
Production of DNA bifilarly substituted with bromodeoxyuridine in the first round of synthesis :
branch migration during isolation of cellular DNA

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ABSTRACT

Incubation of human lymphoid cells with bromodeoxyuridine (BrdUrd) for short periods produces three classes of DNA containing analog: DNA_{HL} (hybrid DNA, density ≈ 1.75 g/cm³), DNA_{int} (intermediate density DNA, density ≈ 1.71 g/cm³), and DNA_{HH} (DNA with both strands containing analog, density ≈ 1.80 g/cm³). Preparations of DNA_{int} yield DNA_{HH} after extensive shearing and/or treatment with single strand specific endonuclease. Cross-linking of pulse-labeled (BrdUrd+³HdT) DNA in cells by treatment with trioxsalen and near UV light before lysis prevents the appearance of DNA_{HH}. Cross-linking after lysis has little effect. A large fraction of DNA_{HH} is obtained after incubation of cells with caffeine. Extraction of DNA at high salt concentration or cross-linking with trioxsalen and near UV light drastically reduced the amount of DNA_{HH} obtained from caffeine-treated cells. We conclude that most DNA_{HH} arises from in vitro branch migration in isolated DNA growing points.

INTRODUCTION

DNA isolated from cells incubated for short periods with radioactive bromodeoxyuridine (BrdUrd) contains growing points which are detected in CsCl gradients by the appearance of radioactivity at a density intermediate between light and hybrid (DNA_{int}) (1). Treatment of mammalian cells with chemical or physical agents which inhibit DNA synthesis increases the proportion of radioactivity observed in the growing point region even though the total amount may be diminished. For example, the slowing of chain elongation resulting from the introduction of methyl methanesulfonate (MMS)-induced lesions resulted in a larger fraction of DNA_{int} (1). Presumably any block to DNA synthesis can result in a larger fraction of label remaining in the growing point region.

Density gradients in which DNA_{int} is observed are also likely to have radioactive molecules appearing at the density characteristic of bifilarly substituted DNA (DNA_{HH}). The proportion of DNA_{HH} is also sensitive to the treatment to which cells have been exposed prior to extraction and is high-

est in cells treated with mutagens and carcinogens including MMS and acetoxy acetyl aminofluorene (AAAF) (2). Both DNA_{int} and DNA_{HH} can be demonstrated most easily after short periods of incubation with labeled BrdUrd or with a mixture of BrdUrd and ³HdT. Incubation of cells with unlabeled BrdUrd after a short pulse of label results in disappearance of the labeled DNA_{int} and DNA_{HH} fractions.

The appearance of DNA_{HH} soon after the start of incubation is difficult to explain since bifilarly substituted DNA should appear only after two rounds of replication. The resistance of DNA_{HH} to single strand specific endonuclease (2), its sensitivity to pancreatic deoxyribonuclease and the frequency with which the material has been reported makes it unlikely that its appearance is due to an artefact of centrifugation. Any explanation which depends on several rounds of replication, e.g. the replication of mitochondrial molecules, requires a special explanation to account for the greater proportion of DNA_{HH} observed as DNA synthesis is inhibited and also fails to account for the disappearance of the DNA_{HH} fraction when cultures are incubated further with non-radioactive BrdUrd.

Doubly substituted DNA is produced as a result of sister strand exchanges between hybrid DNA molecules (3). The proportion of such molecules produced is very low in contrast to the relative amounts of DNA_{HH} routinely obtained and bifilarly substituted molecules obtained by recombination are stable and do not disappear on further incubation. It is therefore unlikely that recombination accounts for the great majority of DNA_{HH} observed. A more likely model to account for the production of DNA_{HH} suggests that reassociation of parental strands by displacement of newly synthesized (BrdUrd labeled) DNA (branch migration) permits pairing of the two daughter strands to produce bifilarly substituted DNA. The model as a possible mechanism for replication repair presupposes that DNA_{HH} originates from DNA_{int} and the demonstration of four pronged structures by electron microscopy of the DNA_{int} fraction was therefore taken as evidence in its favor.

The model for the production of bifilarly substituted DNA by branch migration and strand displacement was based on the assumption that the isolation of DNA_{HH} mirrored in vivo events. However, the very high proportion of DNA_{HH} obtained in the experiments of Higgins et al. (2) compared to the very low proportion obtained when DNA_{HH} was generated by recombination (3) suggested that most DNA_{HH} could have come from in vitro events.

Psoralen and its derivatives are known to intercalate into the DNA structure and form covalent cross-links between the two strands on irradiation.

tion with low doses of near ultraviolet light (4, 5). Such cross-links make DNA renature rapidly after denaturation with heat or treatment at high pH and therefore protect molecules so treated from the degradative action of single-strand specific nucleases. Since cross-linking can take place in vivo as well as in solution (5), it should be possible to detect DNA_{HH} within living cells by the addition of psoralen and irradiation before lysis and extraction of DNA. Any DNA_{HH} existing in vivo and not protected by nucleosomes (6) should be detectable by resistance to single-strand specific nuclease after treatment with heat or high pH. On the other hand, the introduction of cross-links into the DNA structure should inhibit any branch migration subsequent to cross-linking and prevent the formation of new DNA_{HH} structures in vitro. In this paper we report our experiments using the cross-linking technique to determine if branch migration can be detected in vivo.

MATERIALS AND METHODS

Cell Culture

Cell line XPA-3, a human lymphoblastoid line derived from a complementation group C xeroderma pigmentosum patient (7) was used throughout this study. The cells were maintained in RPMI 1640 medium containing 17 per cent fetal calf serum, penicillin and streptomycin (GIBCO, Grand Island, N.Y.). Commercial testing (Microbiological Associates, Walkersville, Md.) for mycoplasma was negative during our study. Cell counts were made with a Coulter counter (threshold 10 μ m, amplification, 0.25; aperture 1).

Chemicals

N-acetoxy-N-acetyl-2-aminofluorene (AAAF) generously supplied by Dr. J. A. Miller was dissolved in sterile dimethyl sulfoxide (DMSO) before use. Trioxsalen (4, 5', 8-trimethylpsoralen) was donated by Paul B. Elder Co. (Bryan, Ohio) and was dissolved in ethanol to give a solution of 250 μ g/ml.

Treatment and Labelling

Rapidly growing cultures ($5 \times 10^5 - 10^6$ cells/ml) were incubated for 2 hr with 33 μ M BrdUrd and 1 μ MFdUrd, treated for 30 min with 36.8 μ M AAAF in the presence of BrdUrd and labeled for 45 minutes by the addition of dT (methyl-³H; 30 μ Ci/ml; 66 Ci/mmol). Thymidine incorporation was stopped by the addition of hydroxyurea (10 mM) and immersion of the flasks

in an ice bath. Cells were washed in phosphate buffered saline (PBS) (8) containing 10 mM hydroxyurea.

Cross-linking DNA

Cells suspended in 10 ml PBS containing 10 mM hydroxyurea and with 5 µg/ml of Trioxsalen were transferred into Falcon Petri dishes (8.7 cm) and irradiated with a Hanovia Type 3600 fluorescent lamp (peak 365 nm) for 20 min at 4° (5). A Corning #7-51 filter was used to filter out wavelengths below 340 nm. The incident light intensity was 2 mW/cm² determined with a YSI Model 65 Radiometer (Yellow Spring Instruments Co., Yellow Spring, Ohio) at a distance of 7 cm. Cell lysates were cross-linked and irradiated for 20 min before or after enzyme digestion.

DNA Extraction

Cells were harvested and washed twice in PBS containing 10 mM hydroxyurea, washed once in 0.15 M NaCl + 0.015 M sodium citrate (SSC) and resuspended in SSC to a density of 10⁷ cells/ml. Sodium dodecyl sulfate (SDS) was added at room temperature to a concentration of 0.2 per cent and the cells were lysed by three freeze-thaw cycles. DNA was prepared by RNase and Pronase treatment, phenol extraction and dialysis as previously described (8).

Equilibrium Centrifugation

DNA samples in SSC were sheared either by three passages through a 22 gauge needle to give a M_n of 1.5×10^7 daltons or by 10 passages through a 26 gauge needle to give a M_n of 5.2×10^6 daltons. Samples sheared 10 times were also treated with N. crassa endonuclease (see below). We centrifuged samples in a CsCl gradient for 48-72 hours at 30,000 rpm in the SW 50.1 rotor of a Beckman preparative ultracentrifuge at 20°. Gradients were collected and the radioactivity and absorbancy of fractions was determined as previously described (8).

Endonuclease Treatment

DNA preparations and cell lysates were analyzed for single-stranded DNA regions by treatment with single-strand specific endonuclease. We used either the S1 nuclease from Aspergillus oryzae (9) or Neurospora crassa endonuclease (10). Both enzymes were obtained from Miles Laboratories. DNA

samples were dialyzed against the appropriate buffer prior to incubation with enzyme. Reaction mixtures for S1 assay consisted of 100 units S1 nuclease/ μg DNA in 40 mM acetate buffer (pH 4.6) containing 4.8 mM ZnSO_4 . Samples were incubated 30 min at 37°. DNA was incubated with Neurospora crassa endonuclease (1 unit endonuclease/20 μg DNA) in 60 mM Tris-HCl buffer (pH 8) containing 6.25 mM MgCl_2 and 0.1 N NaCl at 37° for 60 min.

Electron Microscopy

DNA was spread according to the aqueous technique of Davis et al. (11). Grids were stained with uranyl acetate and shadowed and viewed as previously described (2).

RESULTS

We used the XPA-3 line because it is deficient in excision repair (12) and therefore lesions produced by treatment with chemical carcinogens such as AAF are likely to remain in the DNA. Treatment of XPA-3 cells with AAF followed by labelling with ^3H -dT for 45 min in the presence of BrdUrd and FdUrd resulted in the incorporation of radioactivity into DNA molecules of three different densities (Fig. 1-A): a major peak characteristic of hybrid DNA ($\rho=1.75 \text{ g/cm}^3$), a second peak of intermediate density (DNA_{int} $\rho \approx 1.71 \text{ g/cm}^3$) which includes replicating molecules with single-stranded regions (1) and some repair synthesis, and a third peak banding at 1.80 g/cm^3 characteristic of duplex molecules substituted in both strands with BrdUrd (DNA_{HH}). DNA_{HH} and DNA_{int} were also observed after lysis with the non-ionic detergents Sarkosyl and Triton X 100.

It had previously been shown that treatment of DNA_{int} with E. coli exonuclease I for 1 hr resulted in partial transfer of radioactivity from DNA_{int} into hybrid DNA (DNA_{HL}) (1). We confirmed this observation with single-strand specific nucleases from Aspergillus oryzae (S1) and from Neurospora crassa. Isolated DNA_{int} (Fig. 2-A) after extensive shear (Fig. 2-B) or after treatment with Neurospora endonuclease (Fig. 2-C) yielded both DNA_{HL} and DNA_{HH} . Of the activity lost from the DNA_{int} region, approximately 87 per cent was recovered in DNA_{HL} and 13 per cent in DNA_{HH} (Fig. 2) indicating that DNA_{HH} can originate from DNA_{int} as required by the model of Higgins et al. (2). The combined use of mechanical shear and N. crassa endonuclease digestion resulted in extensive degradation of DNA_{int} and maximized the yield of DNA_{HH} (Table 1). This procedure eliminated the contribution of any single-stranded, analog-substituted DNA ($\rho=1.85 \text{ g/cm}^3$)

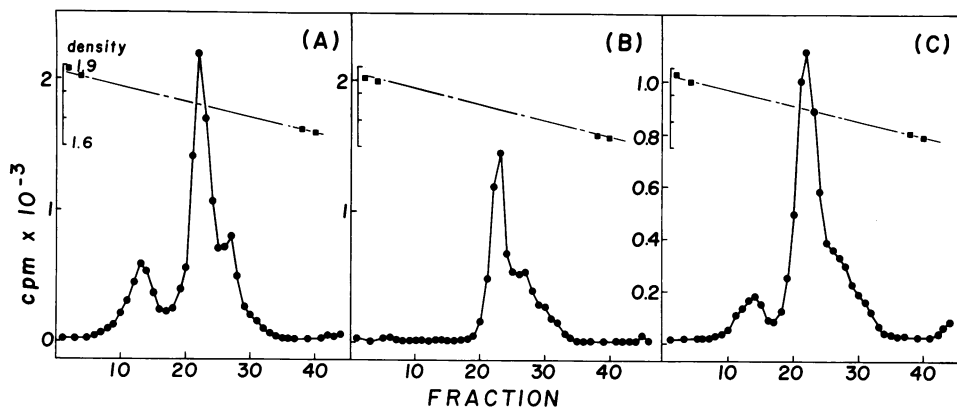


Fig. 1. Neutral CsCl gradients of XPA-3 cell DNA synthesized in the presence of BrdUrd and FdUrd after treatment with AAF. Cells were preincubated with BrdUrd ($33 \mu\text{M}$) and FdUrd ($1 \mu\text{M}$) for 2 hr, treated with AAF ($36.8 \mu\text{M}$) for 30 min (5 per cent residual DNA synthesis) and then labelled for 45 min with ^3HdT (66 Ci/mmol ; $30 \mu\text{Ci/ml}$) in the presence of BrdUrd and FdUrd. Following the labelling, the cell suspension in PBS with 10 mM HU or the cell lysates in 0.2% SDS-SSC were treated with trioxsalen ($5 \mu\text{g/ml}$) and near UV light at 2 mW/cm^2 for 20 min. Prior to centrifugation phenol-extracted DNA was sheared by three passages through a 22 gauge needle. Samples were centrifuged for 72 hrs in CsCl solution as described in Materials and Methods. 8-drop fractions were collected and acid-insoluble radioactivity of individual fractions was measured. Total counts recovered in each profile were (A) without cross-linking; 13,811 cpm (B) cross-linking in vivo; 14,018 cpm (C) cross-linking in vitro after RNase and Pronase treatment; 7,208 cpm.

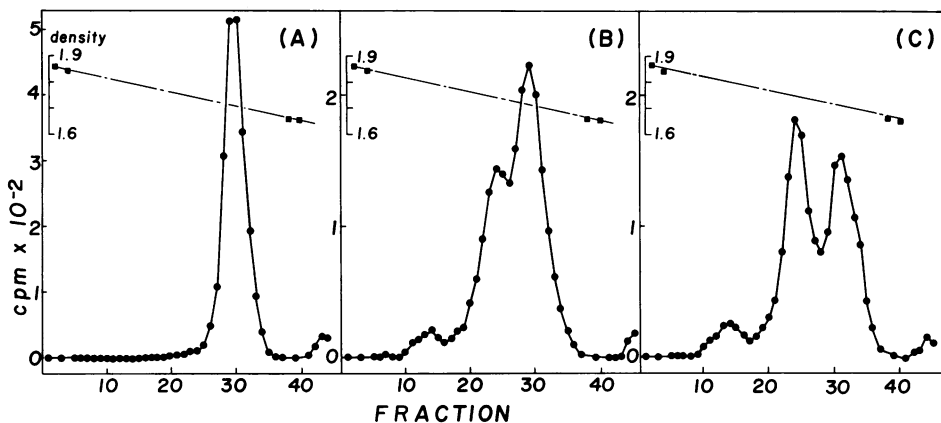


Fig. 2. Transfer of radioactivity from DNA_{int} to DNA_{HH} and DNA_{HL} . DNA_{int} was isolated by rebanding pooled fractions with a density of $1.69\text{--}1.73 \text{ g/cm}^3$ with a peak at 1.71 g/cm^3 from 3 neutral CsCl gradients similar to Fig. 1-A. After dialysis against SSC, DNA_{int} was either sheared by 10 passages through a 26 gauge needle, or reacted with *N. crassa* endonuclease (1 unit/20 μg DNA) for 60 min, and then recentrifuged in neutral CsCl. Total counts in each profile were (A) no treatment; 2,312 cpm (B) sheared 10 passages through 26 gauge needle; 2,068 cpm (C) digested with *N. crassa* endonuclease; 1,936 cpm.

Table 1. Effect of shearing and *N. crassa* endonuclease digestion on the proportion of DNA_{int} and DNA_{HH}

Treatment	Cross-linking	Total cpm	HH %	HL %	int %
A. 3 times through 22 g needle	none	13,811	22	59	19
	<u>in vivo</u>	14,018	0.3	64	34
	<u>in vitro</u>	7,208	11	65	22
B. 10 times through 26 g needle and <i>N. crassa</i> endo- nuclease diges- tion	none	9,742	30	65	4
	<u>in vivo</u>	15,203	0.3	92	6
	<u>in vitro</u>	7,501	18	73	7

Digestion and shearing were carried out as described in Materials and Methods.

which might have contaminated the preparation permitting a more accurate estimate of the yield of DNA_{HH}. Incubation of extracted DNA with *N. crassa* endonuclease caused about 10 per cent solubilization of radioactivity. Although we have observed the complete disappearance of DNA_{int} in some preparations after shearing and treatment with nuclease, results similar to those of Fig. 2, with DNA_{int} remaining, were associated with the highest yields of DNA_{HH} (see Discussion).

Effect of BrdUrd Substitution on Photochemical Cross-linking of DNA

XPA-3 cells were treated with trioxsalen and near UV light after continuous labelling with dT-2-¹⁴C (0.1 µCi/ml; 45 mCi/mmol) for 96 hr in the presence of BrdUrd and FdUrd and also without analog for the production of labelled DNA_{LL}. The resulting labelled DNA_{HH}, DNA_{HL}, and DNA_{LL} were isolated by CsCl gradient centrifugation. The cross-linking procedure did not alter the density profiles. DNA samples in SSC were denatured either by heat at 98° for 10 min or by 10 min incubation at pH 13. At least 85 per cent of the radioactivity of all the cross-linked samples remained acid insoluble after denaturation and S1 nuclease treatment (Table 2). In contrast, less than 10 per cent of the non-cross-linked DNA remained acid-insoluble after similar treatment. DNA substituted in both strands with BrdUrd can therefore be cross-linked to a sufficient extent to make it renaturable and resistant to S1 action.

Table 2. Digestion with S1 nuclease of XPA-3 cell DNA after cross-linking of uniformly labelled cells with trioxsalen and near UV light

Sample	Denaturing condition	Non-cross-linked acid insoluble		Cross-linked acid insoluble	
		cpm	% digestion	cpm	% digestion
DNA _{HH}	---	2743	0	1441	0
	10 min at 98°	266	90	1288	12
	10 min at pH 13	309	90	1411	2
DNA _{HL}	---	1635	0	1058	0
	10 min at 98°	184	90	941	11
	10 min at pH 13	199	88	889	16
DNA _{LL}	---	7810	0	4084	0
	10 min at 98°	416	95	3728	9
	10 min at pH 13	576	93	3872	5

DNA_{HH}, HL, and LL were prepared from cells incubated for long periods with isotope as described in the text. Samples were treated with S1 nuclease as described in Materials and Methods.

Effect of Cross-linking on DNA_{HH} Formation

If DNA_{HH} is formed in appreciable quantities *in vivo*, then cross-linking cells before lysis should result in a detectable fraction of renaturable, S1 resistant, rapidly labelled DNA_{HH}. XPA-3 cells were treated with AAAF, labelled with ³H-dT for 45 min in the presence of BrdUrd and FdUrd as described above, treated with Trioxsalen and near UV light, and then lysed. No DNA_{HH} peak was observed in CsCl gradients of DNA prepared from these cells (Fig. 1-B). DNA_{HH} was not detected even when the sample was extensively sheared and reacted with *N. crassa* endonuclease before centrifugation (Fig. 3). We conclude that the DNA_{int} from cross-linked cells lacks a DNA_{HH} component as part of its structure. Treatment of an SDS lysate with trioxsalen and near UV light either before or after (Fig. 1-C) RNase and Pronase treatment did not greatly affect the recovery of DNA_{HH} (Table 1).

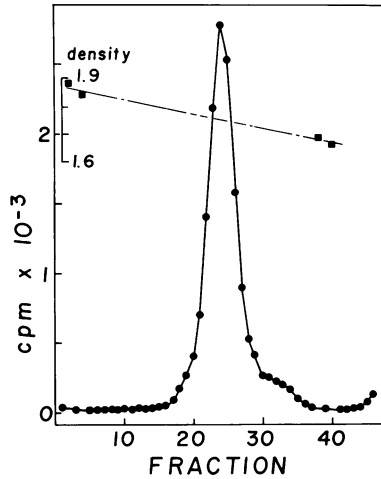


Fig. 3. Effect of a combination of mechanical shearing and *N. crassa* endonuclease digestion on XPA-3 cell DNA cross-linked *in vivo*. XPA-3 cells were incubated with BrdUrd and FdUrd, treated with AAAF, labelled with $^3\text{H-dT}$, and then treated with Trioxsalen and near UV light as described on the legend to Fig. 1. Prior to CsCl centrifugation, phenol-extracted DNA was sheared by 10 passages through a 26 gauge needle and then reacted with *N. crassa* endonuclease for 60 min. The total radioactivity recovered was 15,203 cpm.

We isolated pulse-labelled DNA from trioxsalen cross-linked cells and lysates and then added *Neurospora* endonuclease to heated ($98^\circ - 10 \text{ min}$) or unheated preparations (Table 3). *In vivo* cross-linking protected DNA_{int} from denaturation and extensive degradation by single-strand specific nuclease. Non-cross-linked DNA was hydrolyzed by enzyme digestion after denaturation as expected. Cross-links were therefore sufficiently frequent to protect the fragments of DNA_{HL} obtained in our extraction procedure (about 1.5×10^7 daltons) from denaturation. The DNA_{HH} observed in non-cross-linked samples was not resistant to enzyme after heating and quick cooling, indicating that this material did not originate from a highly repetitious fraction.

Effect of Salt Concentration and of Caffeine

Branch migration increases at temperatures near the T_m (13, 14); increased salt concentration should therefore inhibit the process by increasing the denaturation temperature. Since it has also been demonstrated that caffeine slows the chase of label from DNA_{HH} (15, fig.6) and might therefore stabilize any present, we decided to study the effect of salt concentration during

Table 3. Effect of heating at 98° for 10 min and quick cooling on the yield of pulse-labelled DNA_{HH} and DNA_{HL} resistant to single-strand specific endonuclease

	Cross-linking	Proportion of acid insoluble radioactivity after <i>N. crassa</i> endonuclease digestion (%)	Total acid insoluble radioactivity cpm	Distribution of <i>N. crassa</i> endonuclease resistant radioactivity	
				HH (%)	HL (%)
A.	none	85	9742	30	65
No heat	<u>in vivo</u>	92	15,203	0.3	92
	<u>in vitro</u>	79	7501	18	73
B.	none	6	1293	27	46
Heat	<u>in vivo</u>	85	11,678	0.3	91
	<u>in vitro</u>	63	3946	12	75

DNA from pulse-labelled cells was treated as described in the text. The data in part A are from the same experiment as Table 1B.

extraction and of caffeine during incubation on the yield of DNA_{HH}. A large proportion of the total label was observed in DNA_{HH} when lysates of cells incubated with caffeine were extensively sheared: 28 per cent of the label was isolated as DNA_{HH} when cells were extracted in SSC (Fig. 4-B). Extraction at lower salt concentration resulted in the isolation of molecules which banded at the density of single-stranded analog-containing DNA as reported by Wanka et al. (13) as well as DNA_{HH}. Extraction at high salt concentration (10 X SSC; fig. 4-C) greatly reduced the proportion of DNA_{HH} observed.

We suspect that the different conditions of DNA preparation (no phenol, use of dialysis tubing not pretreated with unlabelled DNA) account for the lack of DNA_{int} in this particular experiment.

Cross-linking DNA in cells incubated with caffeine prior to lysis greatly reduced the yield of DNA_{HH}. DNA from caffeine-treated cells was cross-linked with trioxsalen and near UV light immediately prior to extraction (Fig. 5). In this experiment DNA from non-cross-linked cells had 17 per cent of the label present at the density of DNA_{HH}. In vivo cross-linking reduced this to no more than about 2 per cent of the total.

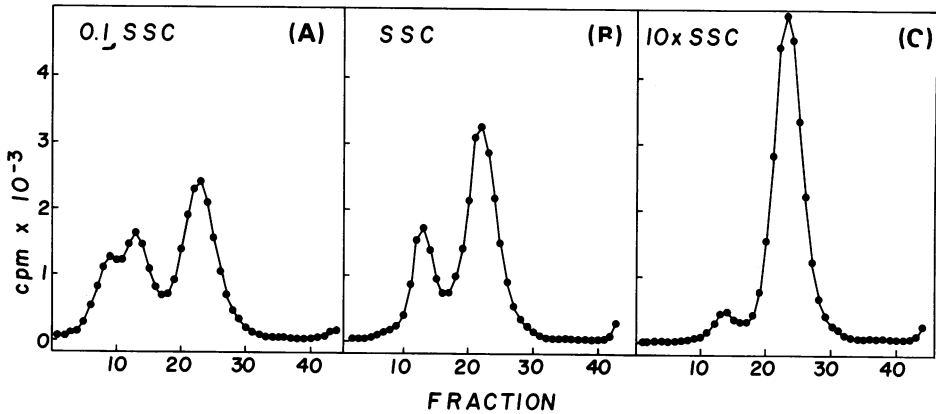


Fig. 4. Effect of salt concentration during cell lysis in 0.2% SDS on the yield of DNA_{HH} from XPA-3 cells treated with 10 mM caffeine. Cells were preincubated with BrdUrd (33 μM) and FdUrd (1 μM) for 2 hr, treated with caffeine (10 mM) for 30 min and then labelled for 20 min with $^3\text{H-dT}$ (66 Ci/mmol, 30 $\mu\text{Ci/ml}$) in the presence of BrdUrd, FdUrd and caffeine. Caffeine incubation reduced the rate of DNA synthesis to one half the control. After washing with PBS, the cells were lysed by the addition of 0.2% SDS at room temperature in either 0.1 SSC, SSC or 10 X SSC. Following RNase and Pronase treatment, the lysates were dialysed against 0.1 SSC, SSC, or 10 X SSC with 5 changes at room temperature and then all were dialysed against SSC with 3 more changes. The lysates were sheared 10 times through a 26 g needle and centrifuged through CsCl without prior phenol extraction. Total radioactivity in each gradient were (A) SDS lysis in 0.1 SSC; 29,313 cpm (B) SDS lysis in SSC; 28,332 cpm (C) SDS lysis in 10 X SSC; 31,126 cpm.

Effect of Cross-linking on Replication Structures in DNA_{int}

Examination of electron micrographs of fractions of intermediate density CsCl fractions has revealed the presence of 4-pronged replication structures in which one arm is substantially shorter than the other three (2). In vivo cross-linking resulted in an extensive reduction in the frequency with which such structures were observed (Table 4), but there was an increment in the frequency with which "Y" type structures were observed (Fig. 6; Table 4). This observation is compatible with the hypothesis that DNA_{int} , with cross-links formed in vivo, lacks an extensive DNA_{HH} component in its structure and also supports the hypothesis that the short fourth prong represents two newly displaced strands of DNA. The four "X" structures seen in the in vivo cross-linked DNA_{int} fraction (Table 4) might be thought of as critical and as indicating the true in vivo proportion of DNA_{HH} . While this interpretation remains possible, these four structures might also be a measure of the error of our procedure, e.g. by indicating

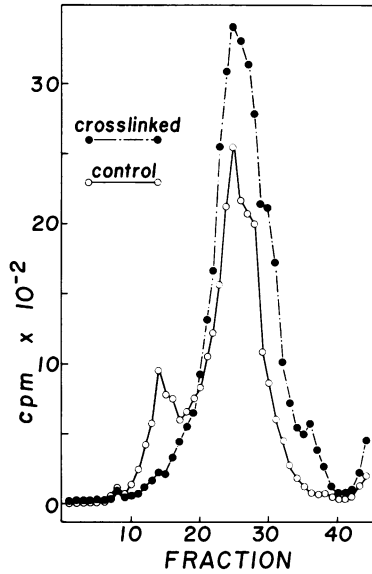


Fig. 5. In vivo cross-linking of DNA synthesized in XPA-3 cells treated with caffeine. Cells were preincubated with BrdUrd and FdUrd, treated with caffeine and labelled with $^3\text{H-dT}$ as described in the legend to Fig. 4. The cell suspension in PBS with 10 mM HU was treated with trioxsalen and near UV light. Prior to centrifugation, phenol-extracted DNA was sheared by 10 passages through a 26 gauge needle. Total acid insoluble counts loaded on each gradient were 35,242 cpm for in vivo cross-linked DNA (●—●) and 25,339 cpm for control DNA (○—○) from cells treated with caffeine but not cross-linked.

Table 4. X and Y structures observed by electron microscopy of DNA_{int} from XPA-3 cells treated with AAAF

	Total number of molecules scored	Type of molecule		
		linear	X	Y
non-cross-linked	1348	1293	38	17
cross-linked	1288	1246	4	38

the number of times a structure in which strands randomly crossed over was classified as an "X". The data indicate that at most, 10 per cent of the DNA_{HH} seen in vitro could have originated in vivo.

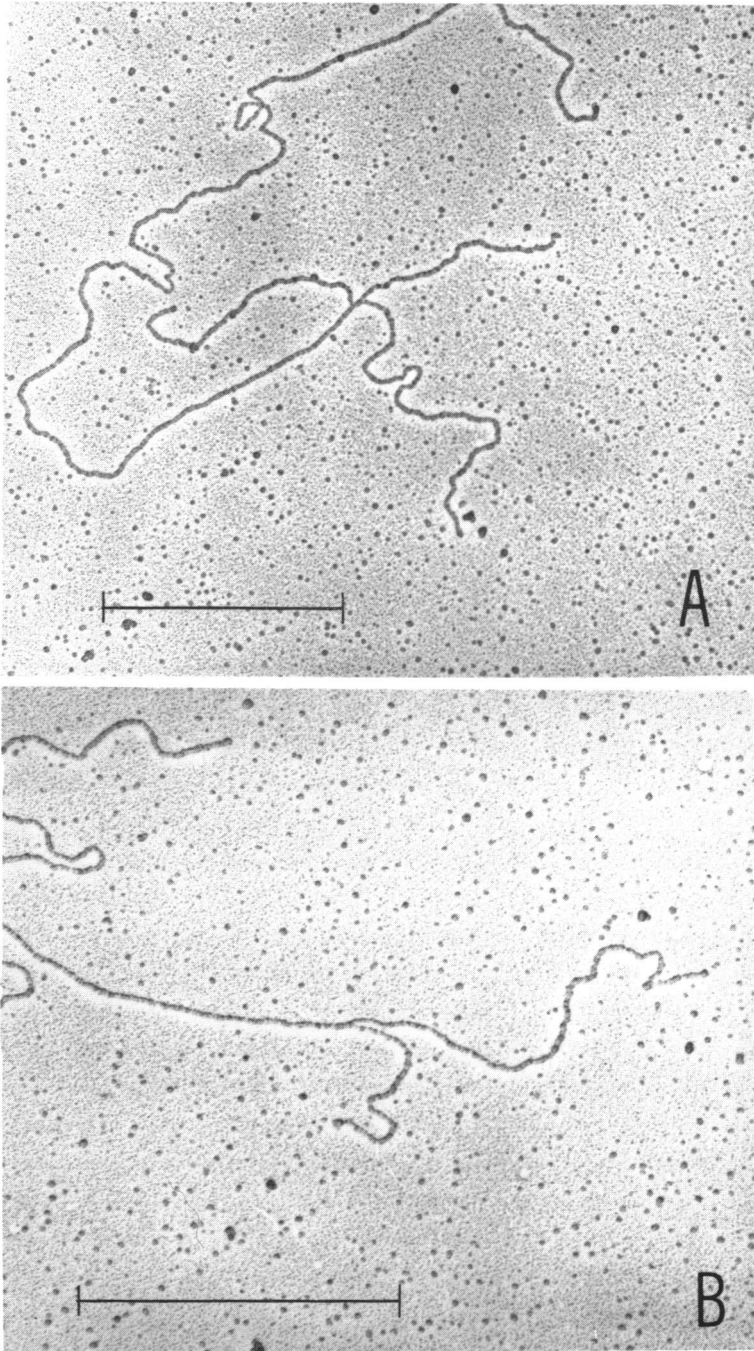


Fig. 6. Electron micrographs of DNA from the DNA_{int} fraction. DNA_{int} either non-cross-linked or cross-linked in vivo was isolated from XPA-3 cells as described in the legend to Fig. 2. Scale indicates 1 μ m. (A) No cross-linking; (B) extracted after cross-linking in vivo.

DISCUSSION

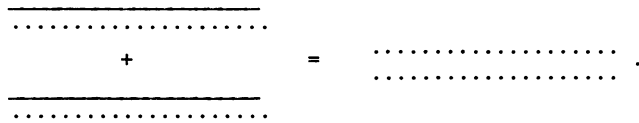
If all DNA_{HH} were formed in vivo, treatment of cells with trioxsalen and near UV light prior to extraction should have no effect on the yield and should protect DNA_{HH} molecules from the effects of *N. crassa* endonuclease action after heat denaturation. The fact is that in vivo cross-linking drastically reduced the radioactivity in the DNA_{HH} region. It might be argued that the cross-linking attaches DNA_{HH} to protein or some other cellular constituent converting it to a non-extractable form or decreasing its density. However, Psoralen derivatives are reported not to react with protein (6), the yield of DNA is approximately the same in cross-linked and non-cross-linked preparations, and the Pronase treatments routinely used should digest any protein attached to DNA molecules. Furthermore, in vivo cross-linking did not decrease the proportion of DNA_{int} observed in our experiments as determined both by density gradient experiments and by counts of "X" and "Y" forked structures in the electron micrographs, as might be expected if there were DNA-protein cross-linking at the replicating region.

We obtained heat-denaturation resistant DNA_{HL} molecules after in vivo cross-linking. We therefore conclude that cross-linking of hybrid DNA in growing point regions is an effective bar to branch migration and that there is little DNA_{HH} present in vivo. Our experiments do not exclude the formation of small amounts of DNA_{HH} in vivo, especially in the presence of caffeine (Fig. 4, 5). At the concentration used, caffeine inhibits DNA synthesis by 50 per cent and therefore cells incubated in its presence should accumulate DNA_{int}. It is not possible to know whether the small shoulder of Fig. 5 represents DNA_{HH} without rebanding. Furthermore, our experiments cannot exclude the production of stretches of DNA_{HH} of less than about 160 base pairs. Treatment of mouse tissue culture cells with Psoralen and near UV light gives cross-links in DNA with a 160-200 base pair periodicity (6). Pieces shorter than this would not be cross-linked if they were protected by nucleosomes. DNA_{HH} fragments originate from DNA_{int} molecules by shear (Fig. 2) and it is possible that short DNA_{HH} stretches would not be as shear susceptible as the 1000 base pair pieces usually obtained (2) and therefore would not be observed separate from DNA_{int} in density experiments nor would short branch-migrated structures be observed by electron microscopy.

The data of Table 1 can be used to estimate the in vitro extent of branch migration assuming that the samples cross-linked in vivo indicate the

unmodified level of DNA_{int}. Comparison of such samples, either cross-linked in vivo or not cross-linked, indicates that permitting branch migration to occur results in a mixture with equal proportions of label in DNA_{int} and DNA_{HH}. The data of Table 1 confirm the assumption that DNA_{HH} is formed at the expense of DNA_{int} and DNA_{HL}.

DNA_{HH} contains two BrdUrd labelled strands; we suppose the simplest DNA_{int} structure to contain only a portion of a strand substituted with analog (1). Since there are equal amounts of label in the two fractions (DNA_{int} and DNA_{HH}) at equilibrium (Table 1), we would expect equal total lengths of analog substituted DNA_{HH} and DNA_{int} strands, i.e.



But since DNA_{HH} arises from DNA_{int} (or at the expense of DNA_{HL} when samples are extensively sheared (Table 1-B)), then it seems likely that after in vitro branch migration about half of the DNA_{int} is found in the migrated configuration (Fig. 7-A) rather than the simplest form which appears most prevalent in vivo (Fig. 7-B). This conclusion helps to account for the observation (Fig. 2-C; 11) that not all DNA_{int} need be nuclease susceptible since the configuration of Fig. 7-A contains no single-stranded regions whose loss would radically alter the density of the fragment. We think that this extensive branch migration must reflect the increase in stability of helices containing BrdUrd. Halogen analog substituted DNA has an increased denaturation temperature indicating increased stability (16). Since branch migration is more extensive at temperatures near the T_m, DNA_{HH} struc-

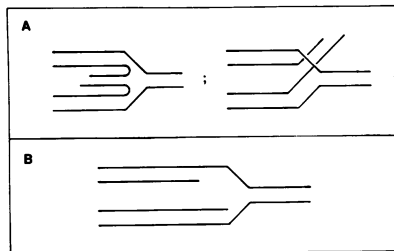


Fig. 7. (A) DNA structures after branch migration (B) A structure of DNA_{int}. Daughter strands in both A and B are partially substituted with BrdUrd.

tures once formed would be "trapped" in that configuration even as compared to the increased stability of DNA_{HL} over DNA_{LL}.

DNA_{int} must actually be considered as a population of molecules of the same density but with structural variations. For example, we have shown that nuclease treatment of DNA_{int} can liberate DNA_{HH} fragments (Fig. 2-C). We can account for the production of a nuclease sensitive structure based on strand displacement of a replicating fork including Okazaki fragments, doubtless there are other possibilities. We consider all these variations as derived from the simplest DNA_{int} type structure (Fig. 7-B).

Our experiments confirm the conclusion that most DNA_{HH} is formed by branch migration in vitro following cell lysis. At least 90 per cent must be formed at this time (Fig. 5; Table 4) and there is as yet no unequivocal evidence for true in vivo formation of DNA_{HH} structure. Our experiments do show that branch migration can occur extensively and they also demonstrate that the cross-linking of DNA is, as expected, an effective bar to branch migration. Since the proportion of DNA_{HH} is not much different if the cross-linking procedure is applied after rather than before RNase and Pronase treatment, we assume that most of DNA_{HH} formation occurs by branch migration during cell lysis. This interpretation is supported by the observation that variation of salt concentration during lysis (Fig. 4) produces major changes in the yield of DNA_{HH}. We suppose that the addition of detergent or the dilution attendant on cell lysis, disrupts and/or removes protective protein from the single-stranded DNA regions at growing points, thereby permitting branch migration to occur. Branch migration apparently does not occur to an appreciable extent in the later stages of extraction after RNase and Pronase treatment since dialysis of sample extracted in 10 X SSC against SSC did not produce large amounts of DNA_{HH} (Fig. 4). These results also indicate that once formed, halogen substituted DNA_{HH} structures are relative stable.

Our study is another demonstration of the usefulness of the technique of cross-linking with trioxsalen and near UV light for the analysis of DNA structure in vivo. Recently this technique was used to determine the in vivo configuration of palindromes in the genome of mouse tissue culture cells (17) and to determine the proportion of DNA in nucleosomes (6). The treatment of cells with trioxsalen and near UV light prior to extraction results in DNA molecules which when isolated are likely to have the structure existing within the cell.

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REFERENCES

1. Kato, K. and Strauss, B. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1969-1973.
2. Higgins, N.P., Kato, K. and Strauss, B. (1976) J. Mol. Biol. 101,417-425.
3. Moore, P.D. and Holliday, R. (1976) Cell 8,573-579.
4. Cole, R. (1970) Biochim. Biophys. Acta 217,30-39.
5. Hanson, C., Shen, C-K. and Hearst, V. (1976) Science 193,62-64.
6. Cech, T. and Pardue, M. (1977) Cell 11,631-640.
7. Andrews, A., Robbins, J., Kraemer, K. and Buell, D. (1974) J. Natl. Cancer Inst. 53,691-693.
8. Coyle, M., McMahon, M. and Strauss, B. (1971) Mutation Res. 12,427-440.
9. Vogt, V. (1973) Eur. J. Biochem. 33,192-200.
10. Linn, S. and Lehmann, I.R. (1965) J. Biol. Chem. 240,1287-1293.
11. Davis, R., Simon, M. and Davison, N. (1971) in Methods in Enzymology. Vol. 21. pp. 413-428. Academic Press, New York.
12. Scudiero, D., Henderson, E., Norin, A. and Strauss, B. (1975) Mutation Res. 29,473-488.
13. Wanka, F., Broans, R., Aelen, J. and Eygensteyn, J. (1977) Nucleic Acids Research 4,2083-2097.
14. Thompson, B., Camien, M. and Warner, R. (1976) Proc. Natl. Acad. Sci. U.S. 73,2299-2303.
15. Fujiwara, Y. and Tatsumi, M. (1976) Mutation Res. 37,91-110.
16. Cullen, B. and Bick, M. (1976) Nucleic Acids Research 3,49-62.
17. Cech, T. and Pardue, M. (1976) Proc. Natl. Acad. Sci. U.S. 73,2644-2648.