat personeel bij een reorganisatie door de instellings- of bedrijfsleiding maar al te vaak als lastpost wordt beschouwd, zou juist niet moeten betekenen dat het in de procedure behandeld wordt als sluitpost.

9. Een eerste aktiepunt van een nog op te richten belangenvereniging van toertochtschaatsers zal ongetwijfeld zijn het afdwingen van een wettelijk geregelde minimum-hoogte voor bruggetjes over tochten en sloten.

10. Als de situatie op de arbeidsmarkt niet drastisch verandert, zal in de toekomst menig cytoloog blijvend verworden tot sollicitoloog.

Nijmegen, 12 februari 1981
P.A. Dijkwel

A C C T C G A A

A T G T G T G T

G C A G A C A T

A A G C G T G G

A G G T G T G T

C T A A T A G C

G A T T G C G C