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Factors That Influence The Size Of The Repair Patch In The Dna Of Cultured Human Cells

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ABSTRACT

The study of DNA repair in human cells has been made difficult by the limited knowledge available on the enzymes that work on DNA, and this problem is compounded by the organization of the DNA into complex chromatin structures. With the exception of the initial incisional event, two or more enzymes have been reported to have the ability to accomplish each of the other enzymatic steps. Furthermore, the number of nucleotides that are replaced to correct each damage site, and how this extent of substitution is regulated, has been an issue of controversy. Because a knowledge of the extent of nucleotide replacement could shed some light on the enzymology of DNA repair in human cells, a study was made of the size of the repair patch in DNA, and the influence of inhibitors on the repair processes.

The buoyant density shift method was employed to measure DNA repair patch sizes. In this method, DNA was extracted from cells that were treated with either UV-light or a methylating agent, and allowed to repair their DNA in the presence of the density label, bromodeoxyuridine (BrdUrd). The DNA was then reduced in size and analyzed in a buoyant density gradient. The small fragment size allowed the repair-incorporated BrdUrd to increase the overall buoyant density of the DNA containing the repair patches. This increase in density over that of

DNA containing no repair patches could be related to the degree of incorporation of BrdUrd, and the patch size could thus, be derived.

The application of the buoyant density shift method to the measurement of repair patch sizes revealed that about 30-40 nucleotides were replaced in the repair of each damaged base, and this extent of replacement was independent of the DNA-damaging agents employed.

In the examination of the role of poly(ADP-ribosyl)ation in DNA repair, the size of the repair patch was not increased by the presence of 3-aminobenzamide (3-AB), an inhibitor of poly(ADP-ribose) polymerase, as had been predicted by other investigators.

An interesting observation was made when DNA repair was allowed to occur in the presence of aphidicolin, a specific inhibitor of DNA polymerase α. Although there was no difference in the extent of repair-incorporation of the label between cells that performed DNA repair in the absence or in the presence of aphidicolin following UV-irradiation, the presence of the inhibitor produced repair patches that were at least twice that of normal. When repair was allowed to occur in the presence of aphidicolin after treatment of cells with DMS, a decrease in repair-incorporation was observed, and again the size of the repair patch was increased.

On the basis of the study done with aphidicolin, several conclusions were drawn. Firstly, DNA polymerase α is involved in the repair of DNA. Secondly, the degree of incorporation of label into DNA following exposure to DNA-damaging agents may not always be a reliable way to quantitate repair events in the cell. Finally, it was proposed that the size of the repair patch is regulated by the relative rates of excision and polymerization in the cell.

The above-mentioned findings, along with the known characteristics of the DNA polymerases, led to the proposal of a model for the participation of polymerases α and β in DNA repair. The model suggests that the size of the single-stranded gap produced by the exonuclease determines the polymerase which fills the gap, ie., a small gap of about 10 nucleotides in length is filled in by polymerase β, while a larger gap of about 30 nucleotides or more is filled in by the α enzyme. Because the α polymerase cannot fill gaps to completion, the β enzyme is required to complete the gap-filling process.

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NOMENCLATURE

AAAF	N-acetoxy-2-acetylaminofluorene
3-AB	3-aminobenzamide
ADP	adenosine diphosphate
AP	apurinic-apyrimidinic
BrdUrd	bromodeoxyuridine
DMS	dimethylsulfate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulfonate
FBS	fetal bovine serum
FdUrd	fluorodeoxyuridine
MMS	methyl methanesulfonate
NAD	nicotinamide adenine dinucleotide
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonylfluoride
SDS	sodium dodecylsulfate
Tris	tris(hydroxymethyl)aminomethane
TTP	thymidine triphosphate
UV	ultraviolet
XP	xeroderma pigmentosum

Chapter 1

GENERAL INTRODUCTION

1.1 Repair of DNA

The DNA in all living cells is constantly undergoing structural changes. The causes of such alterations can be the result of spontaneous reactions such as hydrolysis or deamination, or they can be due to agents that react with the DNA. Spontaneous chemical changes in the DNA in mammalian cells have been estimated to occur at surprisingly high rates. Depurination of DNA can be as high as several thousand residues per genome per day (Lindahl, 1979; Lindahl, 1982). Moreover, deamination of about one hundred cytosine residues, per genome per day, into uracil can also occur under the same condition. Agents which act on DNA to effect changes can come from the external or internal environment of the cell. Examples of the former are ultraviolet (UV)-light, x-rays, and various electrophilic chemicals. Within the cell, such compounds as s-adenosylmethionine, a weak alkylating agent, can methylate DNA nonenzymatically. Oxygen radicals produced by biochemical processes within the cell can also react with and alter the DNA structure.

Alterations in the DNA structure, if left uncorrected, have deleterious consequences, such as lethality, mutations, or, in the case of higher organisms, tumourous growth. A non-lethal mutation, while allowing the cell to survive, will be passed on to subsequent generations, and most of these mutations are harmful rather than beneficial. To ensure survival, several mechanisms for coping with the

presence of the lesions in the DNA have evolved. An example of this is the error-prone replication bypass system, which is inducible in bacteria, and allows DNA synthesis and cell survival at the expense of the integrity of genetic information. On the other hand, to ensure that the accuracy of the genetic content is preserved, several mechanisms for the correction of the damaged bases have evolved. The simplest one involves the direct conversion of pyrimidine dimers back to their monomeric form by a photoreactivating enzyme which, when bound to the dimer in the DNA, absorbs visible light to catalyze the cleavage of the joined bases. Another important corrective process is a transfer of the methyl group from the O^6-position of the guanine residue onto the cysteine residue of a methyltransferase (Waldstein et al., 1982; Pegg et al., 1982; Hora et al., 1983). This transfer of the methyl group results in the regeneration of the guanine, with an accompanying inactivation of the methyltransferase. A third repair pathway is excision repair, which entails the enzymatic removal of damaged bases followed by their replacement using the complementary strand as template.

In human cells, one of the most important repair mechanism is the excision repair pathway, which is the focus of this discussion. The importance of this repair pathway to humans is demonstrated in the disease xeroderma pigmentosum (XP), a rare autosomal recessive hereditary disease. Such individuals show an unusually high tendency to develop skin

tumors on areas that have been exposed to sunlight (Friedberg et al., 1979; Hanawalt et al., 1979), and, except for the XP variants, the defect was shown to be associated with the inability to excise pyrimidine dimers from DNA. In addition to XP, a number of other hereditary diseases conferring predisposition to cancer also appear to involve deficiencies in DNA repair (Friedberg et al., 1979). Because the present study centers around the excision repair of damage in human cells, a brief review of current knowledge on the enzymology of DNA repair is presented.

1.2 Enzymes involved in DNA repair

The enzymology of the excision repair pathway in prokaryotes is better understood than in eukaryotes because of the better defined genetic system in the former, and the ease of isolating various mutants. In contrast, the identification of gene products and the physiological functions of various enzymes involved with DNA repair in human cells have been made difficult by the limited availability of mutants. The most studied mutation is the one found in the cells of individuals with XP. Skin fibroblasts cultured from such individuals have been found to be hypersensitive to UV-light, and the defect was later found to be in the initial endonucleolytic step (Cleaver, 1968; Hanawalt et al., 1979). Since then, eight genetic complementation groups have been reported (Bootsma, 1978; Moshell et al., 1983), all being defective in the initiating

incisional event. Consequently, at least eight genes are believed to be associated, either directly or indirectly, with the first step of excision repair. The identity and function of any of the genes have yet to be determined, demonstrating the complexity of the processes of DNA repair in human cells.

1.2.1 Endonucleases

To study the incisional process, attempts have been made to purify from mammalian cells an endonuclease that recognizes pyrimidine dimers. Initial efforts produced enzymes that recognized photoproducts other than pyrimidine dimers (Friedberg et al., 1977; Nes et al., 1978; Waldstein et al., 1979). Waldstein et al. (1979) reported the partial purification of an endonuclease, from calf thymus, which was claimed to recognize pyrimidine dimers specifically. Further studies of this enzyme were hampered by its lability to freezing. An estimate of the molecular weight of this enzyme was not possible because it eluted from gel filtration columns at or near the void volume, indicative of a high molecular weight protein complex. To date, no further reports about this enzyme preparation have appeared.

Enzymes that initiate excision repair in mammalian cells, but recognize adducts other than pyrimidine dimers, have been isolated more successfully. These are the DNA glycosylases (Lindahl, 1982), which catalyze the cleavage of the base-sugar bonds of damaged nucleosides in DNA, leaving

an apyrimidinic or apurinic site (AP-site). Several glycosylases that have been isolated, eg. uracil-DNA glycosylase, hypoxanthine-DNA glycosylase, and 3-methyladenine-DNA glycosylase, have very similar physical and biochemical properties. They have a low molecular weight of between 18,000 and 31,000 and have no requirements for any cofactors. They act by simple hydrolysis of the glycosyl bond, and show a strong preference for double stranded DNA. Each enzyme exhibits very narrow substrate specificity.

Following depurination or depyrimidination, either enzymatically by the glycosylases or by spontaneous hydrolysis, an endonuclease acts to cleave the DNA to generate a nick. To date, only one AP endonuclease has been reported to exist in mammalian cells (Lindahl, 1979; Lindahl, 1982), which incises on the 5'-side of AP sites to produce 3'-hydroxyl nucleotide and deoxyribose 5'-phosphate termini (Mosbaugh et al., 1983). Unlike the corresponding enzyme in E. coli, the mammalian endonuclease does not have associated exonuclease activity. The endonucleases isolated from HeLa cells (Kane et al., 1981) and other mammalian tissues (Lindahl, 1979) are monomeric proteins with a molecular weight of about 30,000 daltons, and have a strict requirement fon Mg^{+2} for activity. They have no activity with intact DNA, or DNA containing pyrimidine dimers or alkylated nucleotides. The nick left by the action of the

endonuclease then becomes a substrate for the enzymes involved with the removal and replacement of the damaged bases.

1.2.2 Exonucleases

In E. coli, the excision and polymerization steps can be accomplished simultaneously by a single enzyme, DNA polymerase I (Hanawalt et al., 1979), which has associated exonuclease activities. Recently, studies done on the uvrABC gene products of E. coli suggested that they are responsible for the removal of a 12 to 13 nucleotide fragment containing the lesion, leaving a single stranded gap for DNA polymerase I to act on (Yeung et al., 1983; Sancar & Rupp, 1983). In addition to pyrimidine dimers, the uvrABC protein complex has affinity for other adducts that are "bulky", such as benzo[a]pyrene-guanine adducts, acetylaminofluorene-C8-guanine adducts, thymine-psoralen-thymine crosslinks, and psoralen-thymine monoadducts. In mammalian cells, no exonuclease activities have been detected in purified DNA polymerases. However, the excision and polymerization activities were found to operate closely together as coupled reactions (Mortelmans et al., 1976; Hanawalt et al., 1979; Mosbaugh et al., 1983).

Several mammalian exonucleases, with bidirectional or unidirectional activities, have been isolated that can possibly function in the repair of DNA. The two bidirectional enzymes are the human placenta

correxonuclease, and the DNase V from Novikoff hepatoma cells (Grossman, 1981). The correxonuclease shows a slight preference for single-stranded DNA and can act on phosphodiester bonds internal to denatured or UV-irradiated duplex DNA that has been incised, releasing pyrimidine dimers from DNA. The DNase V isolated from Novikoff hepatoma associates tightly with polymerase β with a stoichiometry of 1:1, shows a strong preference for double stranded DNA, and digests DNA to produce 5'-nucleoside monophosphates predominantly (Mosbaugh et al., 1980). Because this enzyme cannot liberate thymine dimers from UV-irradiated DNA, its role may be restricted to AP-sites that have been incised. However, the DNase V isolated from HeLa cells (Mosbaugh et al., 1983) can act on UV-irradiated DNA, and can interact with polymerase β to function in excision repair.

On the basis of substrate specificities, three unidirectional exonucleases have been implicated to function in DNA repair (Grossman, 1981). DNase VI, isolated from rabbit liver, acts in the 5' → 3' direction, and can excise thymine dimers, releasing them in the form of oligonucleotides. DNase VII and DNase VIII were found to co-purify as closely associated proteins from human placenta, but can be separated into individual enzymes. DNase VII acts in the 3' → 5' direction, hydrolyzing terminal phosphodiester bonds of duplex DNA bearing AP sites (Grossman, 1981; Hollis et al., 1981). DNase VIII was mentioned briefly in two reports by Grossman (Grossman,

1981; Hollis et al., 1981), and was reported to require thymine:thymidylate mixed dimers for activity, and the oligonucleotides were released to generate gaps of approximately 10 nucleotides in length in the 5' → 3' direction.

1.2.3 DNA polymerases

After the generation of a gap by the exonuclease, a DNA polymerase follows to close the gap by using the intact complementary strand as a template for accurate synthesis. In mammalian cells, three DNA polymerases, α, β, and γ, have been isolated and are well characterized. Separation and identification of each of the three enzyme activities is made possible by their unique responses to various inhibitors (Scovassi et al., 1980). For example, aphidicolin only inhibits polymerase α; polymerase β is the only one resistant to N-ethylmaleimide; polymerase γ can be distinguished from polymerase α by its sensitivity to dideoxy-TTP, which can also inhibit polymerase β. Through the use of such inhibitors, tentative physiological functions have been assigned to each of the polymerases. The α polymerase is believed to be the major enzyme involved with DNA replication, as replicative synthesis can be inhibited by aphidicolin (Scovassi et al., 1980; Weissbach, 1977; DePamphilis et al., 1980; Ciarrocchi et al., 1978; Pedrali-Noy et al., 1980; Berger et al., 1979). Further evidence to substantiate this belief came from observation that

polymerase α activity is cell-cycle dependent (DePamphilis et al., 1980). However, its role in DNA repair is not quite clear. Most studies have shown that repair synthesis can be blocked by aphidicolin (Hanawalt et al., 1979; Ciarrocchi et al., 1979; Berger et al., 1979; Miller & Chinault, 1982), but some other studies showed an insensitivity of repair to the inhibitor (Giulotto et al., 1981; Pedrali-Noy et al., 1980). A recent proposal suggested that the involvement of polymerase α is related to the amount or types of damages present in the DNA (Miller & Chinault, 1982; Cleaver, 1983; Dresler & Lieberman, 1983). Cleaver (1983) further suggested that if polymerase α is blocked by inhibitors, the β enzyme could intervene to complete the gap-filling process.

Work done on purified polymerase indicated that this enzyme requires a gap of 30 to 70 nucleotides in length for activity, whereas the polymerase, although it has preference for DNA with a gap of about 10 nucleotides, can act on a nick (Korn et al., 1981). This led to the proposal that the α polymerase is involved with the repair of adducts which lead to the generation of a larger gap, such as UV-damage, and the β polymerase is involved with the repair of adducts that produces nicks, such as methylated bases (Cleaver, 1983).

The major role played by polymerase β in the cell is believed to be in the repair of DNA (Hanawalt et al., 1979; Scovassi et al., 1980; Grossman, 1981), either alone or in conjunction with the α enzyme (Cleaver, 1983). It is a small enzyme with a molecular weight of 30,000 to 50,000 daltons, compared with that of polymerase α which has a molecular weight of 130,000 to 280,000 daltons. This size difference could be the reason for the ability of the β enzyme to work on a smaller gap of about 10 nucleotides, and its ability to fill gaps completely (Hanawalt et al., 1979; Wang & Korn, 1980). In contrast with polymerase α, polymerase β activity in the cell is independent of the cell cycle. More direct evidence for the ability of polymerase β to function in repair came from studies on brain and muscle cells (Hanawalt et al., 1979; Waser et al., 1979). These cells, being terminally differentiated, have no α polymerase activity, but still retain the ability to repair damage in DNA after UV-irradiation.

To date, there is no evidence for the participation of γ polymerase in repair (Grossman, 1981; Scovassi et al., 1980; Weissbach, 1977). Its main role appears to be in the synthesis of mitochondrial DNA. A fourth polymerase, δ, has been reported to have associated 3' → 5' exonuclease activity (Hanawalt et al., 1979; Scovassi et al., 1980; Holmes et al., 1983). This enzyme has yet to be characterized further. Its physiological role in the cell is unknown. From its response to inhibitors, which resembles that of polymerase

α, it could be another, yet uncharacterised, form of this enzyme.

1.2.4 Ligases

The final step in the restoration of the integrity of the duplex DNA is the rejoining of the newly synthesized strand to the pre-existing one by a DNA ligase. The number of ligases present in mammalian cells is still an issue of debate. Initial reports claimed that two forms of ligase are present in mammalian cells (Soderhall & Lindahl, 1975; Soderhall & Lindahl, 1976; Creissen & Shall, 1982). DNA ligase I is the dominant activity in actively dividing cells and is the better studied one. It has a molecular weight of between 170,000 and 220,000, depending on the tissue from which it was isolated. Because this activity is cell cycle dependent, its role is believed to be in DNA replication. The other form of ligase, DNA ligase II, constitutes only 5 to 20% of the total ligase activity in growing cells, and consequently, is not as well characterized. It is separable from ligase I on a hydroxyapatite column, and exhibits different properties; the ligase I is more stable to heat, and to storage at 0°C than ligase II; both have a pH optimum of 7.8 for activity, but ligase I retains 60 to 70% of its activity at pH 6.4 while ligase II only retains about 10% of its activity; there is no cross-reactivity of ligase II with antiserum to ligase I. Unlike ligase I, the level of ligase II in the cell remains unchanged throughout the life cycle

of the cell, implying a possible role in DNA repair. Additional support for this belief was presented by Creissen and Shall (1982) who demonstrated a five-fold increase in ligase II activity upon exposure of cells to the DNA damaging agent, dimethylsulfate. According to these authors, the increase is attributed to a covalent modification of the ligase by poly(ADP-ribosyl)ation.

The existence of a second ligase in mammalian cells was questioned by Teraoka et al. (1979 & 1982) and Ohashi et al. (1983). When these authors included the protease inhibitor, phenylmethylsulfonylfluoride (PMSF), in the extraction buffer during the purification of the ligase, the minor form of the enzyme, ligase II, was not detected. In this context, they have presented evidence suggesting that DNA ligase II may be a proteolytic fragment of a single species of DNA ligase. Further purification of the enzyme to homogeneity revealed a single polypeptide with a molecular weight of 130,000 (Teraoka & Tsukada, 1982). They went on to show that any increase in enzyme activity during the cell cycle is due to an increase in the amount of enzyme in the cell, rather than an increase in catalytic efficiency.

Additional evidence for a single ligase in eukaryotic cells was provided by a study using conditional lethal mutants of yeast that lack DNA ligase activity (Nasmyth, 1979). Such cells are defective in their ability to undergo

DNA replication and repair synthesis when incubated at the non-permissive temperature, suggesting that the single form of the enzyme is responsible for both processes.

In summary, the only enzymes that have been isolated which are known with certainty to participate in excision repair in DNA are the glycosylases. For each of the other steps of the excision repair pathway in mammalian cells, several enzymes have been isolated, but it is not known which of these participates in the repair process. Because there are no mutants that are defective in any of these enzymes, definite assignment of roles to these enzymes is not possible.

1.3 Repair of DNA in chromatin

An incomplete knowledge of the organization of DNA and proteins in chromatin, and the effect this packaging has on enzymes that work on DNA, has further complicated the study of DNA repair in mammalian cells. Wilkins and Hart (1974) showed that pyrimidine dimers in chromatin were less accessible to dimer-specific endonuclease than in purified DNA. Upon exposure to high concentrations of NaCl, more sites were made accessible to the endonuclease. Mortelmans et al. (1976) confirmed the possible interference with the action of repair enzymes by chromatin structure through the use of cell extracts made from repair-deficient XP cells. XP-cell extracts from complementation group A were able to excise dimers from purified DNA to the same extent as

extracts made from normal cells. However, when the DNA was in the form of chromatin, extracts from these XP cells could not excise the dimers, whereas the extracts of normal cells retained the ability to excise dimers from chromatin. This observation indicated that the lesions in chromatin were not accessible to DNA repair enzymes in XP cells. Kano and Fujiwara (1983) recently substantiated this finding that XP cells of complementation group A, along with C and G, are not deficient in the dimer-specific endonuclease, but are lacking in certain factors which could be required for accessibility of dimers to nucleases. Furthermore, Ohashi et al. (1983) observed an inhibition of DNA ligase activity by up to 80% when histones are present with DNA, or when the DNA and histones are in the form of chromatin. They then went on to show that the inhibition could be alleviated by poly(ADP-ribose) polymerase that had itself been poly(ADP-ribosyl)ated.

1.3.1 Distribution of repair patches in chromatin

The DNA in eukaryotes is organized as a complex with histones. In mammalian cells, DNA segments of 145 base pairs form a nucleosomal core by winding around octomers of histones which, in turn, are joined to adjacent nucleosome cores by a stretch of linker DNA about 50 nucleotides in length. The linker DNA is more susceptible to cleavage by nucleases than the nucleosomal core DNA, and digestion by

such nucleases liberates nucleosomes containing the histone octomer and the 145 base pair stretch of DNA, still wound around the core.

Initial studies of repair in chromatin showed that soon after treatment of cells with DNA damaging agents, most of the repair incorporated label was more sensitive to nucleases than DNA as a whole (Lieberman, 1982). It was felt, at that time, that this was due to the presence of the repair-incorporated nucleotides primarily in the linker regions of the chromatin. Then, with prolonged incubation, a rearrangement process occurred to distribute the repair patches randomly between nuclease-sensitive and nuclease-resistant regions. The explanation of a biased distribution of repair patches in chromatin could be possible for agents that react with linker DNA preferentially, such as N-acetoxy-2-acetylaminofluorene (AAAF), psoralens plus long wavelength UV-light, and some methylating agents. However, when the study was done with UV-light or 7-bromomethylbenz[a]anthracene which reacts with DNA in both, the linker and core DNA, the repair-incorporated nucleotides displayed the same initial nuclease sensitivity (Oleson et al., 1979; Lieberman, 1982). Although these agents do not discriminate between the regions of the chromatin that the DNA is in, 80 to 85% of the lesions are associated with the core DNA because more DNA is in this region than in the linker region. The assembly of DNA into nucleosome structures was found to have very little effect on the rate

of removal of the adducts from core DNA. In all cases the repair-incorporated nucleotides were more sensitive to nuclease digestion than total DNA at the early stages of DNA repair. Subsequent events occur to redistribute these repaired regions throughout the chromatin (Oleson et al., 1979; Zolan et al., 1982a; Lieberman, 1982).

1.3.2 Models for nucleosome rearrangement

From studies on the distribution of repair patches in chromatin, two models for excision repair in chromatin were proposed (Lieberman, 1982). The first involves a sliding of nucleosome core proteins along the DNA during repair synthesis. According to this model, removal of adducts occur primarily along linker DNA, rendering the newly-incorporated nucleotides nuclease-sensitive. A constitutive, or induced, sliding of nucleosomal cores along the DNA then occurs and eventually, the repaired regions become nuclease-resistant.

The second model suggests that an unfolding-refolding process occurs during repair. The DNA in this model unfolds and becomes partially dissociated from core histones, allowing repair enzymes to have access to the damage, and also causing the repaired regions to become nuclease sensitive. Following the repair synthesis, a refolding of the DNA and histones into its original conformation occurs to render the repaired regions resistant to nucleases. This unfolding-refolding phenomenon would occur for damage in the core regions, but may not have to take place for lesions in

the linker regions. Available data seem to imply that rearrangement occurs regardless of whether the damage is in the linker or core regions. No direct evidence is available to support either the sliding or the unfolding-refolding models that explain the rearrangement of chromatin undergoing repair.

1.4 Aim of the study

The approach I have taken to study the repair of DNA in human cells was to look at the extent of excision and reincorporation of nucleotides into the DNA after treatment with DNA-damaging agents, ie. the size of DNA repair patches. The repair patch is the final product of a series of events that occurs during DNA repair, and by studying the circumstances that give rise to the defined patch size, one would be looking at the enzymology of DNA repair indirectly, especially the exonuclease and polymerase activities. The basic questions that were asked were: (i) How many nucleotides are utilized to repair a single damage site? (ii) What controls, or regulates, the extent of excision and reincorporation processes? One would expect that by replacing more nucleotides than are required for the repair of each damage site, the cell could increase the chance of misincorporation of nucleotides by the DNA polymerases. Studies done on the fidelity of DNA polymerases in human cells showed that they copy synthetic templates with great accuracy (Krauss & Linn, 1980 & 1982). However, when these

workers used cells with a finite life span, like human diploid fibroblasts, that were grown to high population doubling levels, the fidelities of their polymerases decreased, and the level of the α polymerase activity declined. When diploid fibroblasts of relatively low population doubling levels are kept at a confluent state, not only do the DNA polymerase activities decline, but these polymerases incorporate wrong nucleotides to a higher degree than cells that are growing rapidly.

To date, two factors have been proposed that could influence DNA repair patch sizes. The first was the DNA-damaging agent. It was proposed by Regan and Setlow (1974), that simple alkylating agents such as ethylmethanesulfonate (EMS), methylmethanesulfonate (MMS), and propanesultone, as well as ionizing radiation, induce short patch repair involving the removal and reinsertion of only 1 to 4 nucleotides. A second class of DNA damaging agent, eg. UV-light and N-acetoxyacetylaminofluorene, produces a long repair patch resulting in the removal and insertion of 100 to 140 nucleotides per patch. Hence, according to this proposal, repair of DNA damaged by different agents utilizes either a different set of enzymes, or different regulating factors, and will result in the formation of different repair patch sizes.

A second factor that could control the size of the repair patch involves the intervention by DNA ligase II in the excision and polymerization processes. Creissen and

Shall (1982) suggested that the strand breaks induced by the lesion act to stimulate poly(ADP-ribosyl) transferase which then, add poly(ADP-ribose) units onto DNA ligase II. This covalent modification of the ligase functions to increase the enzyme activity by several fold, and this ligase will compete with the exonuclease and polymerase to rejoin the newly synthesized strand to the pre-existing one to terminate the repair process. If the inhibitor of the poly(ADP-ribosyl) transferase, 3-aminobenzamide, is present in the cells, the activation of the DNA ligase is blocked, and the excision-reinsertion processes continue further to produce a larger patch size.

A consideration of the ideas discussed above led me to investigate the influence of the factors described above on the size of the repair patch. In addition, aphidicolin was used to examine the role of polymerase in repair function in human cells.

Chapter 2

MEASUREMENT OF REPAIR PATCH SIZES

2.1 Introduction

At the outset of the study, the number of nucleotides inserted to repair each damage site in human DNA was in question. Several independent measurements using different methods produced patch sizes ranging from about 1 to 4 nucleotides up to 170 nucleotides, depending on the DNA-damaging agent used. Regan and Setlow (1974), using the bromodeoxyuridine (BrdUrd)-photolysis method, arrived at the conclusion that two classes of repair, determined by the class of DNA-damaging agents used, occur in human cells. The first was 'short patch' repair, induced by simple alkylating agents like ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS), and ionizing radiation, and involved an insertion of 1 to 4 nucleotides per repaired site. The second, 'long patch' repair, involved insertion of 100 to 140, and up to 170 (Rosenstein et al., 1980) nucleotides per repair patch, and resulted from treatments of cells with UV-light or AAAF.

When measurements were made using the buoyant density shift method, developed by Edenberg and Hanawalt (1972), a patch size of 30 nucleotides was obtained after exposure of HeLa cells to UV-light. A patch size of about 35 nucleotides was also obtained when an in vitro repair system, using isolated nuclei, was studied (Smith & Hanawalt, 1978).

Regan and Setlow based their conclusion that alkylating agents were repaired by a 'short patch' mechanism on the linear relationship that they observed between single-

stranded breaks and fluence of 313 nanometer light. Examination of their data suggested that the small value for the patch size that they obtained with these agents could be the result of the low doses employed. Furthermore, they showed that when low doses of AAAF, a 'long patch' agent, were used, the relationship between single strand breaks and fluence was linear. In essence, the small number of repaired sites, resulting from the few damaged sites, could have been erroneously translated into a small patch size value through their method of measurement.

Because very little is known about the details of the enzymology of DNA repair in human cells, it appeared to be of fundamental importance to determine whether repair of UV-light damage and alkylating agent damage proceeded by sufficiently different pathways to yield significantly different patch sizes.

To study this question further, the buoyant density shift method was used to investigate the 'long patch' and 'short patch' repair phenomena in human cells. This method was chosen because the laboratory was equipped for it, whereas the BrdUrd-photolysis method requires a specialized high intensity irradiation unit.

In the buoyant density shift method, cells are allowed to repair the damage in the DNA in the presence of the density label, BrdUrd. The DNA is then isolated and broken down to a small fragment size, followed by analysis in a buoyant density gradient. The small fragment size allows the

repair-incorporated BrdUrd to increase the overall buoyant density of the DNA containing the repair patches, over that of normal DNA containing no BrdUrd. This increase in density is related directly to the degree of incorporation of BrdUrd, and the patch size can, thus, be derived.

2.2 Materials and Methods

2.2.1 Cells and culture conditions

KB cells, a strain of HeLa cells (Nelson-Rees & Flandermyer, 1976), were donated by Dr. K.B. Freeman, McMaster University, Ontario. T98G cells of glioblastoma origin were obtained from Dr. P.C. Hanawalt, Stanford University, Cal. All cells were grown in Alpha modified Minimum Essential Medium (Flow laboratories, Rockville, MD), supplemented with 10% (v/v) fetal calf serum (Flow Laboratories, Rockville, MD), penicillin (100 IU/ml), streptomycin (100 µg/ml), and tylocine (60 µg/ml). All cells were grown at 37°C in humidified 5% CO_2 in air.

2.2.2 Treatment of cells and repair synthesis

All chemicals used were of reagent grade and were purchased from Sigma Chemicals, St. Louis, MO., unless otherwise specified. All radioactive compounds were purchased from New England Nuclear, Boston, MA, or from Amersham, Arlington Hts. IL.

The method of Smith et al. (1981) was employed with slight modifications. Cell cultures containing 5×10^6 cells were incubated overnight in medium containing [^{14}C]thymidine (0.0025 μCi/ml, 60 mCi/mmol), in 75 cm^2 plastic petri dishes to prelabel DNA for use as a normal density marker. An hour prior to treatment, the medium was changed to one containing 16 μM BrddUrd and 1 μM FdUrd. This is done so that DNA replicating units that are almost completed will not be labelled. For treatment with UV-light, the medium was replaced by 1 ml of phosphate-buffered saline (PBS), followed by exposure to 20 J/m^2 of UV-light. The source of the UV-light was a General Electric G15T8 germicidal lamp emitting light primarily with a wavelength of 254 nm. The dose of the UV-light was determined with a Blak-Ray Ultraviolet Meter. Irradiation was given for 13 seconds at a distance of 12 inches with a dose rate of 1.54 $J/m^2/sec$. Other KB cells, similarly prelabelled were exposed to 200 μg/ml of the DNA-damaging agent, methyl methanesulfonate (MMS), made up in methanol, for 1 hr. in medium containing BrdUrd and FdUrd. After these treatments, the cells were incubated in medium containing 16 μM BrdUrd, 1 μM FdUrd, and 10 mM hydroxyurea for 0.5 hr. before the addition of a tracer amount of [^{3}H]thymidine (10 μCi/ml, 20 Ci/mmole), and incubation was allowed to proceed for a further 3 hrs. for UV-light, or 6 hrs. for MMS. Following the repair period, a chase period was applied by incubating the cells overnight

in regular medium containing BrdUrd and FdUrd before the DNA was extracted for analysis. This was done to chase any newly replicated DNA into the hybrid density region.

T98G cells, although derived from a glioblastoma, exhibit contact inhibition (Stein, 1979). These cells were grown to confluence in 75 cm^2 petri dishes, after which the medium was changed to one containing 1% fetal calf serum, and incubation was continued for a further 48 hrs. Treatment of cells with UV-light (20 J/m^2) and conditions for repair synthesis were as described for KB cells, with the exception that a 6 hr. repair period was used after all treatments. Repair synthesis was allowed to proceed either in the presence of 5[6-^{3}H]BrdUrd (10 µCi/ml, 20 Ci/mmole) or [^{3}H]thymidine (10 µCi/ml, 20 Ci/mmole). Dimethylsulfate (DMS), another DNA-damaging agent, was made up to 5 mg/ml in methanol, and when desired, was added to the medium to a concentration of 50 µg/ml. After 0.5 hr. incubation, a 6 hr. repair incubation and chase was applied as described. To provide a normal density DNA marker, T98G cells were grown separately in medium containing 0.1 µCi/ml [^{32}P]orthophosphate for 48 hrs. These cells were then harvested and pooled with the UV-treated or DMS-treated cells for isolation of nuclei.

2.2.3 Isolation of total DNA

For each analysis with KB cells, 3 plates of cells were used. DNA was isolated by a modified procedure of Kirby (1968). The cells were scraped off the plates with a rubber policeman and collected by a standard centrifugation procedure. The resulting pellet was resuspended in 20 ml of 5% (w/v) sodium p-aminosalicylate and lysed with 1% (w/v) SDS. The lysate was extracted twice with an equal volume of phenol reagent (950 ml liquified phenol from Fisher Scientific company, 140 ml of m-cresol, and 1.0 g of 8-hydroxyquinoline). The resulting DNA was precipitated by the addition of 2 volumes of ethoxyethanol, spooled onto a glass rod, washed twice with 70% (v/v) ethoxyethanol, and redissolved in 10 ml of SSC/10 (15 mM NaCl, 1.5 mM sodium citrate).

2.2.4 Isolation of nuclei and nuclease treatment

For analysis of nucleosomal DNA, 10 plates of confluent T98G cells were employed for each treatment with either UV or DMS. The cells were trypsinized off the plates and collected by centrifugation. Isolation of nuclei was according to the method of Smerdon et al., (1979). After one wash with cold PBS, the cell pellet was resuspended in cold Nuclei buffer (0.25 M sucrose, 10 mM Tris, pH 8.0, 1 mM $CaCl_2$), containing 0.5% (v/v) Triton X-100, and homogenized with about 10 strokes in a Dounce homogenizer, to release the nuclei. The nuclei were collected by centrifugation at

3000 x g for 10 mins., and resuspended in Nuclei buffer containing Triton X-100, and repelleted. The resulting nuclear pellet was washed once with cold Nuclei buffer, without Triton X-100, recentrifuged, and was then resuspended in 5 ml of Nuclei buffer.

To digest chromatin, Staphylococcal nuclease (Boehringer-Mannheim) was added to the suspension of nuclei to a final concentration of 15 units per OD_{260} unit. The nuclei were digested for 90 mins. on ice to obtain limited digestion, or were digested for 30 mins. at 37°C to achieve complete digestion of chromatin. The reaction was halted by the addition of EDTA to a concentration of 10 mM, and kept on ice for 30 mins.

2.2.5. Preparation of nucleosomal DNA

To the 5 ml suspension of nuclease-treated nuclei, SDS was added to a final concentration of 0.2% (w/v), and proteinase K (Boehringer-Mannheim) was added to a concentration of 100 µg/ml, followed by an overnight incubation at 37°C according to a modification of the procedure of Smith et al., (1981). The digest was then extracted once with an equal volume of the phenol reagent that had been saturated with TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The DNA was precipitated by the addition of 0.1 vol. of 3 M sodium acetate, followed by 2 vol. of ethanol, and kept at -20°C overnight. This DNA was redissolved in 0.5 ml of TE buffer and loaded onto a 6% polyacrylamide gel, and

eletrophoresis was carried out until the bromocresol green marker had just run off the gel. DNA bands were visualized by ethidium bromide staining, and the nucleosomal DNA bands were cut out and eluted from the gel according to the method of Maxam and Gilbert (1980). The DNA eluted from the gel was ethanol precipitated and redissolved in 2.5 ml of TE buffer.

2.2.6 Sizes of DNA fragments

For mechanical shearing, DNA of normal density, in SSC/10 or TE buffer, was brought up to a volume of 10 ml, and passed through an Aminco shear press twice at 48,000 psi. The sheared DNA fragments were concentrated by ethanol precipitation, and were shown to have sizes ranging from about 200 to 800 base pairs, but with an overall average size of 400 base pairs, as judged from their electrophoretic migration in 1.25% (w/v) agarose gel. Reference markers used were prepared by the Bam HI, Hind II, and Hind III digestion of the plasmid pBR322, for which the fragment sizes are 277, 346, 483 and 3256 base pairs (Sutcliffe, 1978). Reference markers used for nucleosomal DNA were digestion products of pBR322 by Hinf I, producing fragments of 154, 220, 298, 344, 396, 506 and 1631 base pairs. Limited digestion of chromatin produced DNA fragments of 160 and 320 nucleotide pairs in length, whereas fragments of 145 nucleotide pairs were obtained from complete-digestion with the nuclease.

2.2.7 Buoyant density centrifugation

To 4.5 ml of DNA solution, 5.9 g of cesium chloride (CsCl) (BDH Chemicals, Toronto, Ont.) was added, mixed, the refractive index adjusted to 1.3965, and centrifuged in a Ti50 rotor of a Spinco centrifuge at 37,000 rpm for 40 hr. at 20°C. The density gradient was fractionated by pumping from the top, and collecting the 39 fractions from the bottom, with a volume of 0.16 ml per fraction. A 20 µl sample was taken from each fraction, mixed with 0.5 ml water, and assayed for radioactivity by liquid scintillation counting. Fractions containing normal density DNA (1.7 gm/cm^3) were pooled and rebanded in a second neutral CsCl gradient. The resulting DNA was dialyzed overnight at 4°C against SSC/10, or TE buffer, to remove CsCl, before fragmentation.

To enhance the influence of the repair-incorporated BrdUrd on the buoyant density of the DNA fragments, the DNA was analysed as single-stranded molecules by centrifugation in an alkaline density gradient. To prepare such a gradient, 2.5 ml of fragmented DNA, or nucleosomal DNA, was mixed with an equal volume of double strength alkaline phosphate buffer, pH 12.5 (27.2 ml of 1.0 N NaOH, 100 ml of 0.2 M Na_2HPO_4, made up to 250 ml). A 4.5 ml portion of this alkaline DNA solution was mixed with 6.2 g of CsCl and centrifuged for 72 hr. as before. The fractions were collected directly into scintillation vials, neutralized

with a drop of glacial acetic acid, and diluted with 1 ml of water before assaying for radioactivity by liquid scintillation counting.

2.2.8 BrdUrd-induced shift in alkaline CsCl

To determine the relation between the increase in buoyant density of unsubstituted and BrdUrd-substituted DNA, DNA samples containing 5, 10, 15 and 20% BrdUrd were prepared as follows. Mixtures of BrdUrd plus [^{14}C]thymidine were prepared by mixing appropriate volumes of 1 mM nucleosides to make up the desired percentages. For instance, 1 ml of 1 mM BrdUrd was added to 9 ml of 1 mM [^{14}C]thymidine to make a solution containing BrdUrd and thymidine in a ratio of 1 to 9. KB cells which had been incubated overnight with 0.1 μM FdUrd were released into S phase by the addition of 0.01 volume of the appropriate BrdUrd/thymidine mixture. Following a 3 hr. incubation, the cells were harvested and the DNA extracted as described earlier. These BrdUrd-substituted DNA samples were centrifuged to equilibrium in an alkaline CsCl solution along with 100 μg of unlabelled, normal density DNA to act as normal density markers. Centrifugation and fractionation of the gradient were as described previously. To each fraction, 1 ml of water was added, and the OD_{260} readings were taken to determine the banding position of normal density DNA, before assaying for radioactivity by liquid scintillation counting.

2.3 Results

2.3.1 Replicative synthesis after DNA damage

Examples of neutral buoyant density profiles of DNA obtained from KB cells treated with UV-light or MMS are shown in Figs. 1A and 1B, respectively. In each case, two DNA peaks are present; the unreplicated DNA of normal density, and the replicated DNA of higher density containing one normal strand and one BrdUrd-substituted strand. In this figure, as with all others hereafter, the higher density region of the gradient is on the left side of the graph.

Although a substantial amount of [^{3}H]thymidine was incorporated by semi-conservative synthesis of DNA, treatment of the cells with DNA-damaging agents, along with the presence of hydroxyurea, act to suppress replicative synthesis, as shown by the ^{14}C-profile. The smaller amount of semi-conservatively replication seen after treatment with MMS, as compared with UV-treatment, is due to the higher dose of the DNA-damaging agent employed. The presence of tritium in the unreplicated DNA is the operational definition of repair replication.

When T98G cells were grown to confluence and kept in medium containing 1% serum, the amount of replicative synthesis was negligible after exposure to UV-light (Fig. 2). For this analysis, a sample of DNA that had undergone repair synthesis was subjected directly to an alkaline buoyant density analysis. All the tritium label was found

Figure 1. Neutral buoyant-density profiles of DNA from KB cells allowed to repair their DNA following treatment with 20 J/m^2 UV-light (panel A), or 200 µg/ml of MMS for 1 hr. (panel B). The brackets show the fractions at normal DNA density that were taken for rebanding. ○, ^{3}H-repair label; ●, ^{14}C-prelabel.

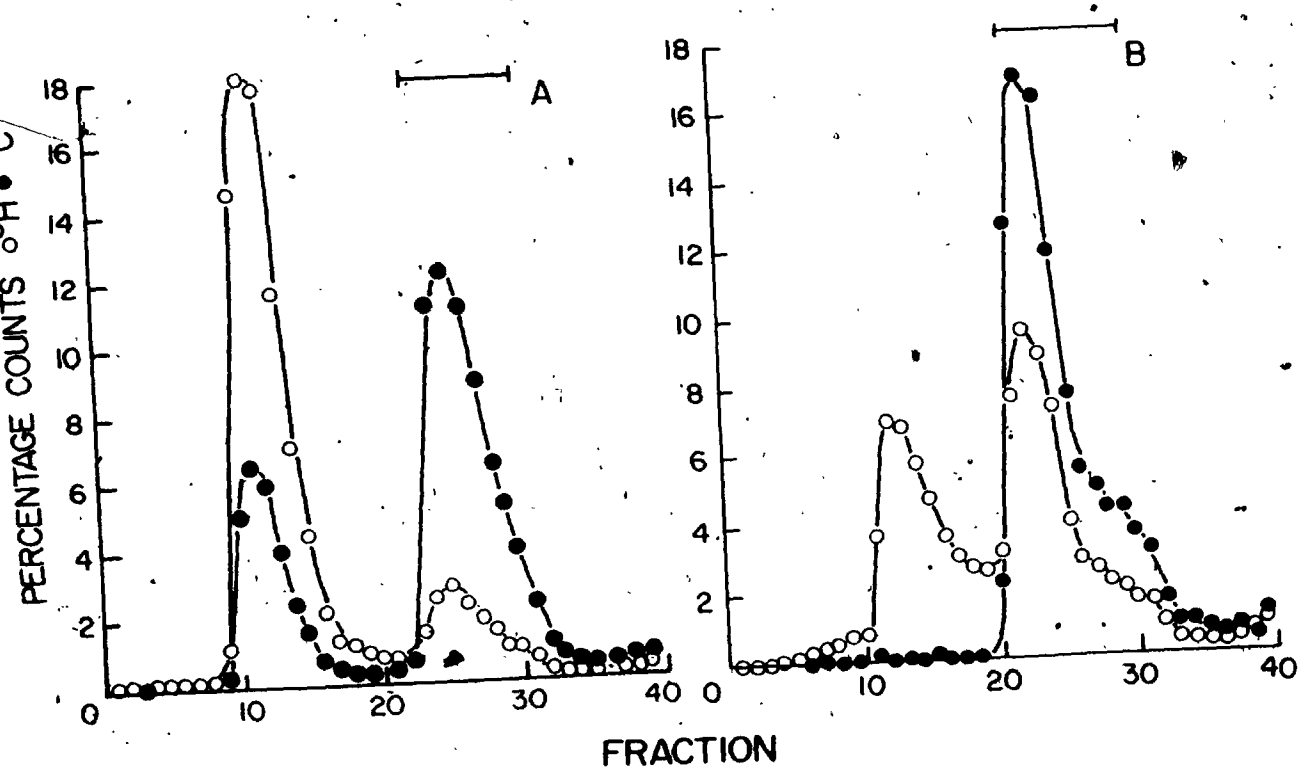
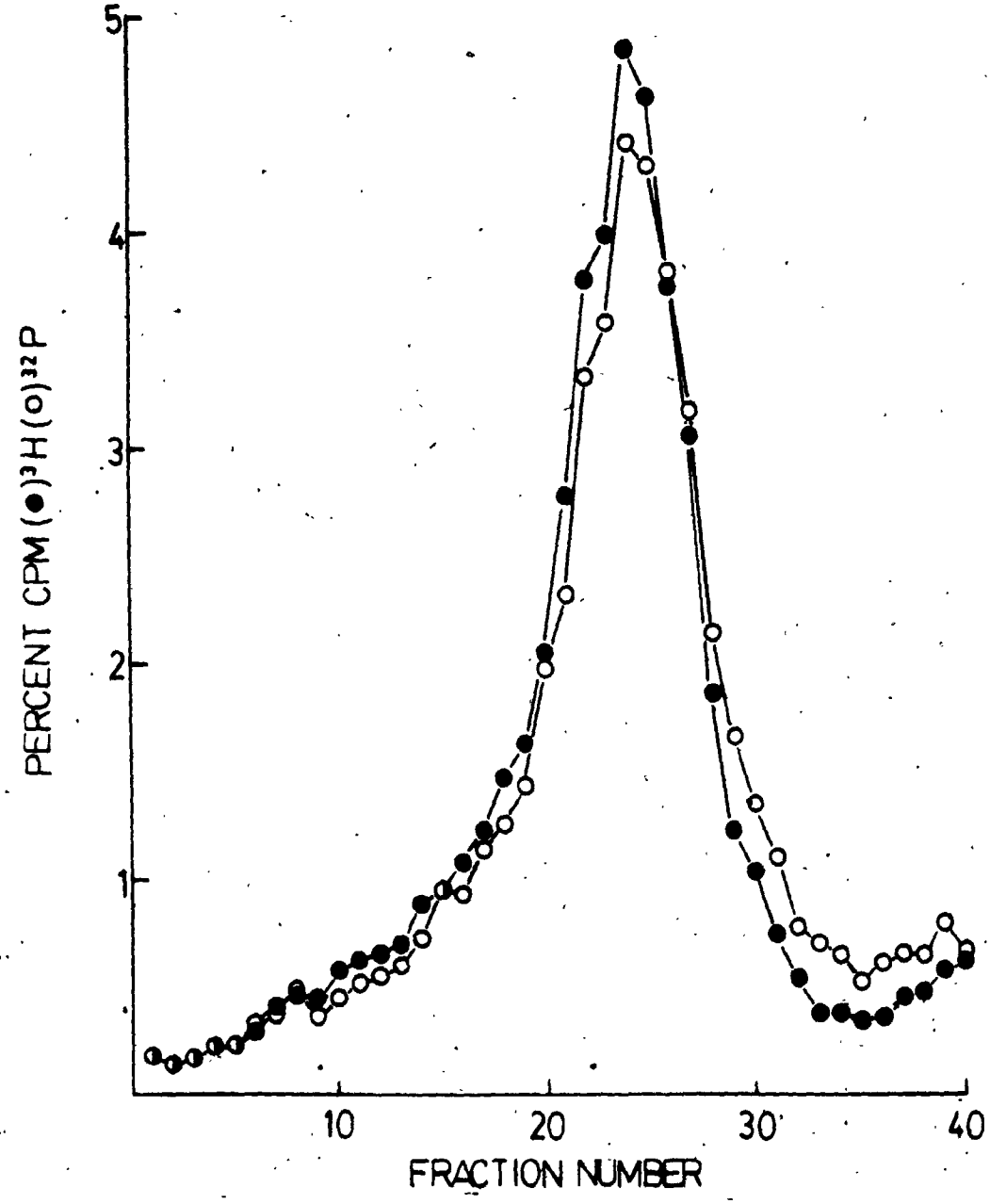

PERCENTAGE COUNTS ○ ^{3}H ● ^{14}C
A
B
FRACTION
0
2
4
6
8
10
12
14
16
18
10
20
30
40

Figure 2. Alkaline CsCl centrifugation of high molecular weight DNA from UV-irradiated confluent T98G cells. ●, ^{3}H-repair label; ○, ^{32}P-prelabel.


PERCENT CPM (●) ^{3}H (○) ^{32}P
5
4
3
2
1
10
20
30
40
FRACTION NUMBER

associated with normal density DNA, indicating that all the label incorporated into the DNA was associated with repair replication. Hence, a preliminary centrifugation in neutral CsCl to separate repair labelled DNA from any DNA labelled by semi-conservative replication was not necessary. This is important because the small fragment size of the nucleosomal DNA does not allow easy separation of semi-conservatively-synthesized DNA from repair-synthesized DNA in a neutral CsCl gradient.

2.3.2. Repair patch size measurements

DNA from KB cells that were treated with UV-light or MMS and contained the repair-incorporated BrdUrd was separated from DNA that was substituted with BrdUrd, and reduced to an average fragment size of 400 nucleotide pairs before banding in an alkaline CsCl gradient. The sedimentation profiles are shown in Fig. 3. The centre of mass of each peak was determined by totalling the radioactive counts in each fraction, and then locating the point where 50% of the total radioactivity was reached. The density shifts due to the repair incorporation of BrdUrd were 2.5 fractions for UV-light treatment, and 3 fractions for MMS treatment.

The banding positions of DNA containing 5, 10, 15, and 20% BrdUrd, relative to normal density DNA, were determined (Fig. 4), and a straight-line relationship between banding position and BrdUrd-content was obtained. This gave a

Figure 3. Alkaline buoyant-density profiles of sheared DNA from normal density fractions. Panel A, UV-light; Panel B, MMS. ○, ^{3}H-repair label; ●, ^{14}C-prelabel.

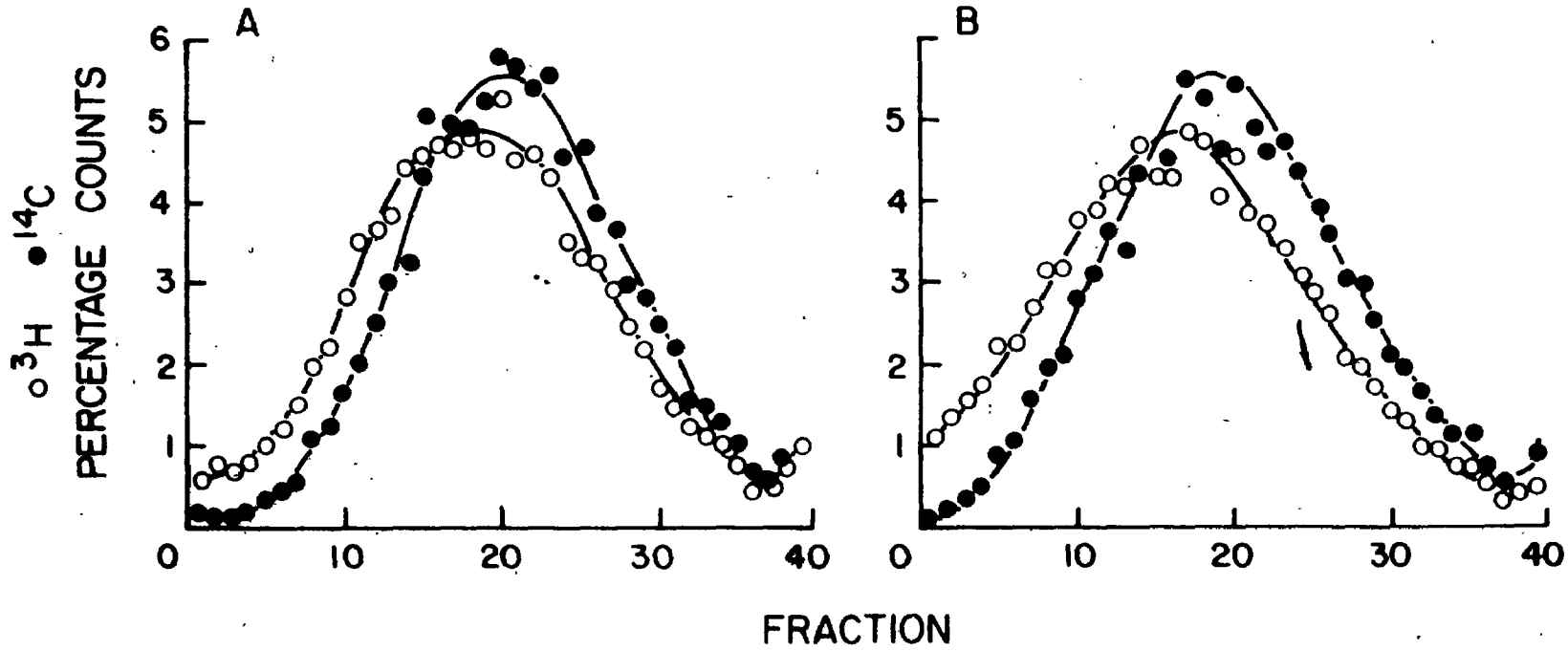
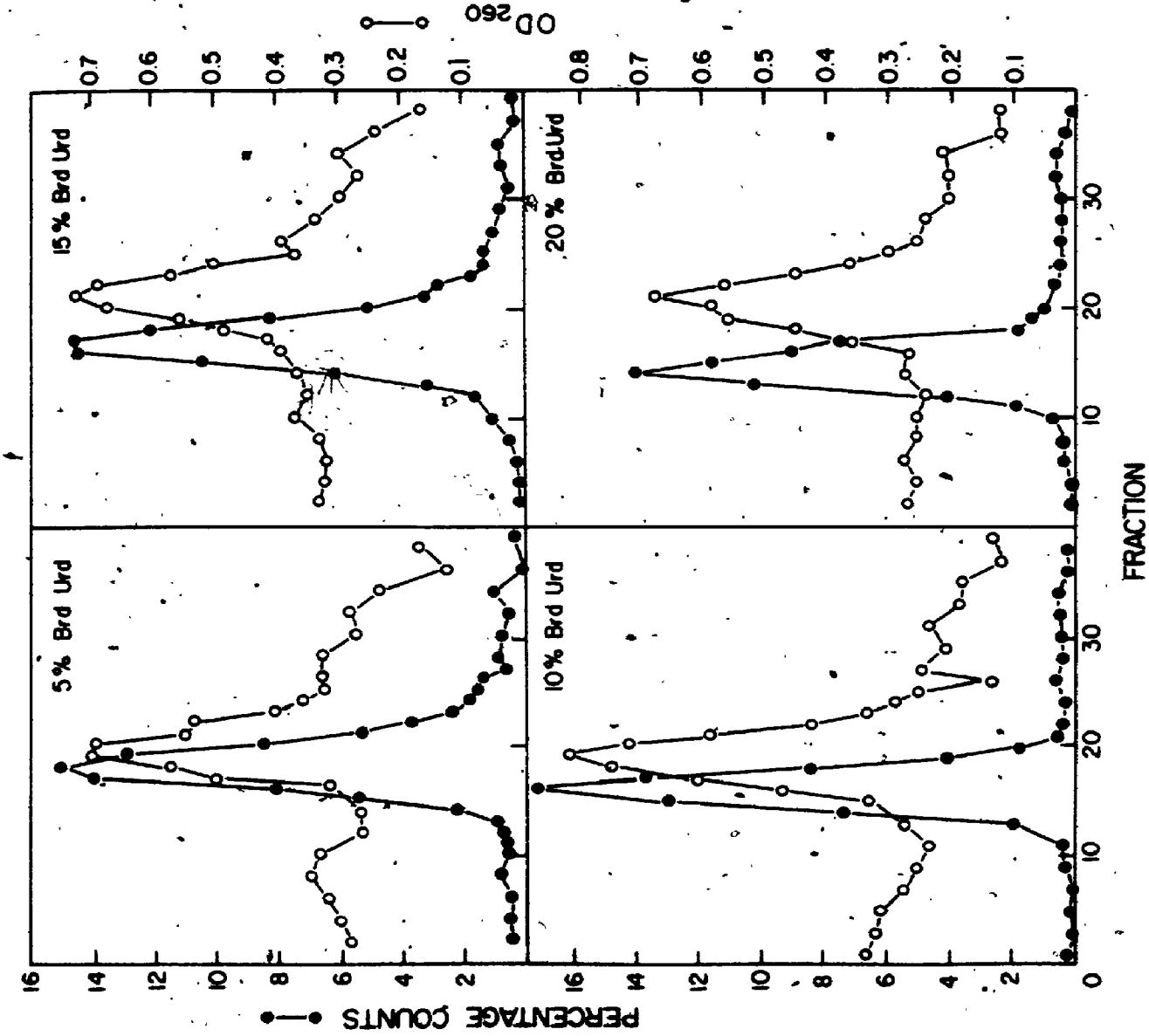

A
B
PERCENTAGE COUNTS
○ ^{3}H ● ^{14}C
0
1
2
3
4
5
6
10
20
30
40
FRACTION

Figure 4. Banding positions in an alkaline cesium chloride gradient of DNA samples with different degrees of BrdUrd substitution. The optical density profiles in each case represent unsubstituted DNA. The ^{14}C-profiles represent BrdUrd-substituted DNA.


5% Brd Urd
10% Brd Urd
15% Brd Urd
20% Brd Urd
PERCENTAGE COUNTS
OD260
FRACTION

density shift of 0.3 fractions for each percentage substitution by BrdUrd. The density shift of 2.5 to 3 fractions for repaired DNA indicates that 8.3 to 10% of the thymidine in the DNA fragments was replaced by BrdUrd. The average single-strand fragment size is presumably 400 nucleotides, and, thus, the average repair patch contains 34 to 40 nucleotides.

2.3.3 Influence of DNA fragment size on density shift

Limited digestion of the chromatin of confluent T98G cells by S. nuclease produced DNA fragments of different, but narrowly defined sizes (Fig. 5) from which the 160 and 320 nucleotide pair fragments were isolated. When these fragments, containing the repair-incorporated BrdUrd, were subjected to buoyant density shift analysis, a shift of 2 fractions was observed with the 160 nucleotide pair fragment, as determined by the comparison of the centres of masses of the DNA profiles, while a shift of 1 fraction was obtained with the 320 base pair fragment (Fig. 6). This provides a pleasing validation of the anticipated linear increase in density of repaired DNA, due to the BrdUrd content, as the fragment size is decreased. Unfortunately, a patch size estimate cannot be made from these data because confluent T98G cells discriminate between thymidine and BrdUrd; in favour of the former (Smith et al., 1981), and the extent of the substitution by BrdUrd into the repair patch is not known.

2.3.4 Analysis of repair in nucleosomal DNA

In the attempt to overcome the selectivity of thymidine over BrdUrd, repair of DNA in T98G cells after UV-light treatment was allowed to occur in the presence of [^{3}H]BrdUrd, rather than the [^{3}H]thymidine/BrdUrd mixture, to label the repair patch. Total digestion of chromatin produced DNA fragments of 145 base pairs, and when this was subjected to alkaline buoyant density analysis, a density shift of 4 fractions was obtained. This translates into a patch size of 20 nucleotides (Fig. 7). The same patch size of 20 nucleotides was obtained when T98G cells were treated with DMS (Fig. 8), allowed to repair in [^{3}H]BrdUrd, and analyzed with nucleosomal DNA.

Smith[1] has recently found that in confluent T98G cells, and in African green monkey cells, but not in diploid human fibroblasts, repair incorporation of [^{3}H]BrdUrd increases with the concentration of BrdUrd in the medium and he has established an empirical relationship between these values. At the BrdUrd concentration used in this study, 16 μM, the incorporation is 45% of the maximum. The repair patch size values reported for the T98G cells are, therefore, underestimated by a factor of ~2. After correction, the patch size value of ~40 nucleotides is the same as that found for KB cells that were treated with UV-light or MMS.

[1]Personal communication, C.A. Smith, Department of Biological Sciences, Stanford University, Stanford, CA.

Figure 5. Size analysis of DNA fragments obtained by digestion of nuclei with Staphylococcal nuclease at 0°C. The DNA fragments eluted from the 6% polyacrylamide gel were analysed in a 1.25% agarose gel. Lane 1; HinfI digestion fragments of pBR322 with their sizes given in base pairs. Lane 2; Staphylococcal nuclease digest of total nuclear DNA. Lanes 3 and 4; the 320 bp and 160 bp fragments obtained by elution from the polyacrylamide gel.

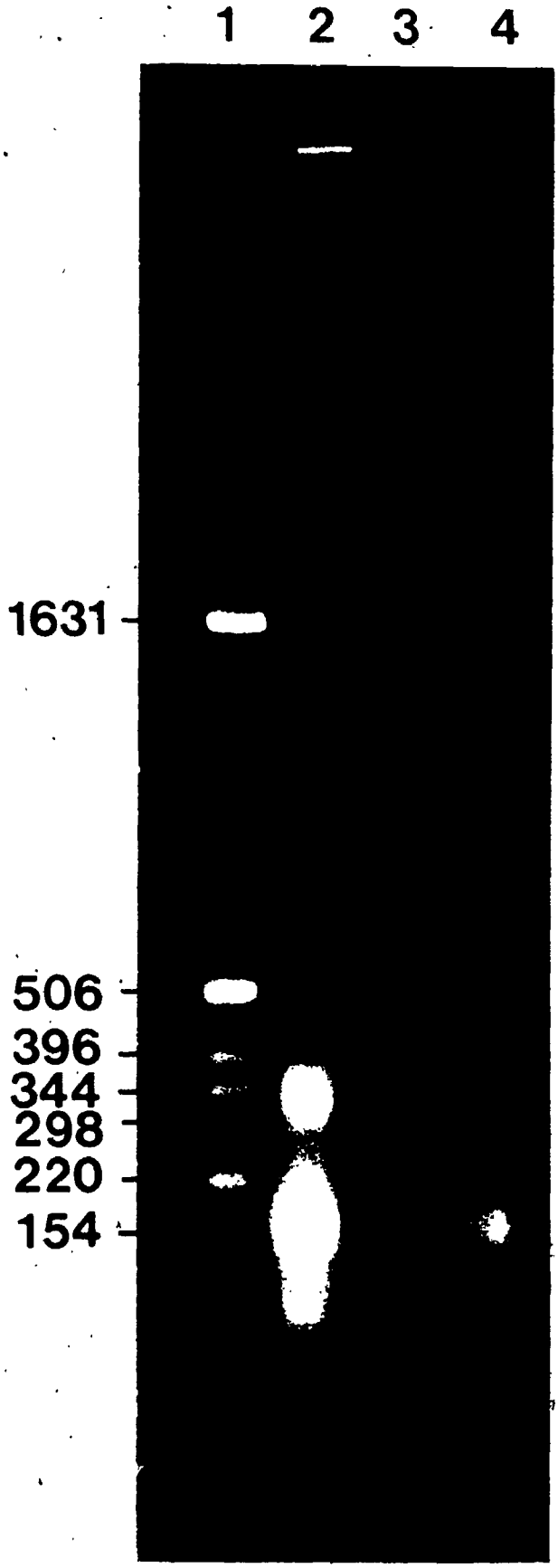

1
2
3
4
1631
506
396
344
298
220
154

Figure 6. Alkaline CsCl centrifugation of 160 and 320 bp DNA fragments prepared by nuclease digestion of nuclei from UV-irradiated confluent T98G cells. Repair took place in medium containing a mixture of [^{3}H]-thymidine plus BrdUrd. ●, ^{3}H-repair label; –O, ^{32}P-prelabel. In panel A the size of the DNA fragments is 160 nucleotides; in panel B the size is 320 nucleotides.

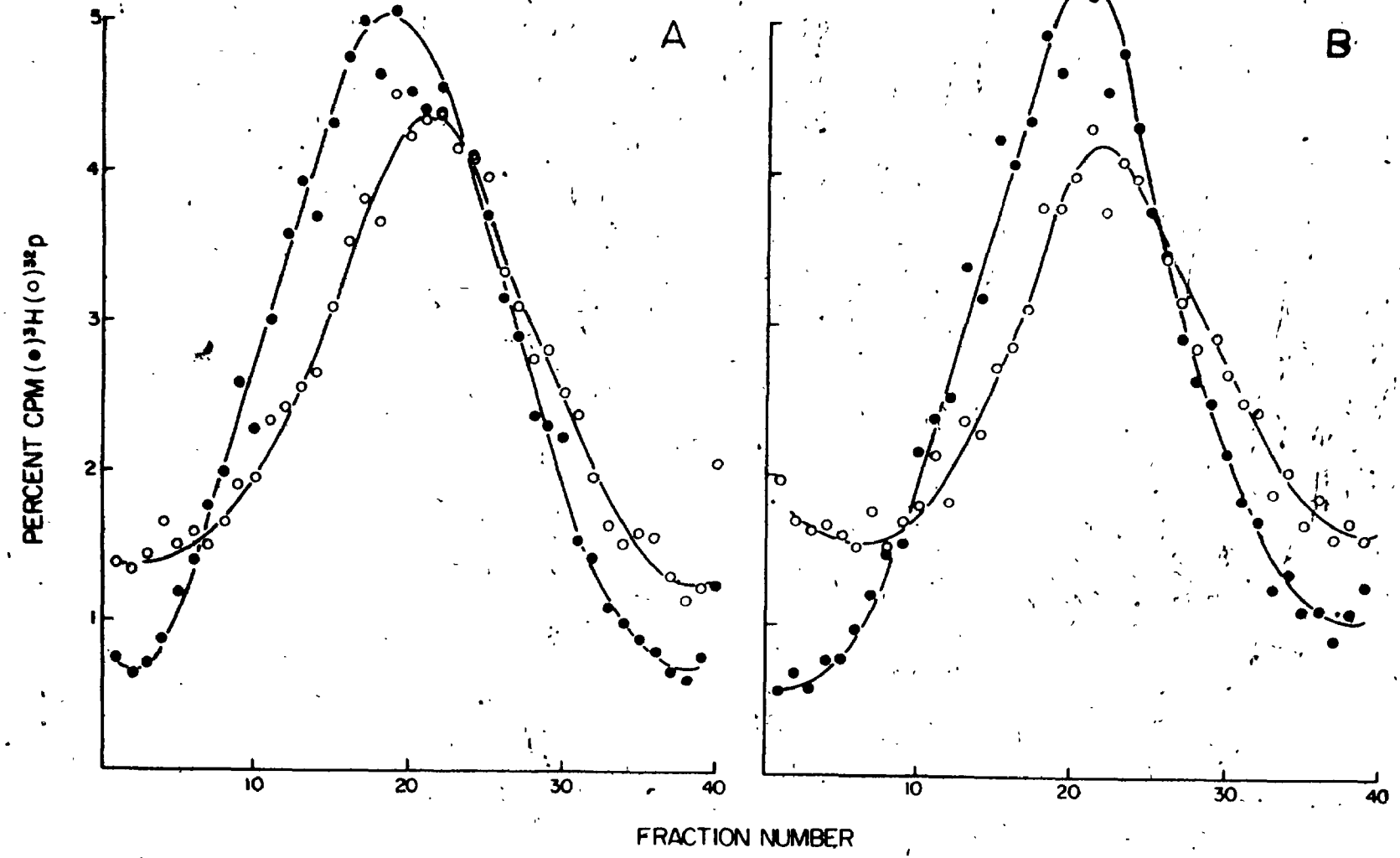

A
B
PERCENT CPM (●)^{3}H (○)^{32}P
1
2
3
4
5
10
20
30
40
FRACTION NUMBER

Figure 7. Alkaline CsCl centrifugation of 145 bp DNA fragments prepared by nuclease digestion of nuclei from UV-irradiated confluent T98G cells. Repair took place in medium containing [^{3}H]BrdUrd. The size of the DNA fragments is 145 nucleotides. ●, ^{3}H-repair label; ○, ^{32}P-prelabel.

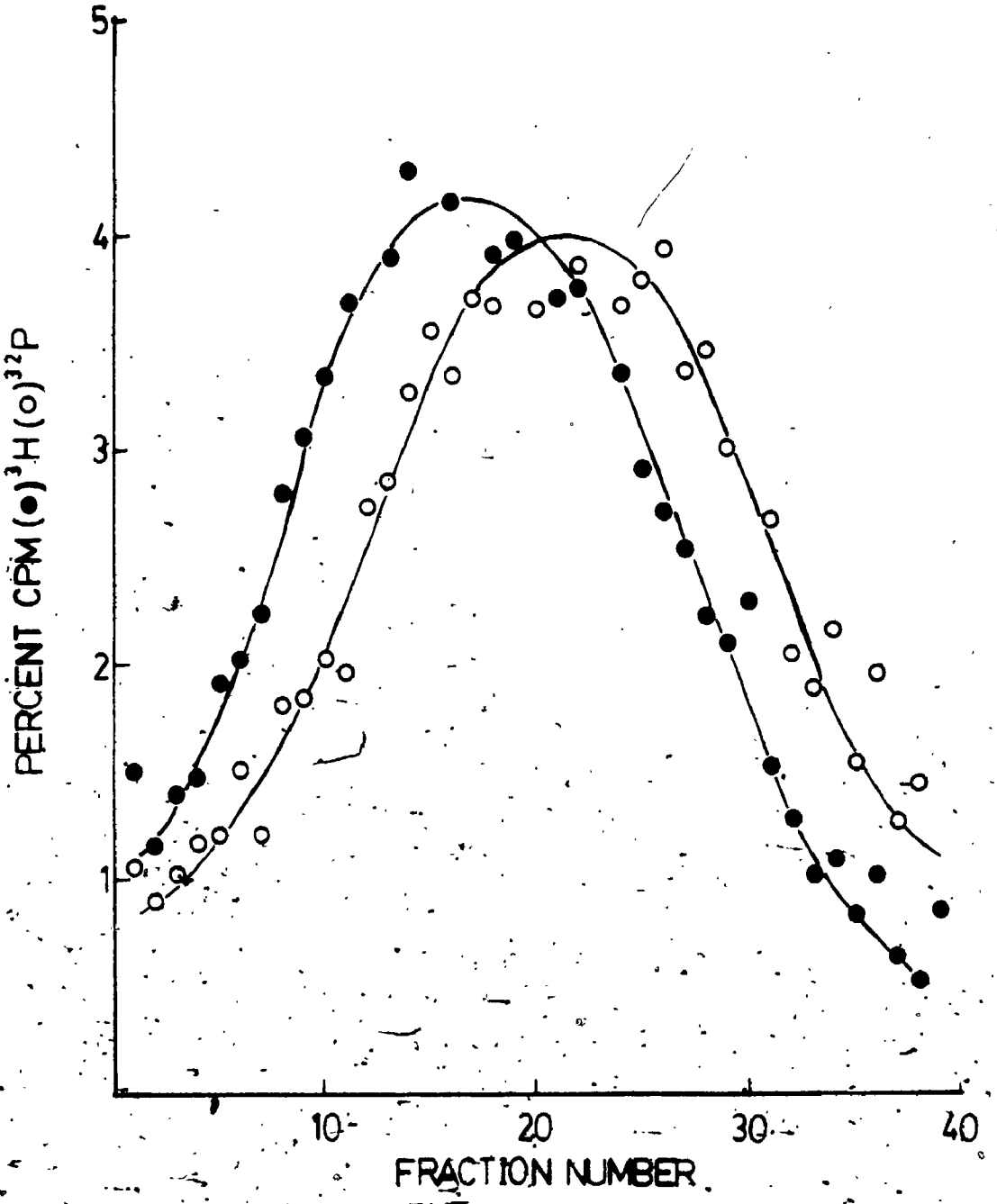
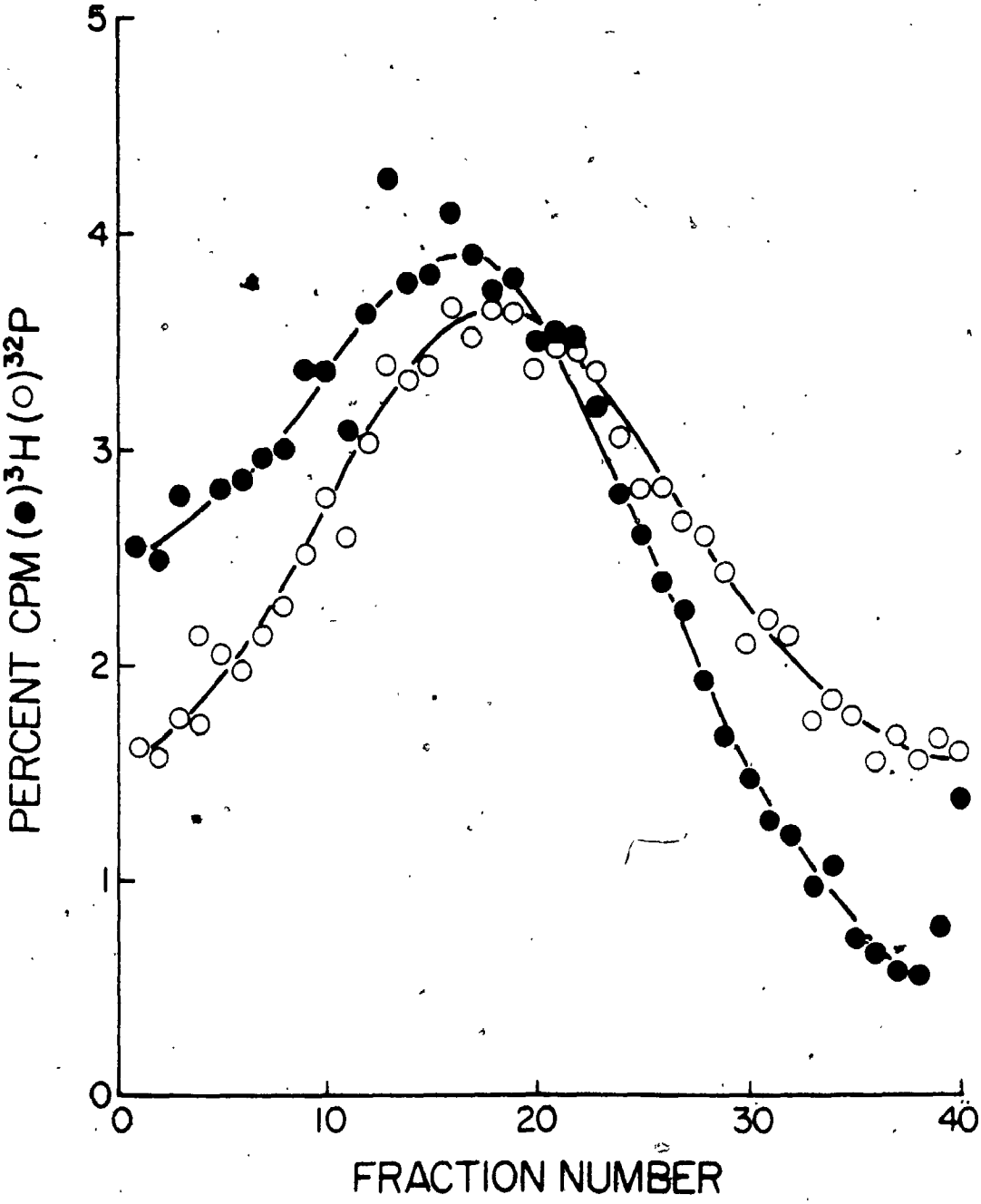

PERCENT CPM (●) ^{3}H (○) ^{32}P
5
4
3
2
1
10
20
30
40
FRACTION NUMBER

Figure 8. Alkaline CsCl centrifugation of nuclease prepared DNA fragments from confluent T98G cells that had been treated with 0.4 mM DMS. Repair took place in medium containing [^{3}H]BrdUrd. The size of the DNA fragments is 145 nucleotides. ●, ^{3}H-repair label; ○, ^{32}P-prelabel.


PERCENT CPM (●)^{3}H (○)^{32}P
0
1
2
3
4
5
0
10
20
30
40
FRACTION NUMBER

2.4 Discussion

The buoyant density shift method of Edenberg and Hanawalt (1972) was used to measure the size of DNA repair patches after treatment of cells with DNA damaging agents, and to study the 'short patch' and 'long patch' repair mechanisms proposed by Regan and Setlow (1974). Exposure of KB cells to UV-light or MMS produced a patch size of between 35 and 40 base pairs. The short patch repair pathway postulated to be involved with the repair of methylating agents was not observed. If present in the cell, the short patch repair system, involving 1-3 base pairs, may not be detectable in the presence of the larger repair patch system involving 35-40 base pairs. The application of the buoyant density shift method by others to measure repair patch size after treatment with other agents, eg. angelicin and black light irradiation (Kay et al., 1980), methoxypsoralen plus black light irradiation (Kay et al., 1980), acetoxysafrole (Phillips et al., 1981), AAAF (Zolan et al., 1982b), and furocoumarins and long wavelength UV-light (Zolan et al., 1982b), produced sizes of between 20 and 25 nucleotides. In all the above mentioned studies, no evidence for a small patch size repair mechanism was shown.

Reassessment of the patch size induced by MMS and EMS using the BrdUrd-photolysis method produced values of between 40 and 45 nucleotides (Snyder & Regan, 1982; Francis et al., 1981). However, these authors did not rule out the

existence of a short-patch component. Nevertheless, these newer findings throw some doubt on the existence of a short patch repair pathway.

Earlier application of the BrdUrd-photolysis technique generally yielded higher estimates for the size of the repair patch, induced in mammalian cells by UV-light or AAAF (Regan & Setlow, 1974; Rosenstein et al., 1980), ranging from 100 to 170 base pairs. However, more recent applications of the BrdUrd-photolysis technique have yielded patch size estimations in the range of 40 to 60 nucleotides for the repair of UV-light damage (Francis et al., 1981; Snyder & Regan, 1982).

The estimation of the repair patch size using the buoyant density method is based on the maximal incorporation of BrdUrd into the repaired region, and the small size of the fragmented DNA. Sonication (Smith et al., 1981) and mechanical shearing, used in this study, were used to reduce the size of the DNA. Because the resulting DNA fragments have a heterogeneous size distribution, the patch size estimate is based on the average fragment size. Hence, it would be anticipated that a more accurate patch size estimate would be obtained by using DNA fragments with a narrowly defined size, and the sensitivity could be increased with a smaller fragment size. Treatment of African green monkey DNA with the restriction endonuclease Hind III produces a homogenous population of DNA, called α DNA, with a fragment size of 172 base pairs (Zolan et al., 1982b), but

it comprises only 15 to 20% of the monkey cell genome and is not transcribed. Repair of certain adducts in α DNA was found to occur at a lower level than in bulk DNA, eg. repair in α DNA after treatment with furocoumarins and long wavelength UV-light was only 30% of that in bulk DNA, while repair of DNA treated with AAAF was 60% of that in bulk DNA. In spite of this characteristic, the repair patch size was found to be around 20 nucleotides in length.

An alternate method for producing DNA fragments with a homogeneous size distribution, and small fragment size, is to digest chromatin with Staphylococcal nuclease. The patch size estimate made on these 145 base pair fragments was the same as that made on sonically produced fragments and on DNA, which is about 20 nucleotides.

The question of whether repair occurs primarily in the linker position of the chromatin, or in the core region as well, is still under investigation (Lieberman, 1982). In the protocol of repair patch size studies, a repair period of 6 hrs., followed by an overnight chase in non-radioactive medium was employed. Rearrangements of the chromatin to distribute repair-incorporated nucleotides throughout the chromatin (Oleson et al., 1979; Zolan et al., 1982a; Lieberman, 1982) would have occured, and patch size measurements using core DNA would be representative of repair in total chromatin. The fact that the patch size obtained was similar to that obtained when bulk DNA was

analysed after they were sonicated to give a fragment size of 200 base pairs (Smith et al., 1981), would indicate that no bias was introduced by analyzing the core DNA only.

Recently, Leadon et al., (1983) reported that the repair patch size in α DNA after treatment of African green monkey cells with aflatoxin B, or MMS was about 10 nucleotides, while that in bulk DNA was about 20 nucleotides. This smaller patch size with α DNA was not observed when these cells were treated with some other damaging agents like UV-light. The result suggests that different repair pathways function in the two DNA species, although the shorter repair patches are not mandated by the structure of DNA and could represent two or more size classes of repair patches. Whether these two classes exist in human cells remains to be determined.

To date, the factor, or factors, that regulate the size of repair patches is not known. One possibility is the structural organization of chromatin which could limit the repair incorporation, or the restriction could be placed by the inherent limitation of the repair enzymes, as suggested by the studies of Wang and Korn (1980).

Chapter 3

POLY(ADP-RIBOSYL)ATION AND DNA REPAIR

3.1 Introduction

One aspect of DNA repair that has generated much interest has been the involvement of poly(ADP-ribosyl)ation in repair. Poly(ADP-ribose) is a homopolymer of repeating ADP-ribose units linked through ribose-ribose (1"-2')glycosidic bonds (Mandel et al., 1982). The polymer is the result of the enzymatic transfer of the ADP-ribose moieties of NAD, with the concomitant release of the nicotinamide moieties, to a growing chain of ADP-ribose units, and is catalyzed by poly(ADP-ribose) synthetase, or polymerase. This reaction occurs in the nucleus and the acceptors for this homopolymer are various nuclear proteins.

Several hypothesis have been formulated regarding the roles of poly(ADP-ribosyl)ation in eukaryotes, including an involvement in DNA replication, cell proliferation and chromatin structure. A substantial number of reports on the stimulation of poly(ADP-ribose) polymerase by DNA-damaging agents have also implicated a role in the repair of DNA (Mandel et al., 1982). A number of such studies have employed the nicotinamide analog, 3-aminobenzamide (3-AB), which inhibits poly(ADP-ribose) polymerase. An observation from these studies was that repair synthesis in cells treated with dimethylsulfate (DMS) was enhanced by the presence of 3-AB (Durkacz et al., 1981; James & Lehmann, 1982). Durkacz et al. (1980) and James and Lehmann (1982) also measured the rate of repair of alkali-labile lesions in DNA following treatment of cells with DMS, and showed that

the repair of such lesions was suppressed by 3-AB. The conclusion from these observations was that the ligation step in the excision repair process was somehow inhibited. Creissen and Shall (1982) subsequently provided direct evidence for the hypothesis by showing that, following treatment of L1210 cells with DMS, the activity of partially purified DNA ligase II was increased 5-fold over that in untreated cells, and that this increase was prevented by 3-AB. From such findings, they proposed that poly(ADP-ribosyl)ation was necessary for the stimulation of DNA ligase II in the repair of DNA, and that in the presence of 3-AB, ligation was inefficient and the repair site would be held open for more nucleotides to be inserted, resulting in a larger patch size. Hence, according to this proposal the size of the repair patch is regulated by the intervention of the DNA ligase to terminate the excision-polymerization process.

To test this hypothesis, I have applied the buoyant density shift method to study the influence of 3-AB on the size of the repair patch that results from the treatment of cells with DMS.

3.2 Materials and Methods

3.2.1 Cells and culture conditions

T98G cells were used and conditions for growth were described in Chapter 2 (section 2.2.1).

3.2.2 Measurement of repair synthesis and patch size

The method employed is described in Chapter 2 (section 2.2.2), with slight modifications. For each condition studied, three, 100-mm Petri dishes of cells were used. Briefly, T98G cells were prelabelled by growth in medium containing 0.4 μCi/ml ^{32}P-orthophosphate for 48 hr. The cells were then allowed to grow in isotope-free medium for 24 hr. when confluence had been reached. The medium was changed to one containing 1% fetal bovine serum (FBS) and incubation was continued for 48 hr. One hour prior to treatment, BrdUrd (16 μM) and FdUrd (1 μM) were added to the cultures. When desired, 3-AB was present with the BrdUrd and FdUrd at a concentration of 5 mM. This pre-incubation with 3-AB is to ensure that its effects in the cell is maximal when repair is initiated. DMS dissolved in methanol was added to the cultures to a final concentration of 0.4 mM. Fifteen minutes later, [^{3}H]-BrdUrd (10 μCi/ml, 20 Ci/mmol) was added along with hydroxyurea to 10 mM, repair was allowed to proceed for 6 hr., then an overnight chase in isotope-free medium (1% FBS, 16 μM BrdUrd, 1 μM FdUrd) was allowed. Irradiation of T98G cells with 20 J.m^{-2} of UV-light and repair incubation were as described in section 2.2.2, and [^{3}H]-BrdUrd (10 μCi/ml, 20 Ci/mmol) was employed to label the repair patches.

DNA was extracted as described in section 2.2.3 and processed by neutral buoyant density centrifugation as in section 2.2.7. The normal density DNA was then sheared,

centrifuged in an alkaline CsCl gradient, and the size of the repair patch was determined as described in section 2.2.7 and 2.2.8.

3.3 Results

Confluent T98G cells were treated with DMS and were allowed to repair their DNA in the presence or absence of 3-AB. DNA was isolated from these cells and was banded in neutral cesium chloride (Fig. 9) in order to separate DNA containing ^{3}H-BrdUrd as a result of semiconservative synthesis from that labelled by repair synthesis. The figure shows that the amount of newly synthesized DNA with a hybrid density and banding at fraction 10 is negligible whether 3-AB was present or not. However, the extent of repair replication occurring in the presence of 3-AB was 1.6 times that occurring in the absence of 3-AB. The peak of ^{32}P-counts that banded at the bottom of the density gradient is due to RNA that had been labelled with ^{32}P. To determine if this increased repair incorporation was due to an increase in the number of nucleotides inserted per repair site, a direct measurement of repair patch size was made by the buoyant density shift method. DNA from the repair peak from each gradient shown in Fig. 9 was pooled, sheared to an average fragment size of 400 bp and banded in an alkaline cesium chloride gradient (Fig. 10). From the data presented in Fig. 10A and 10B it was calculated that the size of the

Figure 9. Neutral cesium chloride gradient analysis of DNA from DMS-treated T98G cells. T98G cells, prelabelled with ^{32}P, were exposed to DMS and allowed to repair for 6 hr. in the absence (Panel A) or presence (Panel B) of 3-AB. ○, ^{3}H-repair label; ●, ^{32}P-prelabel.

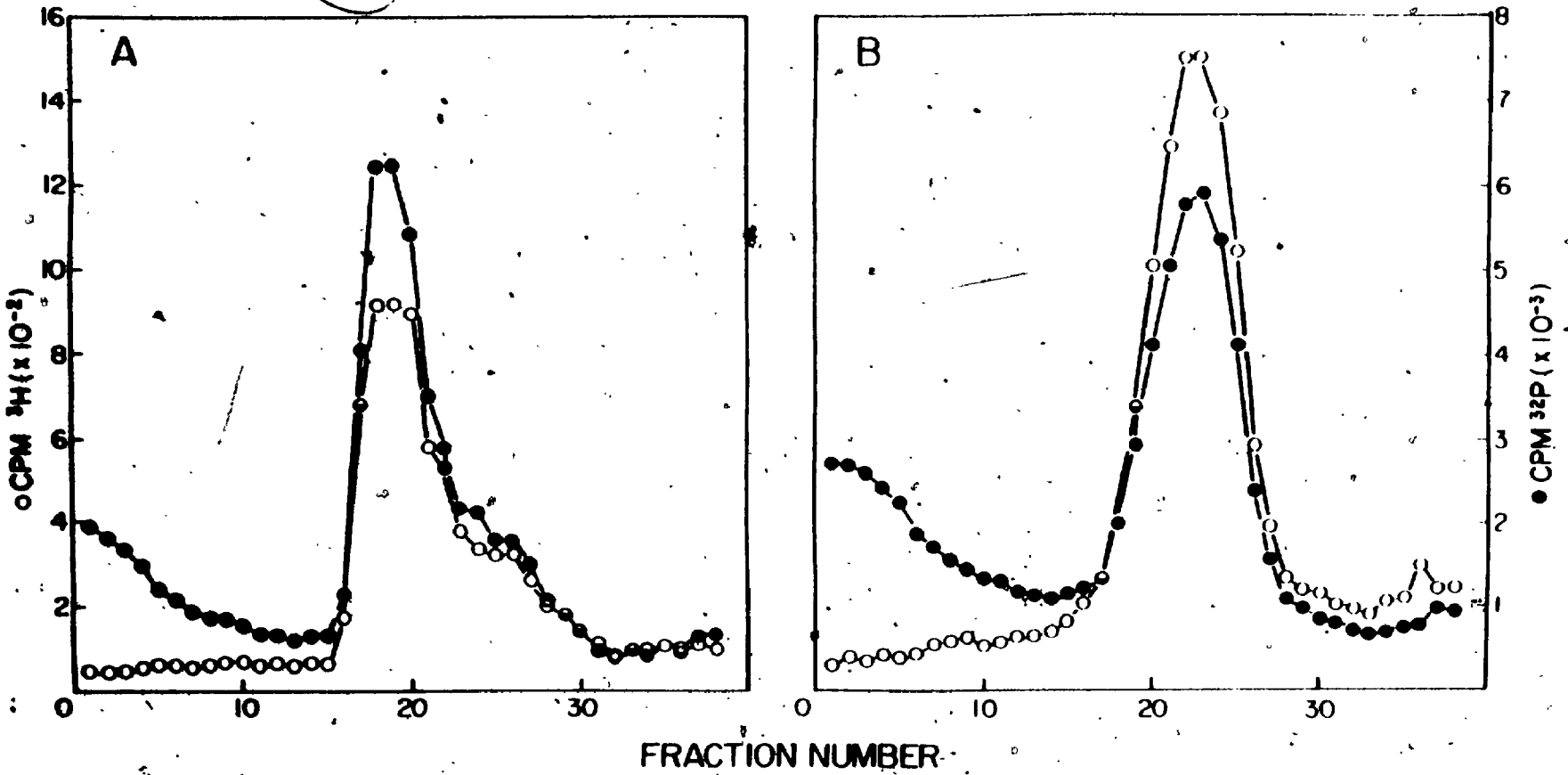
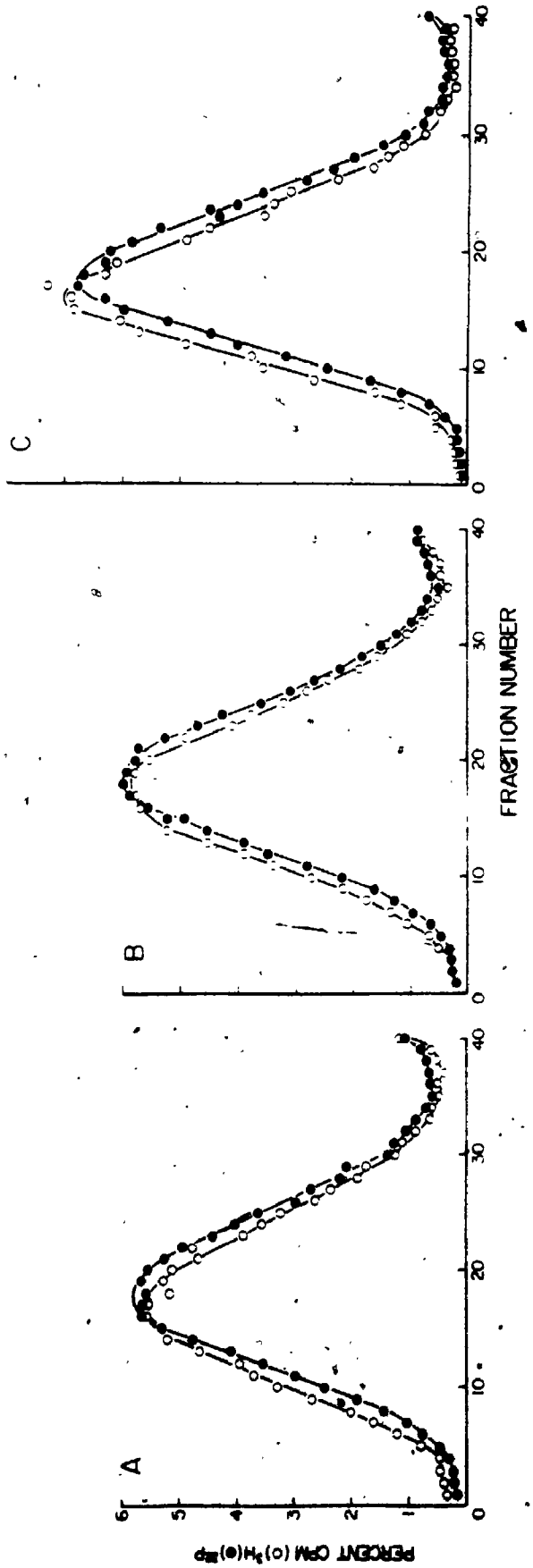

A
B
oCPM ³H(x10⁻²)
● CPM ³²P (x10⁻³)
FRACTION NUMBER
0
10
20
30
2
4
6
8
10
12
14
16
1
3
5
7

Figure 10. Alkaline cesium chloride reband of normal density DNA which was mechanically sheared to an average fragment size of 400 bp. Panel A, DNA from DMS-treated cells which were allowed to repair in regular medium. Panel B, DNA from DMS-treated cells, which were allowed to repair in medium containing 3-AB. Panel C, DNA from UV-irradiated cells that were allowed to repair in regular medium. ○, ^{3}H-repair label; ●, ^{32}P-prelabel.


A
B
C
PERCENT CPM (○) ^3H(●)$_{TCA}$
FRACTION NUMBER

repair patch formed in the absence of 3-AB was 35 nucleotides while that in the presence of 3-AB was 27 nucleotides (see section 2.3.2).

As part of this same experiment, and as a kind of internal control, the size of the repair patch formed during repair of UV-light damage was determined. The alkaline cesium chloride sedimentation profile for this measurement is shown in Fig. 10C. The patch size calculation gave a value of 44 nucleotides.

3.4 Discussion

Direct measurements of the size of the repair patch synthesized in the presence of 3-AB did not show any increase in the number of nucleotides incorporated, as predicted by James and Lehmann (1982), although an increase in repair replication was observed. Hence, another explanation of the phenomenon was needed. A reasonable alternative is that the number of repair sites was increased. However, since it was found by Walker *et al.* (1984) that the removal of the methylation products in the 6 hr. period, during which repair replication and patch size measurements were made, was not altered by 3-AB, this possibility must also be discarded.

In a recent study, Jacobson *et al.* (1983) found that repair replication of UV-light damage was inhibited by hydroxyurea, but in the simultaneous presence of hydroxyurea and 3-AB, there was no inhibition. Thus, when the latter

result was compared to the former, an apparent increase in repair replication was obtained. All of the investigations, including ours, which reported an increased repair replication due to 3-AB have employed hydroxyurea to suppress residual semiconservative synthesis, and this appeared to offer a clue to the explanation of the 3-AB effect.

However, when repair incorporation after treatment with DMS was measured in the presence and absence of both hydroxyurea and 3-AB, either singly or in combination, it was observed that the presence of 3-AB increased repair incorporation regardless of the presence or absence of hydroxyurea (Walker et al., 1984). Hydroxyurea alone caused a decrease in repair incorporation.

These curious results strongly suggested that the enhancing effect of 3-AB on repair incorporation observed here and by other investigators can be explained by some kind of nucleotide pool phenomenon. Sims et al., (1983) have reported that when cells are treated with a methylating agent, ATP levels as well as NAD levels fall dramatically. The presence of 3-AB prevents the fall in levels of both metabolites. It is, therefore, reasonable to suspect that the levels of DNA precursors would be different in DMS-treated cells in the presence or absence of 3-AB and this would affect the amount of labelled precursor incorporated during DNA repair. This idea could also explain the smaller repair patch size that was consistently obtained for DMS-

treated cells that have repaired their DNA in the presence of 3-AB, ie., a value of about 30 nucleotides in the presence of 3-AB and of about 40 nucleotides in the absence of 3-AB. However, the method is not sufficiently sensitive to distinguish unequivocally between these two values.

Cleaver et al. (1983) have reported that 3-AB did not alter the rate at which 7-methylguanine and 3-methyladenine were removed from the DNA of CHO and HeLa cells. However, 3-AB inhibited de novo synthesis of purine and pyrimidine nucleotides from the 1-carbon pathway. This finding was considered to reflect a more general disturbance of nucleotide metabolism which would explain the apparent stimulation of repair replication by 3-AB.

More recently, Milan and Cleaver (1984) showed that 3-AB not only inhibited poly(ADP-ribose) polymerase, but also affected cell viability, glucose metabolism, and DNA synthesis. Hence, any effect of 3-AB on DNA repair could very well be the result of indirect effects of the inhibition on other cellular processes.

The report by Creissen and Shall (1982) that DNA ligase II could be poly(ADP-ribosyl)ated and activated in DNA-damaged cells was not supported by the findings of Ohashi et al. (1983). The presence of the ligase II in the cell was not observed by the latter authors, nor was it found by Teraoka et al. (1982), thereby casting some doubt on its existence (see also section 1.2.4). To further characterize the role of poly(ADP-ribose) in DNA repair, Ohashi et al.

(1983) studied the enzymatic activity of DNA ligase in the presence of histones and chromatin, and the influence of the poly(ADP-ribose) polymer on the enzyme activity. The presence of histones in the reaction mixture resulted in the inhibition of DNA ligase activity, eg., 7.5 µg/ml of histone H1 or whole histones led to an inhibition of activity of 80% or 40%, respectively, and almost complete inhibition of ligase activity was observed with 10 µg/ml histone H1 or 25 µg/ml total histones. This inhibition was dependent on the relative concentrations of histone and DNA. Addition of free long-chain (average of 25 residues) poly(ADP-ribose) to the reaction mixture alleviated the inhibition. Almost total recovery of the ligase activity was obtained when the concentration of the long-chain polymer was 10-times that of histones. This stimulation of the activity of DNA ligase was also obtained when the polymer was synthesized in situ by the poly(ADP-ribose) polymerase. Similar results were obtained when reconstituted chromatin was used as the substrate for DNA ligase.

The interesting observation was made that the long-chain poly(ADP-ribose), the activator of DNA ligase, was found to be exclusively bound to poly(ADP-ribose) polymerase, and that this enzyme-bound polymer was the actual form of the activator. Short-chain (ADP-ribose) polymers, either in monomeric or oligomeric forms, were found to be mostly associated with histones, and had very little stimulatory effect on the DNA ligase.

On the basis of this work, Ohashi et al. (1983) proposed a model in which the poly(ADP-ribose) polymerase, in response to the strand-break in DNA, synthesizes the long-chain poly(ADP-ribose) on itself at the site of the breakage. Because of its affinity for strand breaks, the poly(ADP-ribose) polymerase would link the DNA ligase to the damage site, and the activator-function of the enzyme-bound polymer would alleviate the inhibition of the DNA ligase activity by the histones. It was further proposed that poly(ADP-ribose), being a polyanion, could also act by locally neutralizing the positive charge of the histones, and thus, loosening the DNA-histone interactions.

Relaxation of the chromatin structure by poly(ADP-ribosyl)ation has been demonstrated by Poirier et al. (1982). Through the use of electron microscopy, these authors showed that under high ionic strength conditions (75 mM NaCl), nucleosomes take on a condensed and compact structure. However, polynucleosomes that were poly(ADP-ribosyl)ated remained relaxed and extended. It was suggested, on the basis of these observations, that poly(ADP-ribosyl)ation could produce a relaxation of the chromatin organization, rendering the damaged DNA more accessible to repair enzymes.

In conclusion, direct measurements of the repair patch size did not bear out the prediction of James and Lehmann (1982) that the presence of 3-AB in the cell would produce a larger repair patch. Furthermore, removal of lesions was not

enhanced in the presence of the inhibitor, suggesting that the observed increase in repair replication could be the result of altered endogenous nucleotide pools.

Chapter 4

DNA REPAIR IN THE PRESENCE OF APHIDICOLIN

4.1 Introduction

Inhibitors of DNA polymerase have been employed in the study of the enzymology of DNA repair in human cells, and the results have created a controversy concerning the roles played by polymerases α and β. The commonly used inhibitors have been aphidicolin and cytosine arabinoside, both inhibitors of α polymerase, and dideoxythymidine, the only inhibitor of polymerase β available (Scovassi et al., 1980). DNA polymerase β has been reported with greater consistency to have a role in the repair of DNA (Cleaver, 1983; Dresler & Lieberman, 1983; Giulotto & Mondello, 1981; Hardt et al., 1981; Miller & Chinault, 1982; Pedrali-Noy & Spadari, 1980; Seki et al., 1980; Spadari et al., 1982; Waser et al., 1979). The use of aphidicolin to examine the role of polymerase α in DNA repair has produced conflicting results. Several studies have implicated polymerase α in the repair process by demonstrating a sensitivity of DNA repair to aphidicolin (Berger et al., 1979; Ciarrocchi et al., 1979; Cleaver, 1983; Collins et al., 1982; Dresler & Lieberman, 1983; Hanaoka et al., 1979; Snyder & Regan, 1981; Snyder & Regan, 1982). However, other studies have shown an insensitivity of DNA repair to aphidicolin (Giulotto & Mondello, 1981; Hardt et al., 1981; Pedrali-Noy & Spadari, 1980; Seki et al., 1980; Spadari et al., 1982), implying that the main repair enzyme is a non-polymerase, probably β. More recent studies using aphidicolin, cytosine

arabinoside, and dideoxythymidine, either individually or in combination, yielded results that suggest the participation of both polymerases in DNA repair, depending on the agent used; and the dosage applied (Cleaver, 1983; Dresler & Lieberman, 1983; Miller & Chinault, 1982).

Enzymatic studies done on purified α and β polymerases revealed that the former enzyme requires single-stranded gaps of 30-60 nucleotides in length for optimal activity, but can only polymerize fragments of 20-40 nucleotides in length (Korn et al., 1981). Unlike α polymerase, the β enzyme can work on nicked DNA to incorporate about 15 nucleotides per nick, but is most active on gaps of about 10 nucleotides in length, and is capable of filling gaps to completion.

The reported findings on the effects of inhibitors on DNA repair in the cell, and the in vitro data on purified DNA polymerases, prompted me to investigate the size of the repair patch in DNA in human cells in the presence of aphidicolin to see if there was agreement between the results obtained from work done in vitro and in vivo.

4.2 Materials and Methods

KB cells were used in this study, and were maintained as described in section 2.2.1. Repair synthesis was performed as described in section 2.2.2, with slight modifications. Briefly, KB cells were prelabelled by growth in medium containing 0.4 μCi/ml ^{32}P-orthophosphate for 48

hr. For each condition studied, 5, 100-mm Petri dishes of cells were used. After prelabelling, the cells were grown for a further 24 hr. in isotope-free medium. One hour prior to treatment, BrdUrd (16 μM) and FdUrd (1 μM) were added to the culture. Following an incubation period of 45 min., aphidicolin[1], dissolved in DMSO, was added to give a final concentration of 3 μg/ml. This pre-incubation with aphidicolin is to ensure that the polymerase α in the cell is maximally inhibited when repair processes are initiated after DNA-damage. Control cells received an equal amount of DMSO (final concentration of 0.3%). Treatment of cells with 20 J/m^2 of UV-light or dimethylsulfate (DMS) were as described in section 2.2.2. Repair incubation was performed with growth medium containing 16 μM BrdUrd, 1 μM FdUrd, and [^{3}H]thymidine (10 μCi/ ml, 20 Ci/mmole) for 6 hr. and in the absence of hydroxyurea. Hydroxyurea was omitted to eliminate any influence of altered nucleotide pools on the repair-incorporation. At the end of the repair period, BrdUrd was added to a concentration of 160 μM to dilute the endogenous pool of [^{3}H]thymidine, and incubation was continued for a further 30 min. An overnight chase period was allowed in isotope-free medium containing 80 μM BrdUrd and 1 μM FdUrd.

[1] The aphidicolin was a generous gift of Imperial Chemical Industries, Macclesfield, Cheshire, SK10 4TG, England.

The DNA was isolated and banded in a neutral cesium chloride gradient (section 2.2.3 and 2.2.7). Fractions containing unreplicated DNA were pooled and sonicated, at setting 7, with a Branson model W-350 sonifier equipped with a micro-probe. The DNA solution was cooled in an ice bath and was sonicated for 10, 2-minute intervals, with a 1 minute pause in between. This treatment reduced the fragment size to an average of 350 nucleotide pairs, as judged by electrophoretic mobility in an agarose gel (section 2.2.6). The fragmented DNA was then rebanded in an alkaline cesium chloride gradient for the determination of repair patch size.

Four plates of KB cells were used for the untreated controls. The cells were prelabelled as described for the treated cells. They were then preincubated for 45 min in medium containing 16 μM BrdUrd and 1 μM FdUrd, after which aphidicolin in DMSO was added to 2 of the plates (3 μg/ml final concentration), and DMSO was added to the other 2 (0.3% v/v final concentration), and incubation was allowed to proceed for a further 15 min. [^{3}H]thymidine (1 μCi/ml, 20 Ci/mmol) was then added, and after a 6 hr. incubation period, 2 of the plates of cells, one with aphidicolin and one without, were subjected to an overnight chase period, as described previously. The other two plates of cells were immediately harvested, and the DNA extracted. The DNA from all four samples was subjected to a single neutral CsCl gradient analysis.

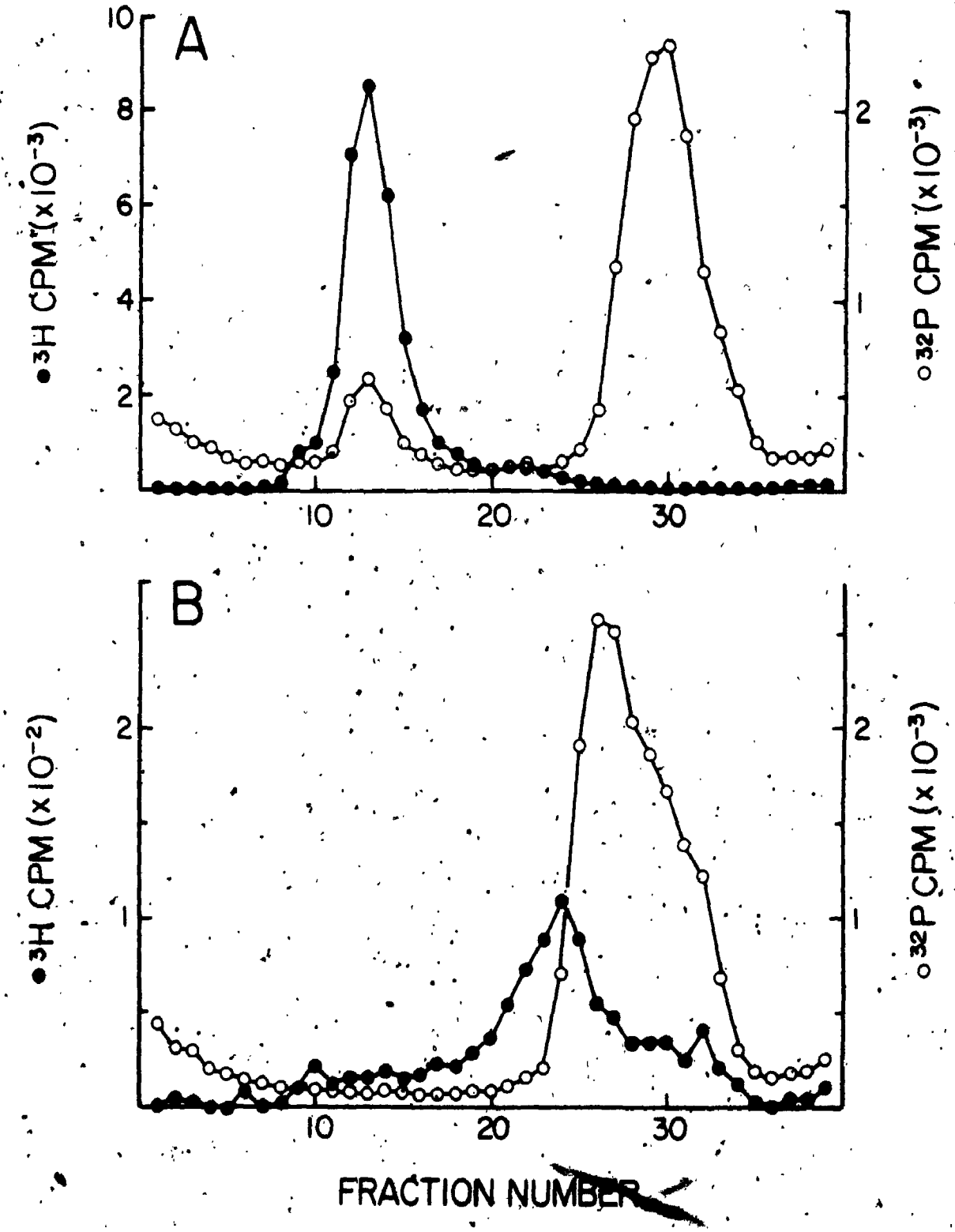
4.3 Results

4.3.1 Synthesis of DNA in the presence of aphidicolin

Neutral buoyant density profiles of DNA synthesized in the absence or presence of aphidicolin are shown in Figs. 11A and 11B, respectively. When DNA synthesized in the absence of aphidicolin was isolated immediately after the 6 hr. incubation period, it sedimented in the denser region of the gradient (Fig. 11A), indicating that replicative synthesis had occurred normally to produce BrdUrd-substituted DNA with a hybrid density. However, in the presence of aphidicolin, replicative synthesis was partially inhibited, and the BrdUrd that was incorporated was only sufficient to produce a small increase in buoyant density (Fig. 11B).

When an overnight chase period was employed following the 6 hr. labelling period, the tritium that was incorporated in the presence of aphidicolin sedimented at the hybrid density region of the gradient (Fig. 12). When no aphidicolin was present, the majority of the tritium label was found at the hybrid density region, and the rest at a greater density, indicating that a second round of DNA synthesis in BrdUrd had begun. These results obtained with aphidicolin show that in a repair experiment, unless an overnight chase period was allowed, some of the tritium found in the normal density peak could have resulted from incorporation into nascent DNA.

Figure 11. Effect of aphidicolin on replicative synthesis. KB cells that were prelabelled with ^{32}P were incubated for 6 hr. in [^{3}H]thymidine and BrdUrd in the absence (panel A) or presence (panel B) of 3 μg/ml aphidicolin, after which the DNA was extracted and subjected to neutral CsCl gradient analysis. ●, ^{3}H-repair label; ○, ^{32}P-prelabel.

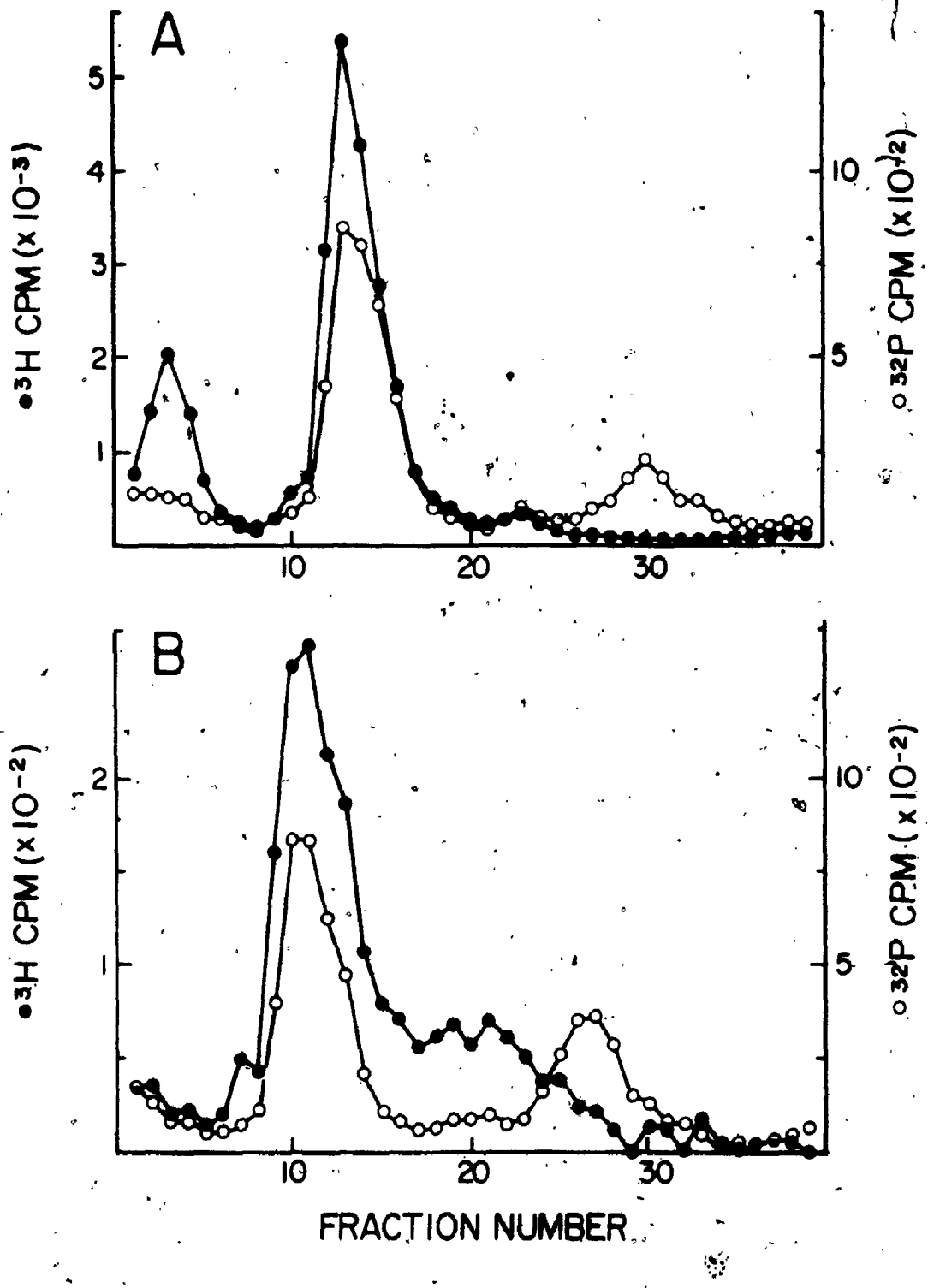

A
B
•3H CPM (x10-3)
o32P CPM (x10-3)
•3H CPM (x10-2)
o32P CPM (x10-3)
FRACTION NUMBER

Under the conditions employed, 3 µg/ml of aphidicolin was effective in reducing the incorporation of tritium by about 95% (Figs. 11 and 12), in agreement with previously reported findings (Hanaoka et al., 1979; Ikegami et al., 1978; Spadari et al., 1982). When the aphidicolin was removed, and DNA synthesis resumed during the overnight chase period, there was no further increase in the amount of label incorporated into hybrid density DNA. This indicated that the 30 min. incubation period with 160 µM BrdUrd just prior to the removal of the aphidicolin, followed by a chase in 80 µM BrdUrd, had diluted the [^{3}H]thymidine pool in the cells sufficiently so that no further incorporation of label occurred upon the removal of the inhibitor.

As illustrated in Fig. 11A, the ^{32}P distribution shows that about 15% of the DNA had undergone replicative synthesis in the 6 hr. period. This proportion of replicated DNA increased to about 80% when the cells were allowed to incubate overnight in the presence of BrdUrd (Fig. 12A). In the presence of aphidicolin, the ^{32}P distribution shows that no significant amount of DNA was replicated in the 6 hr. incubation period (Fig. 11B). However, upon the removal of the inhibitor, followed by an overnight chase, about 65% of the total DNA was found in the region of the density gradient which corresponds to hybrid DNA (Fig. 12B).

Finally, exposure of KB cells to DMSO or aphidicolin plus DMSO did not, of themselves, produce any damage in the DNA so as to induce repair incorporation.

Figure 12. Effect of a chase period on cells that had synthesized DNA in the presence of aphidicolin. KB cells were treated as in Fig. 1, and incubated further overnight in the absence of [^{3}H]thymidine and aphidicolin. The DNA was extracted and centrifuged in neutral CsCl. ●, ^{3}H-repair label; ○, ^{32}P-prelabel. Panel A, no aphidicolin; Panel B, with aphidicolin.


A
B
•3H CPM (x 10-3)
•3H CPM (x 10-2)
o32P CPM (x 10-2)
o32P CPM (x 10-2)
FRACTION NUMBER
10
20
30
1
2
3
4
5
10
5

4.3.2 Repair of UV-light-damaged DNA in the presence of aphidicolin

Neutral buoyant density gradient profiles of DNA obtained from KB cells that were treated with UV-light, and allowed to perform DNA repair in the presence of aphidicolin, are presented in Fig. 13. The ^{32}P profile indicates that irradiation with UV-light reduced the levels of replicative synthesis from about 80% (Fig. 12A) to about 13% (Fig. 13A). The amount of replicated DNA was reduced further, to about 5% when repair synthesis was performed in the presence of aphidicolin (Fig. 13B). A further indication of the inhibitory effect of aphidicolin is that the amount of tritium incorporated into hybrid density DNA was reduced by about 90%. From these observations, we know that the aphidicolin was entering the cell and affecting the cellular replicating systems.

When repair incorporation was examined, aphidicolin did not affect the amount of tritium incorporated into the normal density DNA, as illustrated in Figs. 13A and 13B (note the different scales). The ratios of ^{3}H cpm to ^{32}P cpm were 0.56 and 0.55 for the DNA repaired in the absence and presence of the inhibitor, respectively.

The fragmentation of the DNA down to an average size of 350 base pairs allowed the repair-incorporated BrdUrd to increase the buoyant density of the DNA fragments containing the repair patches, and from these data, the repair patch size can be derived (section 2.3.2). Figures 14A and 14B

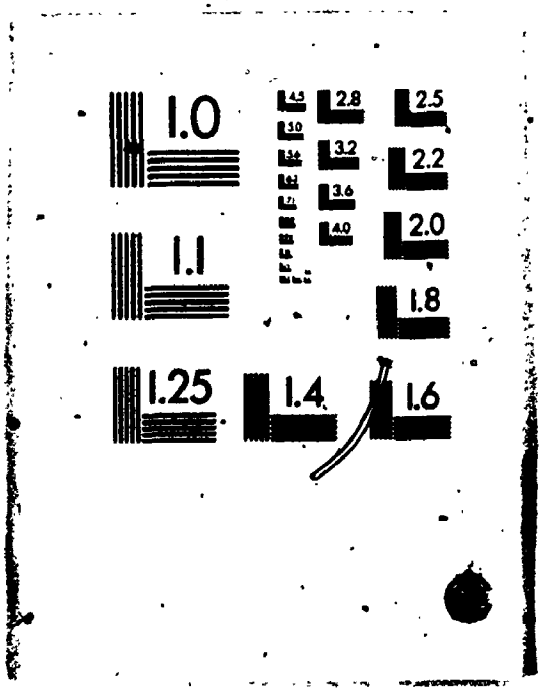
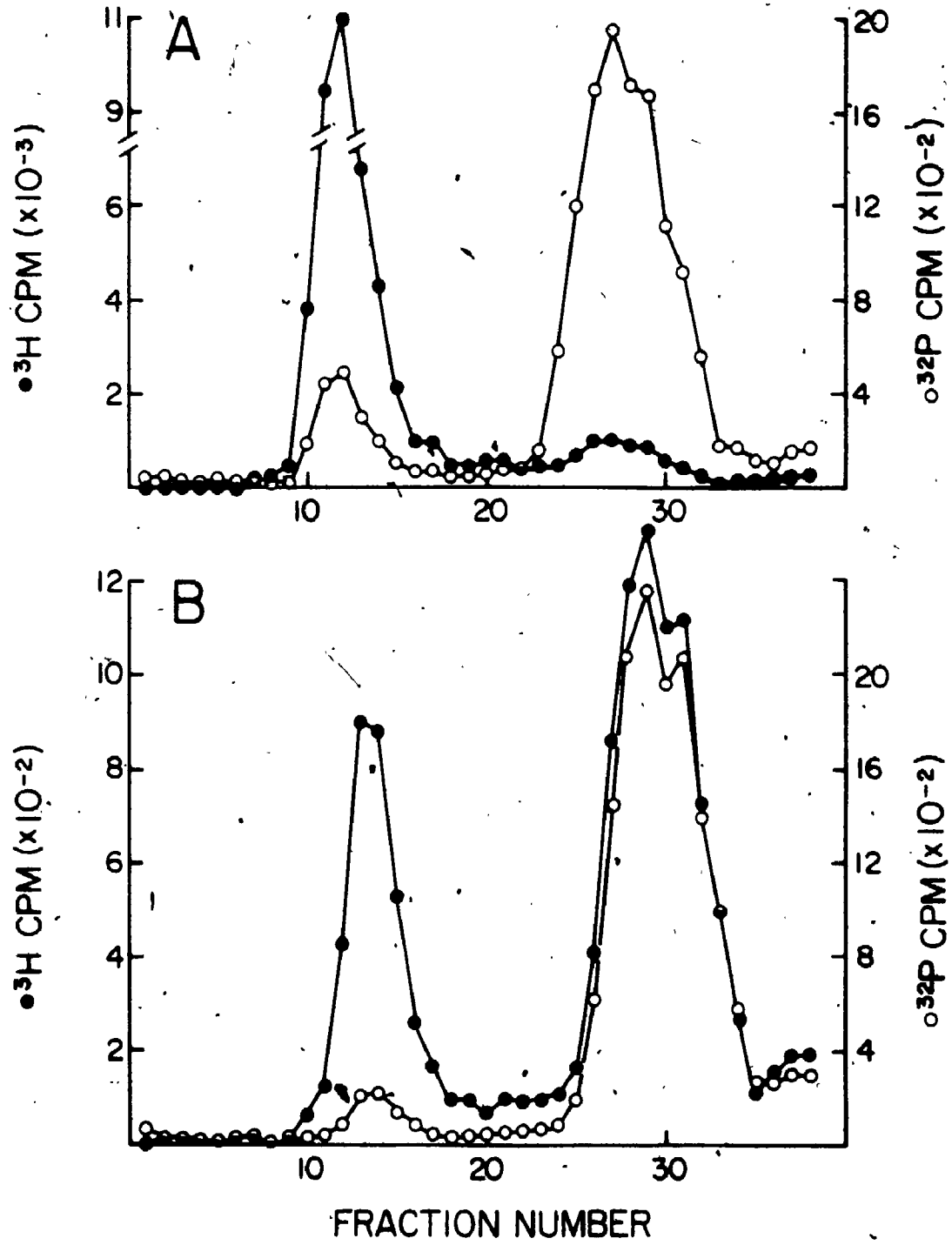


Figure 13. Neutral CsCl gradient profiles of DNA from KB cells that were treated with 20 J/m^2 UV-light, and allowed to repair their DNA in the absence (panel A) or presence (panel B) of 3 µg/ml aphidicolin. The DNA was extracted after an overnight chase period. ●, ^3H-repair label, ○, ^{32}P-prelabel.


A
B
•3H CPM (x10-3)
•3H CPM (x10-2)
o32P CPM (x10-2)
o32P CPM (x10-2)
FRACTION NUMBER

show the sedimentation profiles of repaired DNA relative to normal density DNA in an alkaline buoyant density gradient. In the absence of aphidicolin, the difference between the centres of mass for normal density DNA and repaired DNA is 2.2 fractions; for repair in the presence of aphidicolin, the difference is 4.5 fractions. These values translate into repair patch sizes of 26 nucleotides, for repair done in the absence of aphidicolin, and 53 nucleotides, for repair done in the presence of aphidicolin.

4.3.3 Repair of DMS-damaged DNA in the presence of aphidicolin

When DMS was used as the damaging agent, aphidicolin again caused an increase in the size of the repair patch (Figs. 15A and 15B). These density shifts translate into repair patch sizes of 29 and 70 nucleotides in the absence and presence of aphidicolin, respectively.

The incorporation by replicative synthesis of ^{3}H into hybrid density DNA, in the presence of aphidicolin, following the exposure of cells to DMS was suppressed by 90% (data not shown), as had been observed with UV-treatment. Repair synthesis following DMS treatment was also depressed by aphidicolin. The ratios of ^{3}H cpm to ^{32}P cpm in the normal density DNA peaks were 1.55 and 1.14 in the absence and presence of the inhibitor, respectively, which corresponds to a reduction in repair incorporation of about 30% (data not shown).

Figure 14. Alkaline buoyant-density profile of sonicated DNA from KB cells that were exposed to UV-light and allowed to repair their DNA in the absence (panel A) or presence (panel B) of aphidicolin (3 μg/ml). Arrows indicate the centres of mass of each population of the fragment DNA. ●, ^{3}H-repair label; ○, ^{32}P-prelabel.

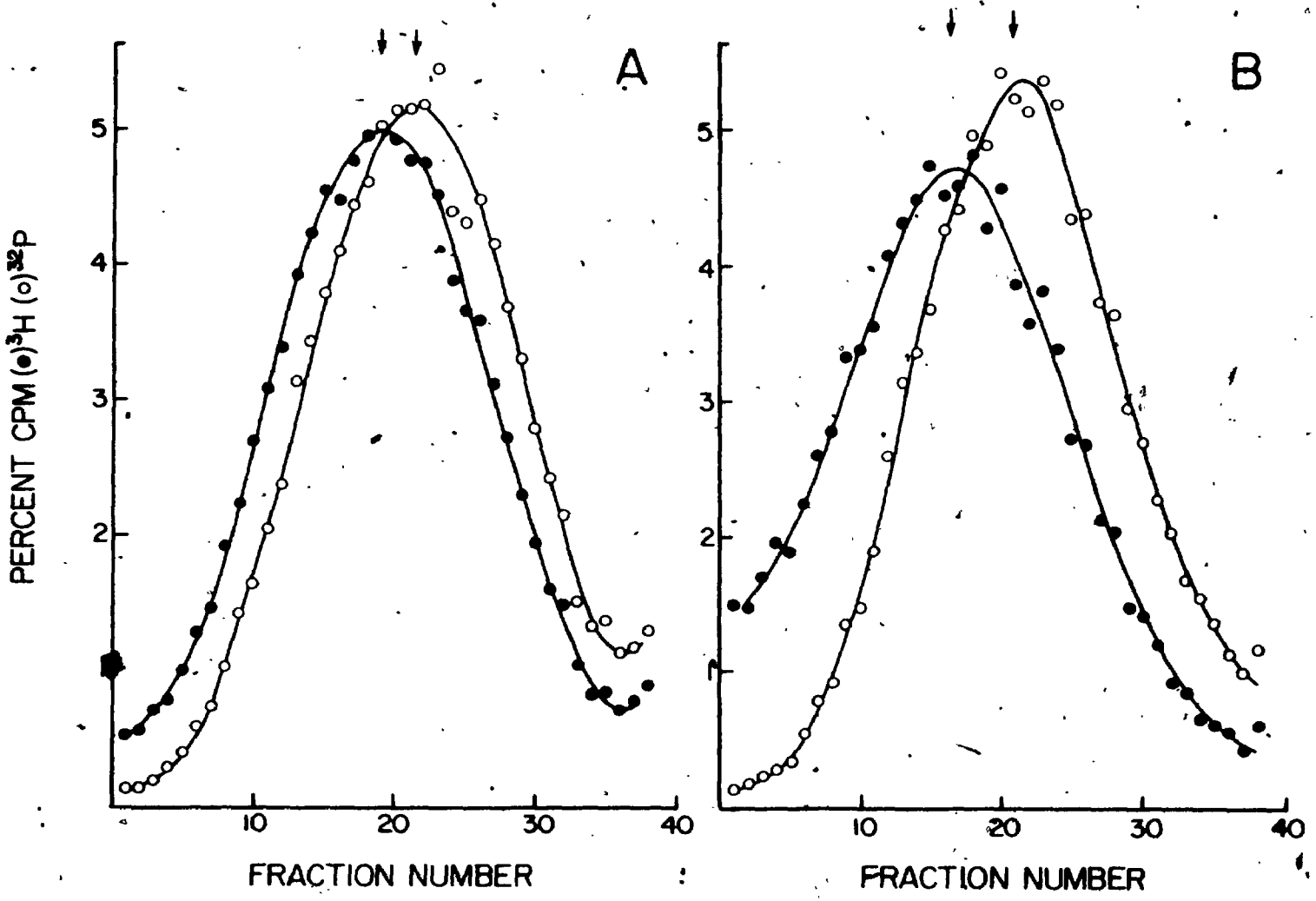
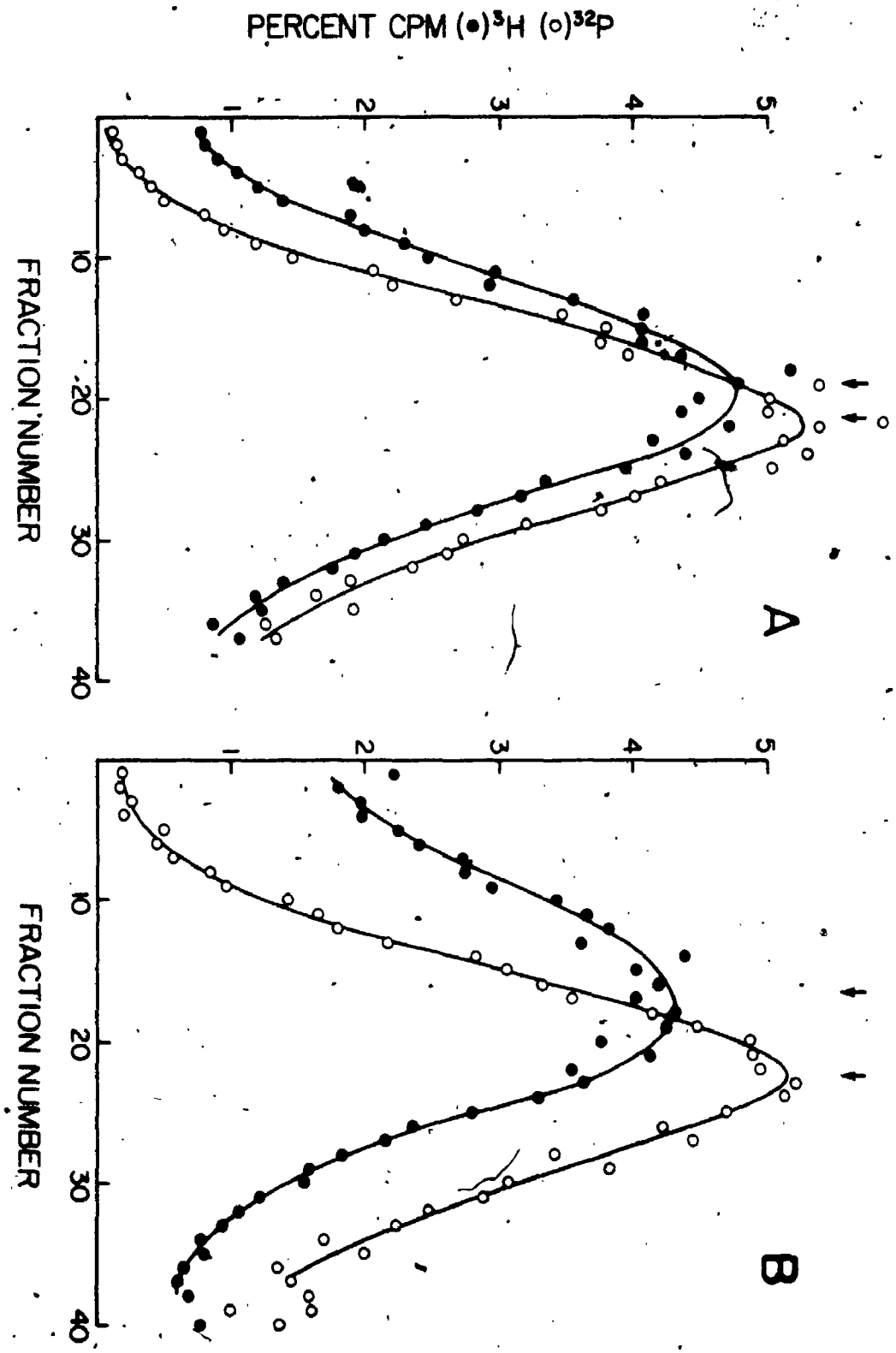

A
B
PERCENT CPM (●)^{3}H (○)^{32}P
5
4
3
2
1
10
20
30
40
FRACTION NUMBER
FRACTION NUMBER

Figure 15. Alkaline buoyant-density profile of sonicated DNA from KB cells that were exposed to DMS and allowed to repair their DNA in the absence (panel A) or presence (panel B) of aphidicolin (3 μg/ml). Arrows indicate the centres of mass of each population of fragmented DNA. ●, ^{3}H-repair label; ○, ^{32}P-prelabel.


PERCENT CPM (•)^{3}H (○)^{32}P
1
2
3
4
5
10
20
30
40
FRACTION NUMBER
A
B

4.4 Discussion

The observation that repair of DNA lesions in the presence of aphidicolin yielded patch sizes that are about twice those formed in the absence of the inhibitor was unexpected. It was anticipated that the α polymerase would be inhibited by the presence of aphidicolin, and whatever repair synthesis that was present in the DNA would have been performed by polymerase β. Because the β enzyme can polymerize a stretch of 10-15 nucleotides (Korn et al., 1981), the resulting repair patch in the presence of aphidicolin, was expected to be of that size.

An explanation for a larger patch size when repair was performed in the presence of aphidicolin could be formulated, based on the report of Pedrali-Noy and Spadari (1979). These authors, using purified enzymes, demonstrated that the polymerizing rate of the α enzyme was decreased by the presence of aphidicolin, whereas the β polymerase was immune to this inhibition. Hence, with a slower rate of polymerization in the presence of the inhibitor, the exonuclease could continue to excise the DNA beyond its usual limit, to generate a larger gap which could be filled in by the β polymerase, eventually, as suggested by Cleaver (1983), or by α polymerase, followed by β polymerase. The repair process is then terminated by the intervention of the DNA ligase.

According to this model, and under the conditions used for damaging the DNA in the cells, the α polymerase plays a prominent role in repair functions, as suggested by Dresler and Lieberman (1983) who showed that the level of participation of polymerase α increased with increasing doses of UV-light. This model further suggests that the size of the repair patch in DNA is determined by the relative rates of the excision and polymerization process, ie. after the generation of a gap, the DNA polymerase will fill in the gap and catch up to the slower-acting exonuclease to terminate excision. A factor that could act to slow down the action of the exonuclease could be the requirement for the DNA to unwind and unfold from the chromatin before repair can occur (Lieberman, 1982).

In contrast to the findings reported by others (Snyder & Regan, 1982; Cleaver, 1983; Dresler & Lieberman, 1983), aphidicolin did not cause any reduction in repair incorporation of label after exposure to UV-light. This could be erroneously interpreted to mean that the aphidicolin-sensitive polymerase α is not involved in the repair of DNA. However, the finding that aphidicolin caused an increase in the repair patch size, and knowing that the inhibitor is specific for α polymerase, suggests that α polymerase was involved. An implication from this study is that the extent of incorporation of label into DNA may not always be an accurate reflection of repair events in the presence of inhibitors.

The lack of an observed increase in the level of repair incorporation to correspond with the increase in repair patch size, after UV-treatment and in the presence of aphidicolin, could be explained by the finding of Snyder and Regan (1981). These authors reported that aphidicolin blocks the removal of pyrimidine dimers in cells that were exposed to UV-light. Thus, the number of repair events could be decreased in the presence of aphidicolin to compensate numerically for the increased incorporation into each repair patch. In a similar vein, the number of repair events following DMS-treatment could be suppressed to a greater degree by aphidicolin to produce the observed 30% decrease in repair incorporation.

Collins (1983) presented a model for the action of aphidicolin in which the inhibited polymerase α acts more slowly, and allows the exonuclease to produce a larger gap than usual. He predicted that a larger repair patch would be formed, and the experimental results described in sections 4.3.2 and 4.3.3 bear out his prediction. The model described in this discussion, although an elaboration of Collin's model, was formulated on the basis of actual repair patch size measurements.

Finally, whether the KB cells were treated with UV-light or DMS, the sizes of the repair patches formed were practically the same, ie. 26 nucleotides and 29 nucleotides, respectively. This suggests that, apart from the initial incisional event which is known to be catalyzed by

different enzymes for UV-damage and methylation damage, the enzymatic processes involved in the repair of various lesions are the same. This concept is further strengthened by the finding that aphidicolin induces similar increases in the size of the repair patch for both DMS and UV-damage.

Chapter 5

DISCUSSION

The buoyant density gradient method of Smith et al. (1981) has provided a way to study the enzymology of DNA repair and its regulation in human cells. It was first proposed by Regan and Setlow (1974) that the size of the repair patch in DNA was dependant on the DNA-damaging agent used (see section 2.1), implying the participation of different enzymes in the repair of lesions produced by different DNA damaging agents. As discussed in sections 2.4 and 4.4, the application of the buoyant density gradient technique in this study has shown that the repair patch size is independent of the agent used to produce the lesion.

Another factor which could regulate the size of the repair patch size was proposed by James and Lehmann (1983). These authors suggested that the intervention of ligase II in the excision and polymerization processes determined the extent of the replacement of nucleotides during excision repair. They further suggested that in the presence of 3-AB, an inhibitor of poly(ADP-ribose) polymerase, the DNA ligase would remain inactive, due to the absence of poly(ADP-ribosyl)ation, and the resulting patch size would increase because the excision-polymerization events could continue uninterruptedly (see section 3.1). Once again, the use of the buoyant density gradient method to measure the size of the repair patch directly has disproved this hypothesis (as discussed in section 3.4). The repair patch size produced in

the presence of 3-AB following treatment of the cells with DMS was the same as that found when repair was performed in the absence of the inhibitor.

The use of aphidicolin, a specific inhibitor of DNA polymerase α, has provided a clue to the regulation of the excision repair process (see section 4.4). In the presence of this inhibitor, a larger repair patch size was found, suggesting that the decreased rate of polymerization allowed the exonuclease to generate a larger gap than usual, which was filled eventually by DNA polymerase α and/or β. Hence, the extent of replacement of the nucleotides during excision repair depends on the relative rates of excision and polymerization (as discussed in section 4.4).

The application of DNA polymerase inhibitors to the study of DNA repair in mammalian cells has resulted in several slightly different proposals on the roles of polymerases α and β in DNA repair. Miller and Chinault (1982) reported that polymerase β was the primary enzyme utilized for the repair of damage induced by bleomycin or neocarzinostatin, while the α enzyme functioned more prominently on damage resulting from N-methyl,N'-nitro, N-nitrosoguanidine or N-nitrosomethyl urea. Cleaver (1983) suggested that polymerase α worked mainly on bulky adducts in DNA. In the presence of inhibitors, the α polymerase could terminate synthesis prematurely, and the resulting single-stranded gap could then become the substrate for the β enzyme. Cleaver further suggested that DNA polymerase β

could be the more prominent enzyme in the repair of alkylated bases, producing shorter repair patches. However, Dresler and Lieberman (1983) proposed that the β polymerase was the major repair enzyme when the dosage of DNA damaging agent applied is low. With increasing doses of the DNA damaging agent, the level of participation of polymerase α would increase.

The finding that aphidicolin increases the repair patch size, and knowing that this inhibitor is specific for polymerase α (Spadari et al., 1982; Lonn & Lonn, 1983), suggests that the α enzyme is involved in the repair of DNA. On the basis of this observation, and those of Cleaver (1983) and Dresler and Lieberman (1983), and along with the studies of Korn et al. (1981) who have delineated the substrate requirements for polymerases α and β, an alternate model on the roles of the α and β polymerases in DNA repair is presented. Immediately after treatment of cells with low doses of UV-light or DMS, a damage-specific endonuclease will produce a nick at the site of the lesion. This then becomes the substrate for an exonuclease which will proceed to generate a single-stranded gap. As soon as a gap of about 10 nucleotides is produced, this becomes the optimal substrate for polymerase β which could fill the gap to completion (Korn et al., 1981) and displace the exonuclease. The whole process is then terminated by the rejoining of the newly synthesized DNA to the pre-existing strand by a DNA ligase. However, if the dose of DNA-damaging agent applied

produced a large number of lesions and hence, more single-stranded gaps than can be handled by the β enzyme, the exonuclease could continue excising without interruption. Eventually, a gap size of about 30 nucleotides will result, and this in turn becomes the optimal substrate for polymerase α (Korn et al., 1981). Because the enzyme lacks the ability to fill gaps to completion, a gap of about 10 nucleotides is left which then becomes the ideal substrate for β polymerase. The β enzyme completes the polymerization, and the ligase terminates the repair process. Support for this contention has come from the report of Mosbaugh and Linn (1983), who used a reconstituted system to show that large gaps produced by DNase V could not be filled to completion by polymerase α alone, but could be if the β polymerase was added to the reaction. Hence, at high doses of DNA damaging agents the major role of polymerase β would be to complete the filling in of gaps that were initiated by the α polymerase. The β enzyme would probably not initiate polymerization at many of the gaps generated by the exonuclease because of the greater polymerase α activity in cycling cells (Falaschi & Spadari, 1977), and also its decreased affinity for gaps of 30 nucleotides in length (Korn et al., 1981). This proposal can explain the dose-dependent participation of polymerases α and β in repair as suggested by Dresler and Lieberman (1983). The events that occur beyond the incision step, comprising excision, polymerization catalysed by the α and β enzymes, and

ligation, must be rapid because it is difficult to detect repair-related strand breaks by centrifugation (Snyder & Regan, 1981) or enzymatic techniques (Cleaver, 1983). This rapidity is further emphasized by the fact that the exonuclease would have about a 30 nucleotide head start on the α polymerase, yet the size of the repair patch is close to 30 nucleotides.

This model agrees with that of Dresler and Lieberman (1983) in that the β enzyme is the major repair polymerase under conditions where the dose of DNA damaging agent is low, and with increasing dosage, the level of participation of α polymerase increases. The model presented here differs in that it calls for the participation of the β enzyme to complete the repair patch that was initiated by the α polymerase. This model also predicts that with a small number of damage sites and the sole participation of polymerase β, the size of the repair patch will be smaller than when α polymerase is required to repair a larger number of damage sites.

One feature of the model is that the β polymerase takes over to complete the gap-filling process from the α polymerase and still does not leave a single-stranded region that can be readily detected. To achieve such close cooperativity, it could be possible that the two enzymes exist as part of a dissociable multienzyme complex, similar to the replitase complex described by Reddy and Pardee (1980, 1983). It would, thus, be of interest to see if a

similar complex, or even the replitase complex, is involved in the repair of DNA in human cells. It whould be of further interest to determine if polymerase β is required to take-over the synthesis of the nascent DNA from polymerase α during cellular replicative synthesis, as in vitro studies of the α enzyme would indicate (Korn et al., 1981; Ikeda et al., 1980).

It has been noted by Chan et al. (1976) that the level of repair replication declined as myoblast cells differentiated into myotubes. It was further observed that lymphocytes exhibited increases in repair functions when they were stimulated to ander'go proliferation by stimulation with phytohaemagglutinin (Scudiero et al., 1976). These observations suggest some correlation exists between the ability of a cell to perform DNA repair and its ability to carry out replicative synthesis. This could be due to the decreased level of activity of the α polymerase in resting cells (Falaschi & Spadari, 1977). If so, whatever repair synthesis that occurs in these terminally differentiated cells would have to be mainly carried out by the β polymerase, along with the residual level of the α enzyme. The α polymerase could be present in different forms as was reported by Krauss and Linn (1982). A possible consequence of this limited repair capability is that the cell would have to leave a portion of the lesions unexcised in the genome. Presumably, for the cell to survive, these lesions would have to be in the part of the genome that is not

transcribed into functional proteins. This allows the enzymes involved with DNA repair to concentrate on the removal of adducts from the regions of the genome that are essential for the production of proteins. The finding that the organization of the chromatin is different between actively transcribing and non-transcribing regions (Weisbrod, 1982) could facilitate this selective excision of adducts. Because the structural organization of the chromatin of actively transcribed genes is more susceptible to digestion by pancreatic DNase I than is bulk chromatin, it could equally be more accessible to DNA repair enzymes than bulk chromatin. As discussed in section 1.3, the masking effect of lesions in DNA by the structural organization of the chromatin has been reported by various investigators.

For the above-mentioned proposals to be elucidated, a better understanding of chromatin structure and organization, along with that of the enzymes that work on the DNA in eukaryotes, is required.

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