Volume 6 Number 1 January 1979 Nucleic Acids Research

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

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Received 28 November 1978

ABSTRACT

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

INTRODUCTION

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

Replicating DNA seemed to be attached to the nuclear protein component by additional sites close to the replication fork (1). The experiments reported here were designed in order to obtain a more detailed insight into the mode of binding of the replicating DNA molecules. Lysates of nuclei labelled in vivo under various conditions were digested with different DNA degrading enzymes and the degradation kinetics of pulse labelled and continuously labelled DNA were compared.

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MATERIALS AND METHODS

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

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

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

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

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

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

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

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

RESULTS

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

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

This finding was confirmed by another experiment carried out after labelling DNA for 1 hour in the presence of 100 µM arabinosylcytosine. This analogue has been shown to cause a strong reduction of the rate of DNA chain growth (7). Thus, in the presence of arabinosylcytosine only a limited region afjacent to the replication fork becomes labelled during one hour incorporation of radioactive thymidine. The results of the DNase digestion of the

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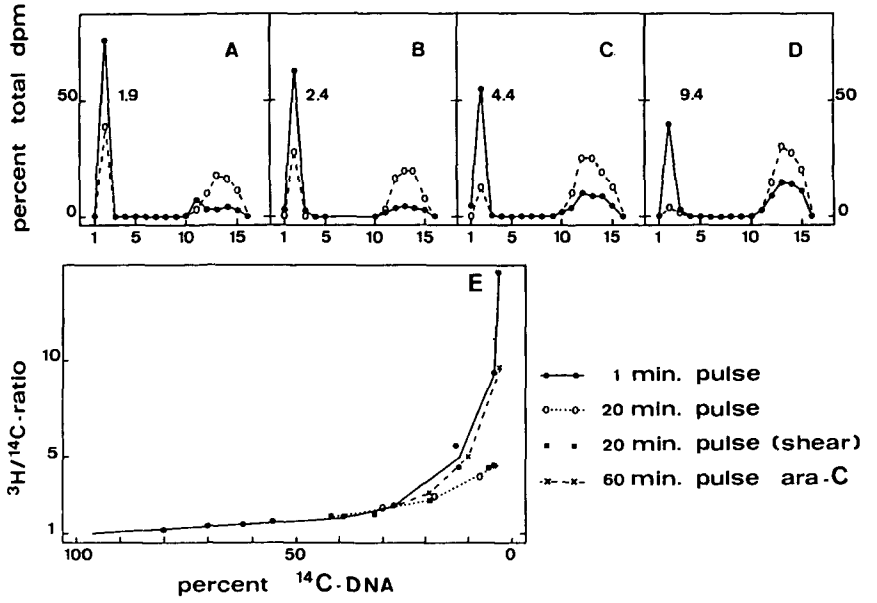

Figure 1. Effect of DNase I digestion on the label distribution in nuclear lysates.
A nuclear lysate was prepared from cells pulse labelled for 1 min with ^3H-dThd. The lysate was divided into 4 portions and DNase I was added to final concentrations of 0 (A), 0.3 (B), 0.6 (C) and 1 (D) µg/ml. After incubation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

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

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

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nuclease S_1. Fig. 2A-D shows that after a 1 min pulse about 30% of the pulse label was removed by enzyme concentrations which did not significantly affect the amount of bound pre-labelled DNA. The almost exclusive release of pulse label at low enzyme concentrations is also indicated by the abrupt decrease of the $^3H/^{14}C$ ratio of the complex (fig. 2E). Similar results were obtained using cells pulse labelled for 1 hour in the presence of arabinosylcytosine. The decrease in the ratio was only slightly greater when high enzyme concentrations were used which also released marked amounts of pre-labelled DNA. Apparently, only a limited portion of the most recently synthesized DNA is removed from the complex by the action of the single strand specific enzyme activity. The pulse label released after a 20 min pulse was only 10% or even less (fig. 2E).

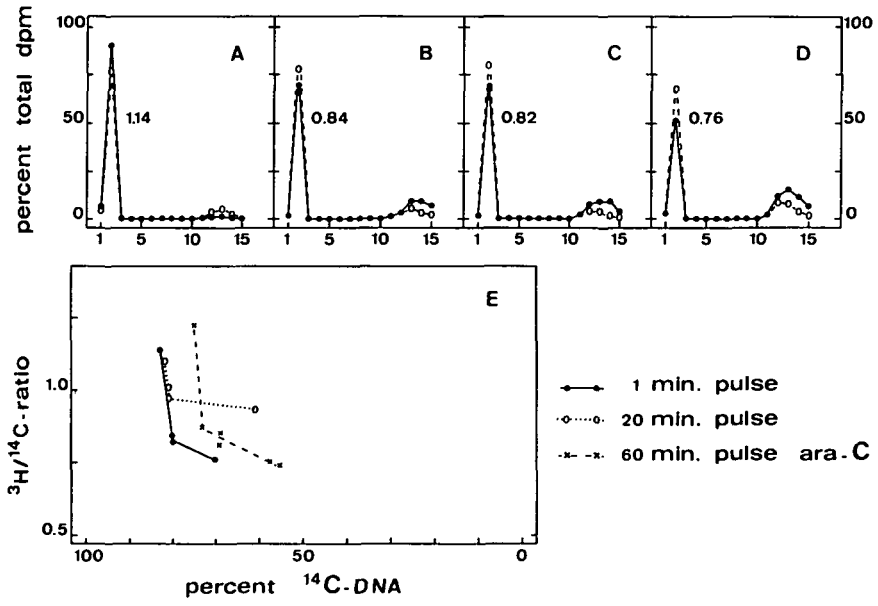

Figure 2. Effect of nuclease S_1 digestion on the label distribution in nuclear lysates.
Portions of a nuclear lysate prepared as described in fig. 1, were digested with 0 (A), 1 (B), 3 (C) and 10 (D) μg/ml nuclease S_1. For further details see fig. 1. The average total dpm per gradient were 12 000 for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

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

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

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

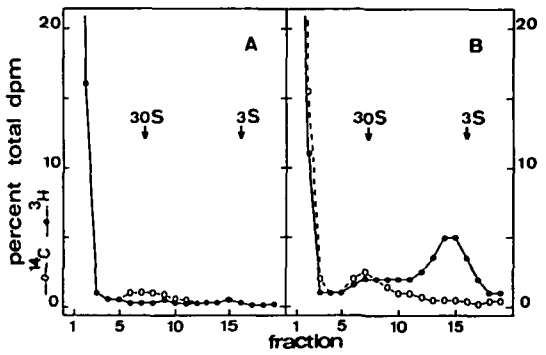

Figure 3. Sedimentation analysis of the pulse labelled DNA fragments released by nuclease S_1 digestion.
A nuclear lysate was prepared as described and a portion of it was incubated with 3 µg/ml nuclease S_1. The sample (B) and an undigested control (A) were centrifuged for 17 hours at 24 000 rpm. The total dpm per gradient were:
A. ^{3}H: 7 050 dpm (78% in fraction 1); ^{14}C: 5 200 (73% in fraction 1).
B. ^{3}H: 9 050 dpm (52% in fraction 1); ^{14}C: 5 600 (67% in fraction 1).

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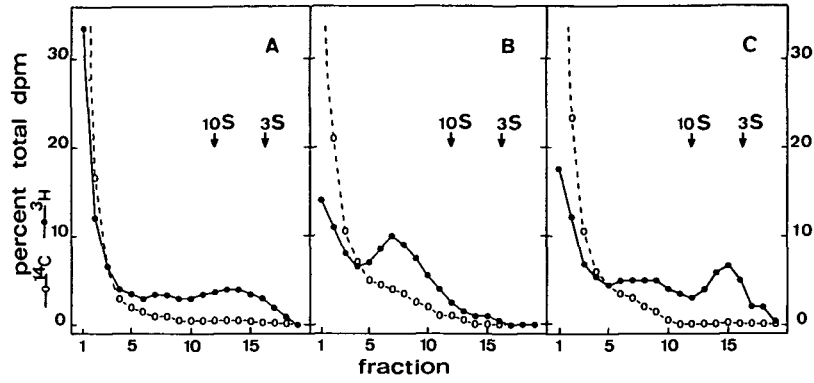

Figure 4. Analysis of the size distribution of pulse labelled DNA, present in the various nuclear lysates.
Nuclei, isolated from cells, pulse labelled for 1 or 20 minutes, or for 60 minutes in the presence of ara-C, were lysed in alkaline SDS. The SDS lysates were analysed in alkaline sucrose gradients.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

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

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

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

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

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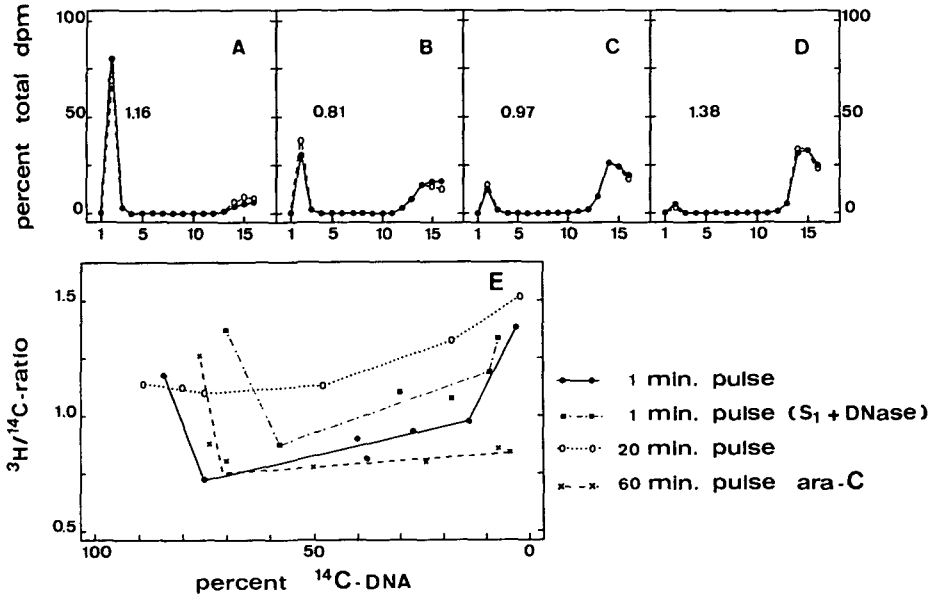

Figure 5. Effect of Staphylococcus nuclease digestion on the label distribution in nuclear lysates.
Portions of a nuclear lysate prepared as described in fig. 1 were digested with Staphylococcus nuclease at the following concentrations: 0 (A), 0.5 (B), 1.0 (C)and 2 µg/ml (D). For further details see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

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

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

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

DISCUSSION

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

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

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

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

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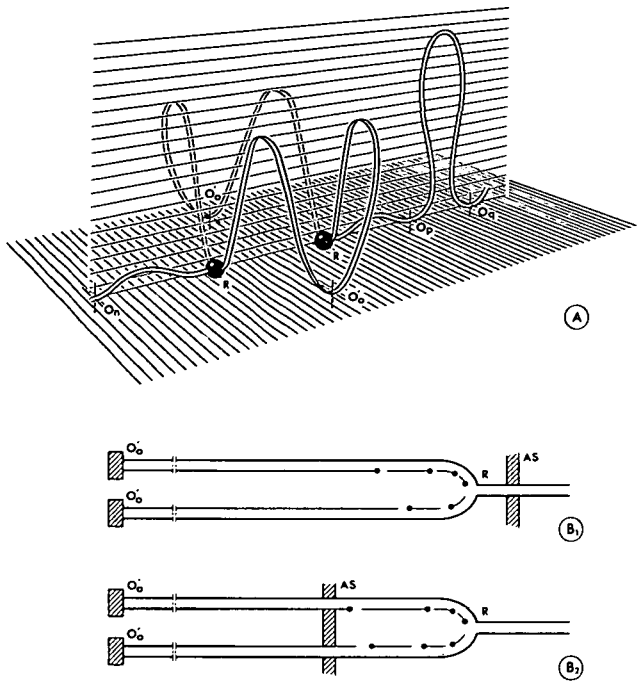

Figure 6. A: Model of the spatial organization of the DNA in the nucleus (according to Dingman). DNA is attached to the matrix at the origins of replication (O_n; O_o''; O_p and O_q). O_o' and O_o' represent replicated origins. Additional binding to the matrix takes place at the replication forks (R).
B: B_1 and B_2 represent two possible modes of attachment of the replication forks to the matrix. Our results are compatible with the occurrence of an attachment site (AS) behind the branch point.

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

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

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

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

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

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

ACKNOWLEDGEMENT

We thank Dr. K.R. Mitchelson for critical reading of the manuscript. This investigation was supported in part by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid of the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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