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The origin of nascent single-stranded DNA extracted from mammalian cells

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ABSTRACT

In vitro cultured bovine liver cells were labelled with radioactive thymidine and dissolved in 0.5% sodium dodecyl sulphate. Centrifugation of the lysate through sucrose gradients in a zonal rotor revealed a slowly sedimenting fraction of preferentially pulse labelled DNA. The DNA of this zone was further analysed by chromatography on hydroxy-apatite, banding in CsCl density gradients, and sedimentation in neutral and alkaline sucrose gradients. It contained, besides small amounts of fragmented bulk DNA, single-stranded nascent DNA and single-stranded pre-labelled DNA which could be separated from each other by using BrdU as a density label. The density labelling also revealed small amounts of nascent-nascent DNA duplexes. The slowly sedimenting fraction was practically absent from cell lysates which were prepared in 2 M NaCl - 50 µg/ml pronase. The results suggest that nascent single-strands and nascent-nascent duplexes are released from the forks of replicating DNA by branch migration. Pre-labelled single strands may be released by the same mechanism, but the *in vivo* structure from which they originate has yet to be elucidated.

INTRODUCTION

Knowledge of the physical state of the replicating DNA is an important facet necessary for a full understanding of cellular DNA synthesis. However, appropriate studies are hampered by more or less severe structural changes occurring during the isolation of replicating DNA from cells, depending on the extraction and purification procedures employed.

Centrifugation of newly synthesized DNA of prokaryotes (1) and eukaryotes (2) through alkaline sucrose gradients has revealed short single strands, indicating that the synthesis of new DNA chains proceeds discontinuously. Rapidly labelled DNA fragments with single strand properties have also been obtained under non-denaturing conditions (3 - 12). As they usually consists of short nascent chains, it has been suggested that the most recently

Abbreviations: SDS, sodium docecyl sulphate; ss, single-stranded; ds, double-stranded.

synthesized DNA strands might not yet be hydrogen bonded to the parental template and, therefore, might be released from the replication fork when inappropriate isolation procedures are used (7,9). In other studies (10,11) a DNA fraction was observed consisting of single-stranded parental DNA with short duplex regions formed through hydrogen bonding of complementary nascent polydeoxynucleotide chains. This material is supposedly derived from the replication fork as a result of unavoidable fragmentation during the extraction of the DNA.

In most of the previous studies the rapidly labelled DNA fragments were obtained either after deproteinization by phenol, or after chloroform extraction. Such ss DNA, however, can become artificially associated with proteins and then be lost during deproteinization (12). For this reason, experiments which include deproteinization by one of the usual phenol or chloroform extractions should be interpreted with caution. However, DNA with similar properties has also been obtained from mammalian cells by gentle lysis in detergent solution and centrifugation through sucrose gradients at neutral pH (13). Using a zonal rotor this method yields sufficient amounts of such intermediates to allow studies on their structural properties. In this investigation we attempted to gain a better insight into the structural changes that occur during the isolation of replicating DNA.

MATERIALS AND METHODS

Cell culture and labelling procedure. The experiments were carried out with an established line of bovine liver cells (14). The cells were grown in monolayer as described elsewhere (15), except that a serum concentration of 10% was used in the growth medium. The cellular DNA was pre-labelled by addition of 0.1 or 0.5 μC/ml 2-^{14}C-dT (NEN; sp act 52.8 mC/mmole) for 40 h (approx. 1.2 cell cycles). The cells were then grown for 1 h in label-free medium and, finally pulse labelled for 15 min by addition of 10 or 20 μC/ml Me-^{3}H-dT (NEN; sp act 20 C/mmole). for density labelling experiments BrdU was added for 20 min at a final concentration of 10 μM together with 10 μC/ml ^{3}H-BrdU (NEN; sp act 25.2 C/mmole).

Separation of the replicative intermediates by zonal centrifugation. The method used has already been described in detail (13). When larger quantities of lysates were needed, cells of 2 Roux bottles were dissolved in 325 ml of 0.5% SDS and the separation was suitably adapted: The lysates were centrifuged at 25 000 r.p.m. in a B-29 zonal rotor (I.E.C. model B-60

ultracentrifuge) for 15 hours at 15°C. The rotor content was composed of 150 ml 60% sucrose shelf, 790 ml linear volumetric 20 - 45% sucrose gradient, 325 ml sample and 125 ml overlay. All solutions were in 50 mM Tris-HCl buffer, pH 7.5. Fractions of 15 ml were collected after the run and samples of 1 ml were used for the analysis of the radioactivities.

Fractionation on hydroxyapatite. Selected fractions of the zonal runs were combined and digested for 30 min at 37°C with 50 µg/ml RNase A (Sigma). Pronase (Calbiochem, B grade) was then added at a final concentration of 50 µg/ml and the incubation was continued for another 30 min. Subsequently, 1 g hydroxyapatite (DNA grade Bio-Gel HTP), pre-washed and equilibrated in 50 mM phosphate buffer, pH 6.8 was added per 20 ml of sample and kept in suspension for 30 min by gentle stirring. The adsorbant was then poured into a suitable column and the DNA was eluted by a phosphate gradient (16) with a concentration increase of 0.5 M per 100 ml. Single-stranded (heat denatured) and calf thymus DNA were eluted at 0.19 and 0.26 M phosphate concentration respectively. Fractions of 2 ml were collected and samples of 0.2 ml were used for the determination of the radioactivities.

Sedimentation analysis in sucrose gradients. Centrifugations were carried out in a Spinco SW 40 rotor. 1 ml samples of the eluates from the hydroxyapatite column were layered on top of 12.5 ml of a 5 - 20% sucrose gradient. Neutral sucrose gradients were made in 0.3 M phosphate buffer, pH 6.8, and contained 0.9 M NaCl. Alkaline sucrose gradients contained 0.45 M NaOH/0.3 M phosphate, pH 12, and 0.9 M NaCl. After centrifugation for 8 hours at 40 000 r.p.m. and at 15°C, 0.5 ml fractions were collected and used to determine the radioactivity. Centrifugation coefficients were estimated according to McEwen (17). If the phosphate was omitted from the sucrose gradient the majority of the radioactively labelled DNA precipitated to the bottom of the tube. This is probably caused by binding to traces of hydroxyapaptite remaining in the eluate, as similar precipitation of DNA with calcium phosphate has been observed by Pirie (18).

Isopycnic centrifugation in CsCl density gradients. 200 µg of calf thymus DNA (Boehringer) was added to selected fractions of the hydroxyapatite eluates and precipitated by 2.5 vol 96% ethanol containing 1% K-acetate. After storing over night at 15°C the DNA was collected by centrifugation and re-dissolved in 20 mM Tris-HCl, pH 8. The sample volume was brought to 4.4 ml and 5.5 g CsCl were dissolved in it. Centrifugation according to Flamm et al.

(19) was performed with minor modifications (20) and the absorbancies of the fractions were measured at 260 nm.

Determination of the radioactivities. Samples of appropriate size were taken from the various fractions and processed as described previously (13). Radioactivities were counted in a Philips Liquid Scintillation Analyser.

RESULTS

Evidence of single-stranded DNA. Zonal centrifugation of the cell lysates in SDS yielded a slowly sedimenting DNA fraction (fig. 1; fractions 21 to 30). This fraction, which was previously nominated R1, showed partial single-stranded properties upon centrifugation in CsCl density gradients (13). The proportion of ^{3}H-pulse label in R1 was found to be between 10 and 17.5% of the total ^{3}H-labelled DNA after labelling for 15 min (5 experiments) and

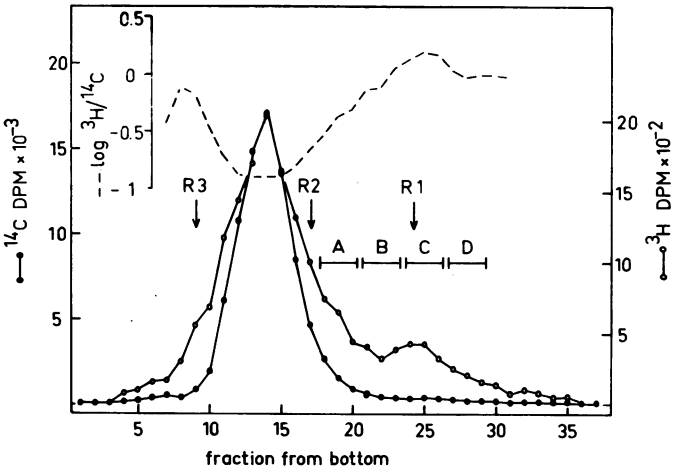

Fig. 1. Sedimentation profile of the DNA of a cell lysate in SDS. Cells were pre-labelled with 0.5 µC/ml ^{14}C-dT and pulse labelled for 15 min with 20 µC/ml ^{3}H-dT. The SDS lysate was centrifuged in a B-30 zonal rotor. R1, R2 and R3 indicate the zones containing replicative intermediates (12).

this proportion decreased to less than 2% when the labelling period was extended to 2 hours. We first aimed to verify the single-stranded nature of the pulse labelled DNA by chromatography on hydroxyapatite in the following way: Groups of 3 successive fractions designated A - D (fig. 1) were combined and the samples were successively digested with RNase and pronase.

Subsequently, the DNA was adsorbed to hydroxyapatite and chromatographed as described in the methods section.

Elution patterns of the samples A – D are shown in fig. 2. Sample A, which was collected from a zone mainly containing a ds intermediate nominated R2 (13), showed more than 95% of the newly incorporated ^{3}H-label eluting as ds DNA. In contrast, samples B, C and D contained large proportions of newly synthesized ss DNA which eluted at a concentration of 0.19 M phosphate. Fraction D also contained a considerable amount of ^{3}H-labelled DNA, which was eluted at lower phosphate concentrations.

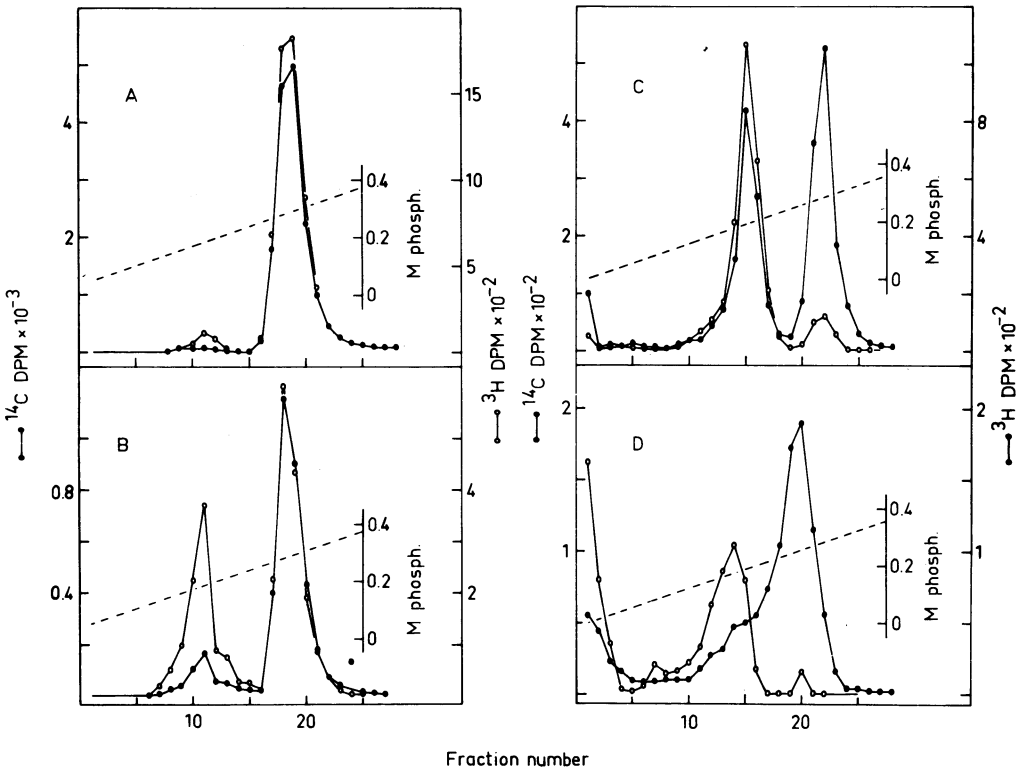

Fig. 2. Fractionation of the slowly sedimenting DNA on hydroxyapatite. Elution patterns A, B, C, and D are of the combined fractions of the corresponding zones indicated in fig. 1.

This is due to its small size (21) as indicated by the low rate of sedimentation in the original gradient (fig. 1).

While most of the ^{14}C-pre-label of R1 was present in ds DNA, a small but significant amount was generally associated with the ss DNA peak (fig. 2B and 2C). The ^{3}H / ^{14}C ratio of the ss DNA fraction was roughly 2.5. This means that, taking into account the higher label density in the ^{3}H-chains

resulting from the 40 fold higher concentrations of radioactivity applied as compared to the ^{14}C-activity, the amount of pre-labelled ss DNA was about 16 times that of the nascent chains. The proportions were variable, but the amounts of pre-labelled ss DNA in R1 were always several times greater than the amounts of pulse labelled DNA.

Zonal fractions 30 and above (fig. 1) usually contained small amounts of label which were not bound to hydroxyapatite. Therefore, a zone corresponding to fractions 21 to 28 of fig. 1 was routinely collected for the experiments described below. In these experiments between 36 and 66% of the pulse label was eluted from hydroxyapatite with the ss DNA peak.

The DNA which eluted at a concentration of 0.26 M phosphate is double-stranded. However, the elution position does not eliminate the possible presence of ss regions of considerable length, as these would not affect the binding to hydroxyapatite. On the other hand, these regions should cause a shift towards higher buoyant density in CsCl gradients. Therefore, ss and ds DNA previously separated by hydroxyapatite chromatography and a sample of bulk DNA corresponding to fraction 14 in fig. 1 were banded in CsCl. When compared to the peak of absorbance of calf thymus DNA only the ss fraction showed the buoyant density shift expected from its ss nature (fig. 3). The

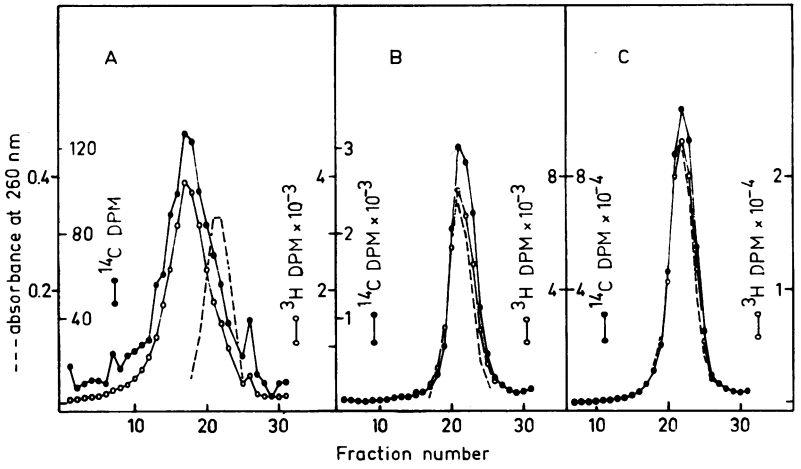

Fig. 3. Banding patterns in CsCl of ss and ds DNA fractions isolated from the slowly sedimenting material. Cells were pre-labelled with 0.1 μC/ml ^{14}C-dT and pulse labelled with 10 μC/ml ^{3}H-dT. The SDS cell lysate was centrifuged in a B-29 rotor and the slowly sedimenting DNA (see text) was fractionated on hydroxyapatite. Panels A and B represent buoyant density patterns of the ss and ds DNA fractions, respectively. Panel C represents the buoyant density of DNA from the top fraction of the original gradient (comparable to fraction 14 in fig. 1). Calf thymus DNA was present as an optical density marker (---). Other symbols are as in fig. 1.

broadening of this band is due to the low molecular weight of DNA fragments, but it may be partially due to a heterogeneity in base composition and/or secondary structure.

Our observations of the properties of ss DNA during hydroxyapatite chromatography and isopycnic banding do not eliminate conclusively a DNA structure in which very short duplexes might be formed by hydrogen bonding of ^{3}H-labelled nascent pieces to very long ss template chains. It has been shown that very short DNA duplexes are eluted from hydroxyapatite at much lower phosphate concentrations than normally expected (21), and thus the binding and elution of ss DNA with short duplex regions might be determined by the long ss sections. Also, the contribution of such very short duplex regions to the buoyant density in CsCl might not be sufficient to cause a recognizable shift of the band in the direction of ds DNA.
However, such a structure should be easily identifiable by the different rates of sedimentation of the short nascent and the long parental strands in alkaline sucrose gradients.

Neutral and alkaline sedimentation patterns of the ss DNA isolated from another preparation of R1 material are shown in fig. 4. The ^{3}H- and ^{14}C-radioactivities coincided closely under both conditions. This indicates that both labels were localized in chains which had a very similar distribution of sizes and excludes a structure in which short nascent strands might be hydrogen bonded to long parental strands.

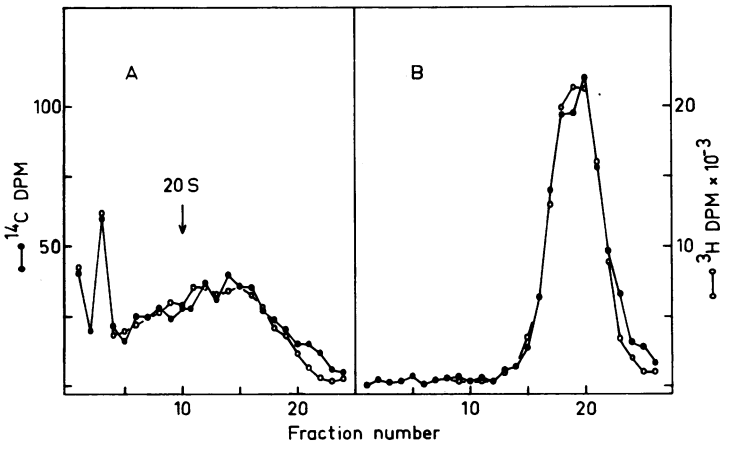

Fig. 4. Sedimentation of the ss DNA fraction in (A) neutral and (B) alkaline sucrose gradients. Sedimentation is from right to left. The ss DNA fraction was isolated by hydroxyapatite chromatography of the slowly sedimenting fraction of a SDS lysate. Labelling was as described in fig. 3.

The sedimentaticn coefficients of these chains in neutral gradients varied from virtually 0 to at least 30S with a majority of material having a sedimentation coefficient close to 20S. This is in agreement with the previous observation that ribosomal RNA sediments together with the R1 fraction (13). In addition, slight differences in the sedimentation distributions have been observed in various experiments owing to some unavoidable arbitrarines in the selection of samples from the R1 zone. The molecular weights estimated from these experiments ranged up to at least $2 \cdot 10^6$. There was no clear lower size limit.

Pre-labelled and pulse labelled DNA chains are of different origin. In view of the similarity of size distribution of the ^3H and ^{14}C labelled ss DNA it seemed possible that both labels are present in the same chains. Such a result would indicate repair synthesis. This possibility was studied by density labelling experiments as described in fig. 5. Comparison with calf thymus DNA shows that ^{14}C-label was present at the same ss position as in fig. 3 (fig. 5C), while the ^3H-band was displaced towards higher density by about 2/5ths of the gradient. For comparison the banding pattern of a ds DNA sample from the top of the original zonal gradient is shown in fig. 5A. The ^3H-label was displaced by less than 1/20th of the gradient towards higher

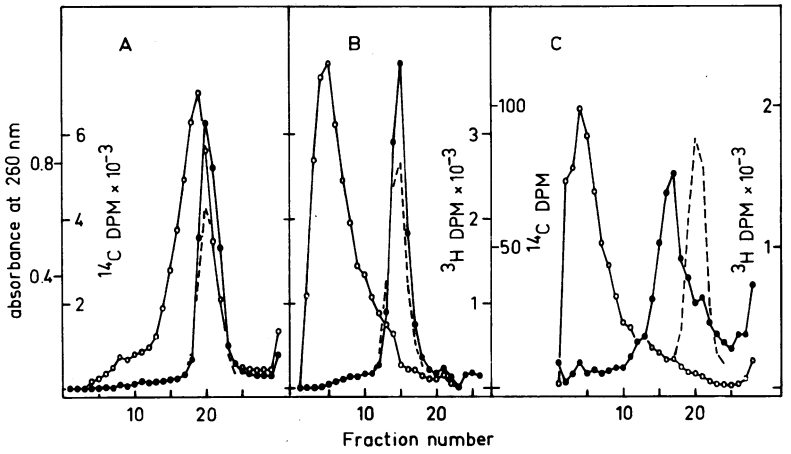

Fig. 5. Buoyant density profiles of DNA labelled with BrdU. Cells were pre-labelled with 0.1 μC/ml ^{14}C-dT and pulsed for 20 min with 10 μC/ml ^3H-BrdU at a BrdU concentration of 10 μM. The SDS lysate was centrifuged in a B-29 zonal rotor and the slowly sedimenting DNA was fractionated on hydroxyapatite. Samples A and B are equal parts of the top fraction of the original gradient (comparable to fraction 14 of fig. 1). Before centrifugation sample B was heat-denatured at 100°C for 5 min in 20 mM Tris-HCl buffer, pH 8. Sample C represents the ss DNA fraction collected from hydroxyapatite. Symbols are the same as in fig. 3.

density. After heat denaturation the newly synthesized strands of this sample were also separated from the pre-labelled DNA strands by about 2/5ths of the gradient (fig. 5B). This result shows clearly that ^{3}H and ^{14}C-label of the ss DNA of the R1 fraction are localized in separate molecules.

Occurence of nascent-nascent duplexes in the R1 fraction.

Isopycnic centrifugation of the BrdU labelled double-stranded fraction of R1 yielded 3 bands. The pre-labelled DNA band coincided with that of the calf thymus marker DNA. One pulse labelled band was shifted to higher buoyant density by about 1/5th of the gradient and a second one by 2/5ths (fig. 6A).

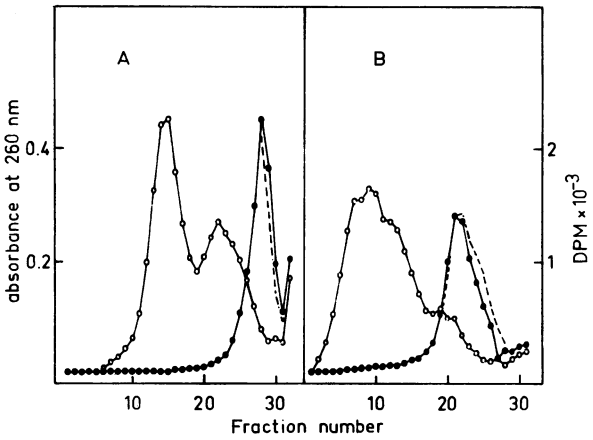

Fig. 6. Buoyant density profiles of the BrdU labelled ds fraction of the slowly sedimenting DNA. A and B are samples of the ds DNA from the same hydroxyapatite fractionation as shown in fig. 5C. Sample B was heat-denatured at 100°C for 5 min before centrifugation. Symbols are the same as in fig. 3.

As the large density shift is equal to that obtained with BrdU substituted single strands (fig. 5A) one can conclude that the high density band represents duplexes of two density labelled nascent strands, while the intermediate band represents density hybrids of low density parental and BrdU labelled nascent strands. This conclusion was confirmed by an experiment in which another part of the same ds material was heat-denatured prior to the density centrifugation. All pulse label banded about 2/5ths away from the pre-labelled and the calf thymus marker DNA, but no band of intermediate density was present (fig. 6B). It should be mentioned that the bands of BrdU substituted ss DNA, in particular those of heat-denatured DNA (figs. 5B and 6B), were skewed in the direction of lower buoyant density. This indicates the presence of DNA chains which have been synthesized during the time in which the intracellular BrdUTP concentration was increasing towards its final level,

and therefore, were less densely labelled. Accordingly we found that the symmetry of the bands was much improved when BrdU incorporation was continued for 4 hours (results not shown).

Unsubstituted duplexes and density hybrids are probably fragments of the bulk DNA. As shown in fig. 5A, partially substituted DNA obtained after a short pulse of BrdU exhibited only a minor density shift, but fragmentation of such material by shearing gives rise to short pieces of unsubstituted and full hybrid duplexes, as shown by Rommelaere et al. (22). The occurence of short nascent-nascent duplexes after a brief exposure to BrdU has not been reported previously. The possible origin of this DNA will be discussed below.

The relationship between double-helix stability and the release of nascent strands. The T_m of calf thymus DNA in the 0.5% SDS solution used in the preceding experiments was found to be 67°C. In 2 M NaCl the T_m should be higher than 100°C, owing to the stabilization of the double helix by NaCl (23). We have recently been able to prepare nuclear lysates in 2 M NaCl-pronase solution (24). It seemed reasonable, therefore, to investigate whether the high NaCl concentration would affect the release of the nascent strands from the replicating DNA.

A sedimentation pattern of a 2 M NaCl lysate which was extensively digested with pronase prior to centrifugation is shown in fig. 7. The pro-

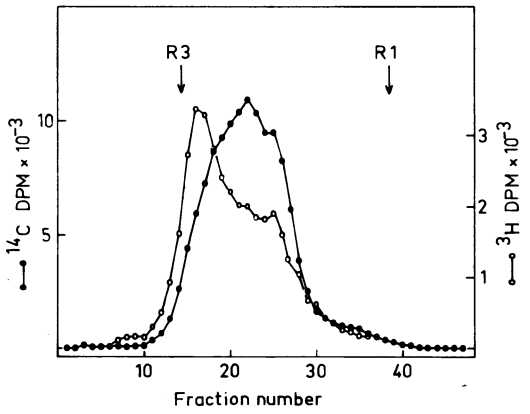

Fig. 7. Sedimentation profile of labelled DNA of a cell lysate prepared with NaCl-pronase. Cells were pre-labelled with 0.1 µC/ml ^{14}C-dT and pulsed with 10 µC/ml ^{3}H-dT. The cells were then homogenized in 50 mM Tris-HCl, pH 8, containing 0.1% Triton X100. The homogenate was brought to 150 ml and mixed with an equal volume of 4 M NaCl in the same detergent-buffer solution. Pronase was added to a final concentration of 50 µC/ml and the lysate was incubated for 2 h at 20°C. The centrifugation was performed in a B-29 rotor with a sucrose gradient containing 2 M NaCl. Fraction volumes were 30 ml.

portion of pulse label in the R1 zone was about 1.6%. This is 85 to 90% less than what was usually recovered from centrifugation performed with SDS lysates. Instead, a marked amount of pulse label remained associated with a high molecular weight DNA fraction which sediments slightly faster than the top of ^{14}C-pre-labelled bulk DNA. This zone corresponds fairly well with that of a replicating intermediate fraction called R3 (13). Apparently the release of ss nascent fragments, as well as nascent-nascent duplexes, from high molecular replicative DNA had been prevented by the stabilizing effect of the high NaCl concentration.

DISCUSSION

The results of this investigation confirm the previous observation that ss fragments (R1) are released from replicating DNA of mammalian cells when they are dissolved in 0.5% SDS solution at 37°C (13). Furthermore, the detailed analysis shows that at least the 5 following qualitatively different types of DNA can be recovered from the R1 zone:

1) Nascent single strands ranging from practically a few nucleotides up to about $2 \cdot 10^6$ dalton mol. weight,
2) pre-labelled single strands of a similar size range,
3) nascent-nascent DNA duplexes,
4) nascent-parental DNA duplexes, and
5) non-replicating pre-labelled DNA duplexes.

Parental ss DNA with short duplex regions formed by hydrogen bonding with nascent chains, as obtained when other isolation procedures are used (10,11), could not be detected in the slowly sedimenting fraction. Repair synthesis does not contribute to any significant extent to the labelling pattern, as pre-label and pulse label are localized in separate strands of DNA. The short duplexes of type 4 and 5 are most likely fragmentation products of pre-labelled bulk DNA that has been partly replicated during the limited pulse experiments with density label (22).

The origin of the 3 other types of low molecular weight ss and ds DNA have not been explained satisfactorily to date. Release of nascent single strands at neutral pH is not restricted to eukaryotic cells but has also been observed in bacteria(3,4) and phages (5). Habener et al. (7) and others (9) have suggested that the most recently synthesized DNA chains might occur in a destabilized state and, with certain extraction conditions might be converted to the ss form; but at the present time there is no experimental evidence for this view. Considering the stabilizing effect of high NaCl

concentrations on the replicating DNA, it seems possible that nascent and pre-labelled single strands as well as nascent-nascent duplexes may have been released as a result of branch migration.

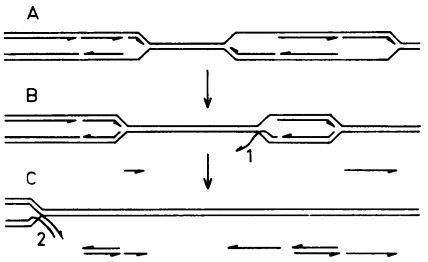

Fig. 8. Diagrammatic representation of the release of nascent DNA by branch migration. A represents part of a replicating DNA molecule based on models proposed by other authors (1,28,35). The same molecule at different extents of branch migration is represented by B and C together with the released nascent single strands and nascent-nascent duplexes. Growing 3'ends of the nascent chains are indicated by arrowheads. Single-stranded and ds whiskers are marked by 1 and 2, respectively.

Branch migration, which was first described by Lee et al. (25), is supposed to be involved in the formation of so-called whiskers which have been observed in electron micrographs of replicating DNA molecules (26,27,28). Such ss whiskers will be released from the fork as soon as branch migration has proceeded up to a gap, where successive Okazaki pieces (1,2) have not yet been sealed by ligase (fig. 8A - C).

Branch migration is enhanced at temperatures not too far below the melting point of DNA. This has been shown by either increasing the ambient temperature to T_m - 25 (27) or by changing to an ion concentration at which the T_m is markedly decreased (29). A similar relation has been observed for the release of nascent DNA in the present study. In 0.5% SDS (T_m = 67°C) where an ambient temperature of 37°C corresponds to T_m - 30°C, the release was much greater than in 2 M NaCl at 20°C (corresponding to T_m - 80°C) (23). This enhancement of chain release at submelting temperatures close to the T_m suggests that branch migration is caused by temporal local denaturations at the replication fork. Such transient conformational changes have also been shown to occurr over a temperature range from just below T_m to temperatures 30 to 40°C lower (30). Each time such a local denaturation takes place at the growing end of the nascent strand in the replication fork (fig. 9, step 1) there will be equal chances to either restore the original conformation by reannealing (step 1'), or to shift the fork backwards by annealing of the

parental strands leaving the unpaired daughter strand as ss whisker (fig. 9, step 2). In this way daughter strands will be displaced step by step from the parental template. In D-loops the displacement of the extra strands can start from both ends (27).

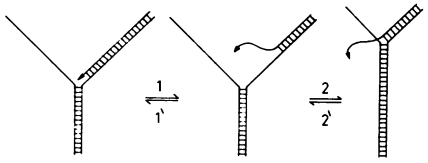

Fig. 9. Diagrammatic representation of the mechanism of branch migration based on local denaturations at the replication fork. Step 1 represents the local denaturation which can be either followed by renaturation (step 1') or by annealing of the parental strands (step 2) giving rise to formation of a whisker. The growing 3'end is indicated by the arrowhead.

Obviously such a mechanism would not stop after a ss gap has been eliminated by reannealing of the parental strands. Further branch migration, however, requires coincident denaturation in both branches of the replication fork (fig. 8), and therefore, will only proceed at a greatly reduced rate. Because daughter strands are complementary to one another, they can subsequently form a ds whisker which can grow by further branch migration and eventually be released as a nascent-nascent duplex. Thus, branch migration provides a basically common explanation for the release of nascent single strands and nascent-nascent duplexes.

It has to be mentioned that the extent of branch migration and the release of nascent DNA after BrdU substitution need not to be the same as in unsubstituted DNA. In view of the increased stability of BrdU substituted double helix DNA (31,32) a change of the free energy may be associated with the formation of, for example, a light-light (parental) duplex from a newly synthesized heavy-light density hybrid. This means that one direction of branch migration might be favoured thermodynamically against the other. The data available so far do not permit a founded conclusion about the way in which the over all process may be affected by the BrdU substitution.

Branch migration might also be the cause of the release of pre-labelled ss fragments if one assumes that D-loops, comparable to those described for mitochondria (27,29,33), exist in nuclear DNA. In mitochondria they seem to represent an early "hold-point" of the replication process which is maintained for several hours (29). Inappropriate experimental conditions during

the isolation of mitochondrial DNA leads to a marked loss of D-loops. This loss has been ascribed to the release of the extra strand by branch migration. Extra strands released from mitochondria are likely to be present in the pre-labelled ss DNA fraction. However, the great mass of this material has a size of several times that obtained from mitochondria (29,33). This suggests that it has been derived from larger D-loops which would be expected to occurr in nuclear DNA. Possibly, displacement loops in nuclear DNA have not been observed so far, because they are lost by the inappropriate extraction conditions usually employed.

A previous interpretation based on labelling kinetics concluded that the pulse labelled material of the R1 fraction contains the most recently synthesized DNA chains, while the rapidly sedimenting R3 represents a late intermediate of the replication process (13). The results of the present study show that the more recently synthesized chains from lysates produced in high NaCl also appear in the zone corresponding to the R3 of the SDS lysates. It obviously contains the majority of the replicating molecules which are either not altered or are only slightly altered under these conditions. In SDS lysates, however, R3 obviously represents a structure which remains after the most recently synthesized DNA chains have been released by branch migration. Significant incorporation of label into R3 was only observed after pulse times of more than 5 min (12), indicating that a similar time is required for the transformation of newly synthesized strands into a more stable conformation. As fork movement is understood to proceed at a rate of 0.2 to 0.6 μm/min (34,35), the pieces of DNA synthesized within 5 min may vary between 1 and 3 μm. This is compatible with the finding that pulse labelled nascent chains released by branch migration can reach a length of up to $2 \cdot 10^6$ daltons.

Finally it can be concluded that the lysis procedure with high NaCl-pronase (23) is superior to others in that it yields a replicating DNA in which the original *in vivo* structure is better preserved.

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