ASSOCIATION OF NUCLEAR DNA WITH A RAPIDLY SEDIMENTING STRUCTURE

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SUMMARY: Nuclei were isolated from monolayer cultures of bovine liver cells by use of a Triton containing Tris buffer, and dissolved in 1 M NaCl. Analysis of the lysate by sedimentation through sucrose gradients revealed that a variable proportion of the DNA, sometimes as much as 90%, remained associated with a rapidly sedimenting structure. The rapidly sedimenting material was degraded to smaller fragments by pronase digestion, indicating that proteins are essential to maintain the structural properties conferring the high rate of sedimentation. DNA could be released almost quantitatively by DNAase digestion without causing a significant change of the sedimentation rate of the supporting structure. A limited digestion showed that newly synthesized DNA was more resistant against release from the support. It is suggested that DNA molecules are attached to the rapidly sedimenting structure by one binding site per replicon, and that during replication additional attachment sites are generated by DNA regions at the replication forks.

The eucaryotic genome consists of a number of discrete DNA molecules. For the purpose of genetic continuity each individual molecule has to be duplicated during the nuclear cycle, and a set, consisting of one of the 2 sister molecules of each pair, has to be transmitted to each of the 2 daughter nuclei during mitosis. Clearly, this can only be achieved by the involvement of a regulatory device that controls the chronological and spatial order of the various contributing events. At least some of these processes, in particular the separation and sorting of the daughter molecules, can hardly be envisaged without assuming the participation of some morphologically defined structure, possibly the nuclear envelope (1).

An apparent association of replicating DNA with nuclear membranes has been reported on several occasions (2), but most of these findings may be disregarded as they are probably due to experimental artifacts (3,4). In this paper we report results on the association of nuclear DNA with a rapidly sedimenting component.

MATERIALS AND METHODS

Cell culture and labelling procedures. Monolayer cultures of bovine liver cells were grown in Carrel flasks as described elsewhere (5) except that a serum concentration of 10% was used in the growth medium. DNA was prelabelled by

addition of 0.2 μC/ml of 2-[^{14}C] dT (52.8 mC/mmole; NEN) for about 40 h. After growing the cells for another h in label free medium 10 μC/ml methyl-[^{3}H] dT (20 C/mmole; NEN) were added for 20 min. Simultaneous labelling of DNA and proteins was achieved by adding 1 μC/ml [^{14}C(U)] leucine and lysine (260 mC/mmole; NEN) and 0.1 μC/ml [^{3}H] thymidine for 20 h.

Preparation of the nuclear lysate. If not stated otherwise the following standard procedure was performed at room temperature: The monolayers were briefly rinsed with Triton-Tris (0.1% Triton X 100 in 5 mM Tris-HCl buffer pH 8). The cells were then washed off from the glass surface and homogenized in a single step by vigorously forcing 3 ml Triton-Tris 10 times through a hypodermic needle with a diameter of 0.7 mm onto the cell lawn. After 2 to 3 times all cells were dispersed in the Tris-Triton solution, and no whole cells were detected after completion of the shearing. The homogenate was then made up to 20 ml by addition of Triton-Tris and centrifuged for 2 min at 1,000 x g. The nuclear pellet was thoroughly resuspended in 10 ml 50 mM Tris buffer pH 8, and 10 ml 2 M NaCl solution, pre-warmed to 60°C, were added. The solution was placed in a 60°C water bath for 10 min and subsequently cooled in tap water. The homogenization of the lysate was completed by passing it 5 times through a 1 x 100 mm glass capillary at a pressure of 0.5 atm.

Sucrose gradient centrifugation and determination of the radioactivities. The centrifugations were carried out in a Spinco L2 65 B centrifuge using the 27.2 swinging bucket rotor. 20 ml 15 - 40% sucrose gradients containing 1 M NaCl were prepared on a 5 ml cushion of 65% sucrose containing 1 M NaCl and 0.5 g/ml CsCl. Samples of 4 ml were placed on the top and centrifuged for 2 h at 25.000 r.p.m. and a temperature of 15°C.

Preparation and denaturation of homologous [^{3}H] labelled DNA. A monolayer culture was labelled for 40 h with 0.1 μC/ml tritiated thymidine. The cells were dissolved in 0.5% SDS and the DNA was isolated by zonal centrifugation (6) and chromatography on hydroxyapatite. Finally, it was dissolved at a concentration of 3.5 μg in 5 mM Trisbuffer pH 8 (spec. act. 8,000 d.p.m./μg), heated for 10 min at 100°C and cooled in an ice-bath.

Determination of the radioactivities. Radioactive DNA was measured as described previously (7). When DNA and protein were labelled simultaneously, the procedure was as follows: DNA (1 mg/ml) and bovine serum albumin (2 mg/ml) were added to each sample to a final concentration of 0.2 and 0.4 mg/ml respectively. The samples were thoroughly mixed with an equal volume of cold 20% TCA (trichloracetic acid) and kept at 0°C for 30 min. The precipitates were collected by centrifugation, washed with 96% ethanol and dissolved in 0.9 ml 0.2 M NaOH. 10 ml scintillation fluid (75% toluene, 25% Triton X-100 and 4 g/l Scintimix) were added and the radioactivities were determined in a Philips Scintillation Analyser

RESULTS

Characterization of the rapidly sedimenting DNA. When a nuclear suspension was brought to 0.5 M NaCl or beyond, the nuclei disintegrated after a rapid increase of their volume. In the phase contrast microscope the lysates showed some finely dispersed material, but no nuclei, nuclear fragments or ghosts. Sedimentation of the lysates through sucrose gradients generally yielded 2 completely separated fractions of [^{14}C] labelled DNA (Fig. 1A). The fast sedimenting fraction was recovered from the top of the bottom layer. Its proportion varied between 30 and 70%, but in some experiments it amounted to almost 90%. The [^{3}H]/[^{14}C] ratio was always several times higher than that of the slowly sedimenting band. In many experiments only traces of [^{3}H] label were present in the latter, indicating that the newly synthesized DNA was preferentially associated with the rapidly sedimenting material.

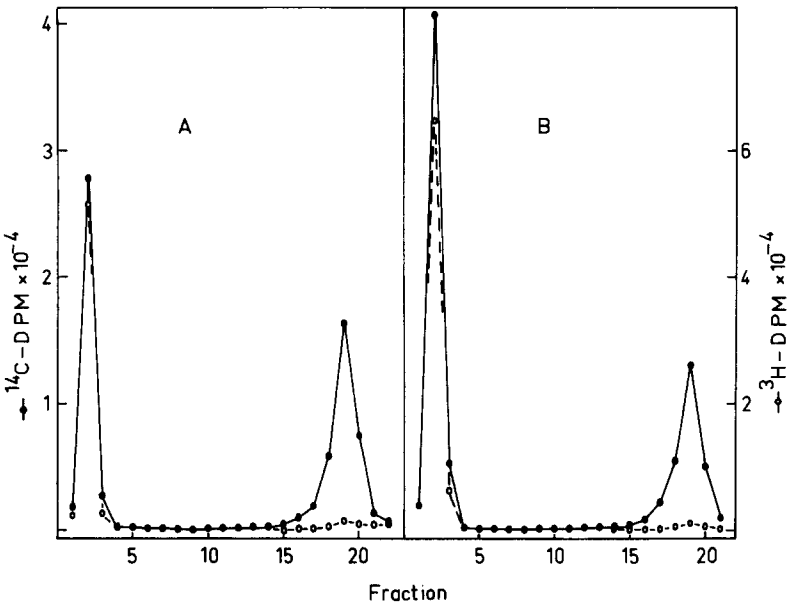

Fig. 1. Association of nuclear DNA with rapidly sedimenting material. A nuclear suspension was prepared and half of it was processed according to the standard procedure (A). To the other half (B), heat denatured calf thymus DNA (Boehringer; heated for 10 min at 100°C) was added to give a final concentration of 0.12 mg/ml. All other treatments were identical to A. Direction of sedimentation in all figures is from right to left.

To make sure that the association of the DNA with this material is not due to an experimental artifact, the following 2 experiments were performed: (1) A roughly 500-fold excess of heat-denatured calf thymus DNA was added to the nuclear suspension before lysis. Fig. 1B shows that the addition did not affect the sedimentation pattern. Thus heat denatured DNA does not compete for the attachment sites for endogenous DNA. (2) A nuclear suspension was prepared from cells which had not been pulse labelled with tritiated thymidine. Homologous [^{3}H] labelled DNA was heat-denatured and added to the nuclear suspension before lysis with NaCl. Fig. 2A shows that all tritium label sedimented as a separate peak and did not become associated with the rapidly sedimenting material. The insignificant number of [^{3}H] d.p.m. coinciding with the latter are due to insufficient correction for the [^{14}C] contribution in the [^{3}H] channel as is shown by the control experiment in Fig. 2B, in which only [^{14}C] labelled DNA was analysed.

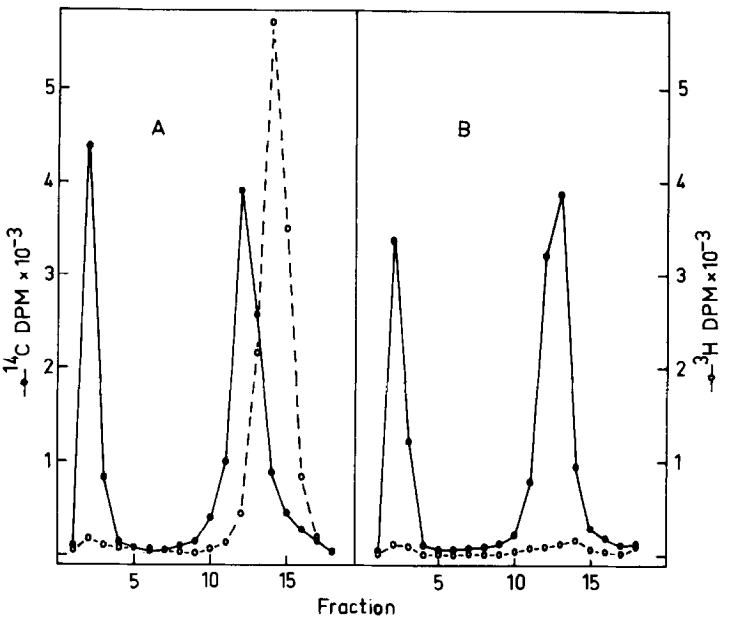

Fig. 2. Failure of single stranded DNA to bind to the rapidly sedimenting material. A nuclear suspension was prepared from a monolayer culture labelled with [^{14}C] thymidine only. Homologous [^{3}H] labelled DNA (15,000 d.p.m.), prepared and denatured as described under methods, was added to a portion of the nuclear suspension (A). Further treatment was according to the standard procedure. B is a control experiment without addition of [^{3}H] labelled DNA.

Characterization of the fast sedimenting material by enzymic digestion.

It was then investigated whether DNA is merely attached to the rapidly sedimenting complex or whether it is also required for its structural integrity. Samples were therefore digested with varying concentrations of DNAase I. Fig. 3 showns that with increasing DNAase concentration more label was removed from the rapidly sedimenting material and appeared in the slowly sedimenting fraction. No degradation products of intermediate sedimentation rates were found between the two labelled bands. This indicates that the DNA fragments detachted from the rapidly sedimenting material by a limited digestion were of about the same size as the DNA of the slowly sedimenting band. From preliminary sedimentation studies we estimated a molecular weight of approxymately 10^8 for this fraction. A significant decrease of the sedimentation rate of the upper band was only observed at higher DNAase concentrations (0.5 and 1.5 mg/l), which caused more than 60% release of the DNA from the rapidly sedimenting structure. These results suggests that the rapidly sedimenting structure consists of a DNAase resistent core from which at least 99% of the DNA can be detached without

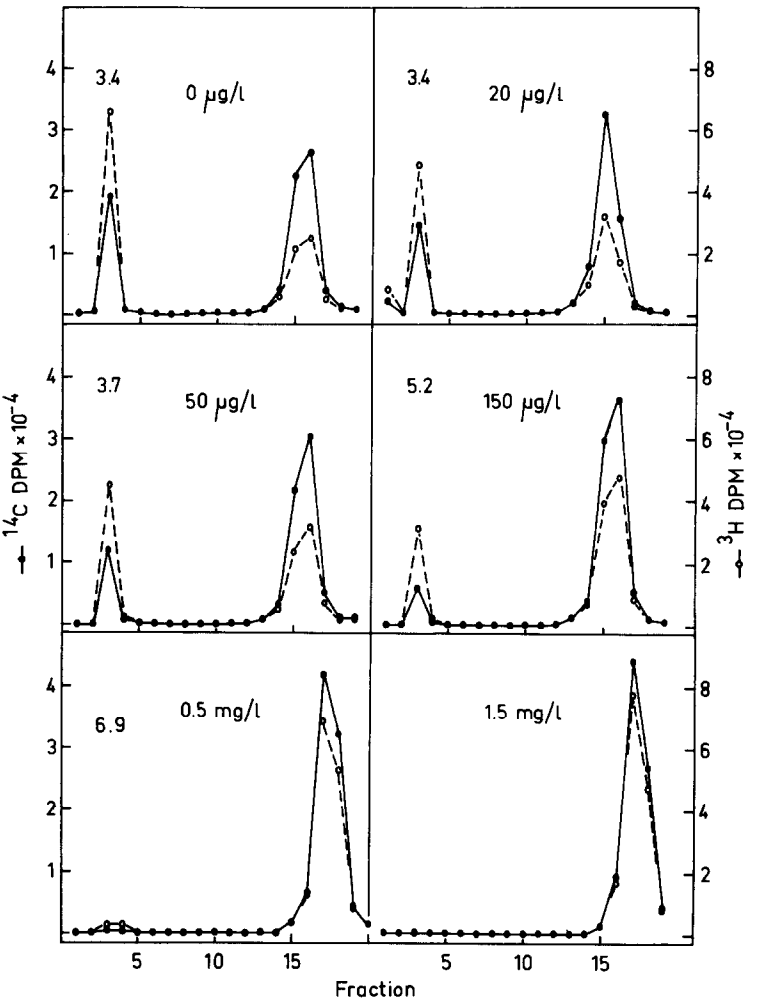

Fig. 3. The release of DNA by DNase digestion. A lysate was prepared according to the standard procedure and $MgCl_2$ was added to a final concentration of 7.5 mM. The sample was divided into 6 portions and DNase I (Sigma) was added to the final concentrations indicated in the figure. The samples were incubated for 10 min at 37°C and the DNAase was inactivated by re-heating for 10 min at 60°C. Sucrose gradient centrifugation was performed immediately afterwards. The numbers above the rapidly sedimenting peaks indicate the $[^{3}H]/[^{14}C]$ ratios.

disintegration of the component that confers to it the high rate of sedimentation.

The $[^{3}H]/[^{14}C]$ ratio which was always several times higher in the lower than in the upper band increased 2 to 3 fold during the course of digestion by DNAase (Fig. 3). Obviously, replicating DNA is more closely associated with the rapidly sedimenting structure, than bulk DNA.

Digestion by pronase also caused a shift of label from the rapidly

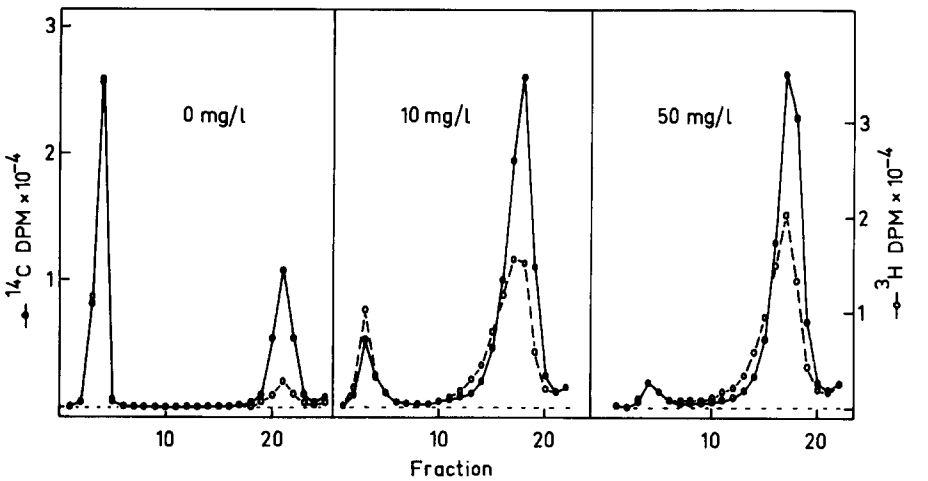

Fig. 4. Degradation of the rapidly sedimenting complex by pronase. A lysate was divided into 3 parts and incubated for 15 min at 37°C with pronase (Calbiochem, B grade) at the concentrations indicated in the figure. The samples were then re-heated for 10 min at 60°C and analysed immediately by sucrose gradient centrifugation.

to the slowly sedimenting band, but the degradation differed from that by DNAase in the following ways (Fig. 4): (a) the $[^{3}H]/[^{14}C]$ ratio of the rapidly sedimenting fraction did not increase; (b) a small but significant amount of label appeared between the 2 peaks, indicating that larger fragments than just DNA pieces were detached from the rapidly sedimenting complex; and (c) the sedimentation rate of the slowly sedimenting fraction did not decrease at high enzyme concentrations.

Evidence for the presence of proteins in the rapidly sedimenting structure. Cells were labelled with $[^{3}H]$ thymidine and $[^{14}C]$ amino acids and the nuclear lysate was centrifuged for different times at different rotor speeds. After centrifugation for 60 min at 4 000 r.p.m. the rapidly sedimenting complex, containing 20% of the labelled proteins and 23% of the labelled DNA appeared as a broad peak in the lower half of the gradient (fig. 5A). The $[^{3}H]/[^{14}C]$ ratio was constant over the entire peak suggesting that DNA and protein were bound to each other. A second sample of the same lysate was centrifuged for 15 h at 25 000 r.p.m. The rapidly sedimenting material, which was now accumulated on the concentrated sucrose shelf, still contained 20% of the labelled proteins and 23% of the DNA (fig. 5B). Similar results were obtained with lysates prepared in 2 M NaCl (fig. 5C). In contrast, the slowly sedimenting DNA which coincided with the main peak of labelled proteins after a limited cen-

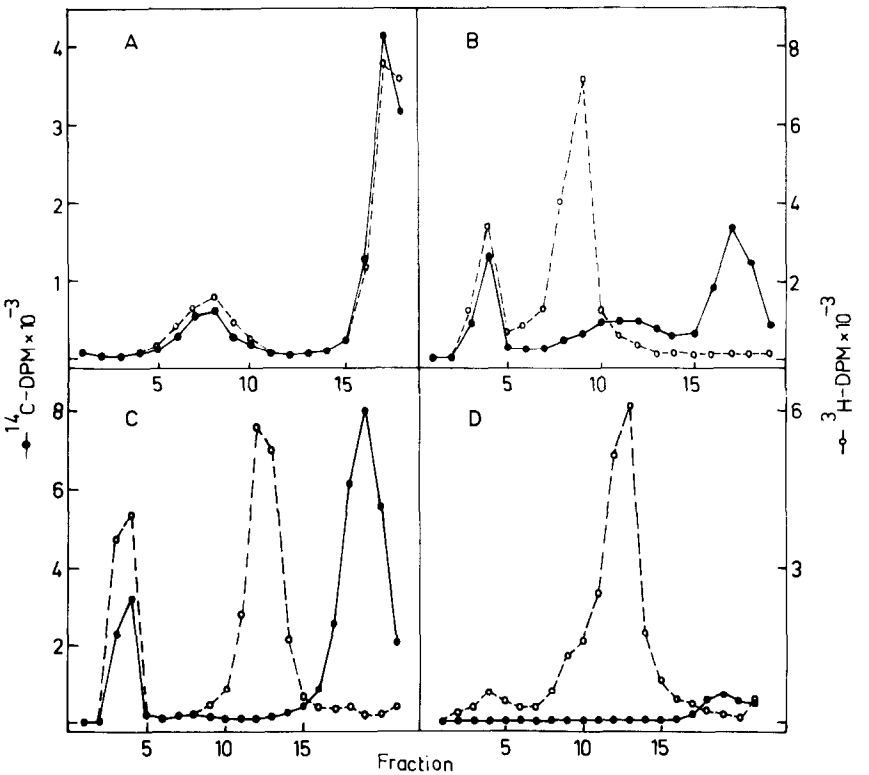

Fig. 5. Incorporation of amino acids into the rapidly sedimenting structure. A nuclear lysate in 1 M NaCl was prepared from cells labelled with [^{14}C] amino acids and [^{3}H] thymidine. 5 ml samples of the lysate were centrifuged through sucrose gradients for 60 min at 4 000 r.p.m. (A), and for 15 h at 25 000 r.p.m. at 15°C (B). - A similar lysate was prepared in 2 M NaCl. 5 ml samples (C and D) were centrifuged for 15 h at 20 000 r.p.m. and 15°C through sucrose gradients containing 2 M NaCl. Sample D was incubated with 100 µg/ml pronase (Calbiochem, B grade) for 30 min at 37°C before centrifugation.

trifugation, could become completely separated from this peak by centrifugation for 15 h at 25 000 r.p.m. (figs. 5B and 5C).

Fig. 5D shows the sedimentation pattern of a sample of a 2 M NaCl lysate which was extensively degraded with pronase prior to centrifugation. The [^{14}C] label of the proteins has become acid soluble except for about 10% imcompletely degraded peptides which remained at the top of the gradient. The degradation of the proteins caused a shift of the rapidly sedimenting DNA to the upper DNA peak. Incubations of lysates with pancreatic ribonuclease A had no effect on the sedimentations patterns of labelled DNA and proteins.

DISCUSSION

The results show that nuclear DNA remains attached to a rapidly sedimenting structure which is stable under conditions that dissociate the bulk of the chromatin into its constistuents. The data obtained with enzymatic degradation reveal that proteins are the main components which stabilize the complex structure. Observations on the protein composition (L. Mullenders, unpublished) suggest that the structure is related to what was described as a structural protein matrix of the nucleus (8,9).

The proportion of DNA attached to the rapidly sedimenting structure varies but can amount to almost 90%. It has been shown that on the average about 40% of the cells are in S-phase (7). If only replicative DNA is attached, then the proportion of rapidly sedimenting DNA should not excede 40% to any marked extent. Therefore, we conclude that essentially all nuclear DNA, whether it is replicating or not, is permanently attached to a structural component. That routinely smaller amounts of rapidly sedimenting DNA are found must be ascribed to unavoidable breakage by shearing and by endogenous DNAase activity during the isolation procedure. A limited DNAase digestion of the large complex detached fragments of roughly 10^8 molecular weight. Since a chromatid contains a single DNA molecule (10) this molecule must be attached several times and the attachment sites should be spaced by about 50 μm along the DNA double helix. This is of the same order of magnitude as the size of the replicon in mammalian cells (11). The finding that newly synthesized DNA is more closely associated with the rapidly sedimenting structure than the bulk DNA indicates that either the replication forks or adjacent regions also are attached.

Our data fit well with the model for chromosome replication proposed by DINGMAN (1), according to which each replicon is attached to the nuclear membrane at its origin and further attachment sites become available for the replication forks during DNA synthesis.

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