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ISOLATION AND CHARACTERIZATION OF A MAMMALIAN NUCLEAR DNA-PROTEIN COMPLEX

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Isolation and characterization of a mammalian nuclear DNA-protein complex

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Aan mijn ouders,
Riet en Jasper

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CHAPTER I

General Introduction

1. General Introduction

1.1 Organization and replication of eukaryotic DNA.

In eukaryotes the genetic information is stored in a number of discrete DNA molecules within the nucleus. Generally the amount of DNA in the various species increases in an evolutionary sequence and reaches, for example in mammalian cells, lengths of up to several meters (1). In the nucleus the DNA occurs as a fibrous complex in association with histones, non-histone proteins and a small amount of RNA and is called chromatin.

During mitosis the chromatin condenses into a discrete number of chromosomes. Based on viscoelastic data (2) and DNA fiber autoradiographic data (3) it has been suggested that each chromosome contains a single, continous DNA molecule. Electron miscroscopic examination of purified chromatin (4) or nuclei (5) shows that the structure of chromatin is a flexible chain of spherical particles. Biochemical evidence for such a periodic structure of chromatin comes from nuclear digestion studies (6). In both interphase chromosomes and metaphase chromosomes about half the nuclear DNA is rendered acid soluble by DNA degrading enzymes, while the remaining DNA is found in small nuclear particles. The nuclease resistent particles are called nucleosomes or ν-bodies and contain 140 base pairs of DNA wrapped around an octameric protein core (7). The protein core consists of two molecules each of the histones H_{2a}, H_{2b}, H_3 and H_4 (8). In addition evidence has been presented that histone H_1 interacts with the spacer DNA found between two adjacent nucleosomes (9), resulting in a higher order structure of DNA in chromatin (10,11).

For the purposes of genetic continuity the nuclear DNA is duplicated once per cell cycle, the majority during a well defined interval of interphase. The duplication of chromosomal DNA occurs through a high number of tandemly arranged replication units or replicons (12). Within each replicon, DNA synthesis starts at an origin of replication and proceeds bidirectionally (12), forming a loop containing replicated DNA (12,13). During replication the chromatin subunit structure is transmitted to one daughter strand, while the other remains temporarily deficient of histones (14). Folding of this strand into nucleosomes occurs within a short time by the addition of the required histones of pre-existing histones (15,16).

Despite intensive investigation the process of chromosome duplication remains a formidable problem. It implies a large degree of organization for the replication of DNA, packaging of DNA into chromatin, the condensation of chromatin in methaphase chromosomes and segregation of chromosomes during mitosis. It is likely that well regulated temporal and spatial controls are required for the various contributing events. At least some of the processes can hardly be understood without assuming the participation of some morphologically defined structure, possibly the nuclear envelope (17,18).

1.2 Association of the nuclear envelope with chromatin and DNA.

Evidence for the attachment of chromatin and DNA to the nuclear envelope comes mainly from ultrastructural studies. The apparent association of chromatin fibres with the pore complexes of the nuclear envelope was first observed in whole mount preparations of honeybee embryonic cells (1). Similar observations were made in mammalian interphase cells (19-22). In addition, Wray and Stubblefield (23) reported the association of fragments of the nuclear envelope with chromosomes during mitosis. Purified nuclear envelopes contain up to 10% of the nuclear DNA, depending on the isolation procedure employed (24,25). Although the nature of the binding is unknown, it is clear that DNA is firmly attached to the nuclear envelope and is resistent to shearing and to high concentrations of salt (23, 26-28).

1.3 DNA replication and the nuclear envelope.

Jacob et al. (29) suggested the involvement of the cell envelope in the process of replication of bacterial DNA. In their 'replicon' model the replication starts at a specific site (called the replicator or origin) which is attached to the cell envelope and proceeds until the entire chromosome has been duplicated. The available data support the postulate that the bacterial chromosome is attached to the cell envelope and that the attachment sites include the origin of replication (30,31).

Analogous to replication in bacteria, it has been suggested that the nuclear envelope of the eukaryotic cell provides structural attachment points in DNA replication. This idea was supported by the apparent attachment of

chromatin to the nuclear envelope.

Much of the evidence that DNA is synthesized at the nuclear envelope comes from cell fractionation studies. According to Tremblay et al. (32) the detergent sodium lauroyl sarcosinate, when crystallized with magnesium ions, forms complexes with membranes which can be isolated at their buoyant density in sucrose gradients (M-band). Using this technique, preferential association of replicating DNA with the M-band was observed in several mammalian tissues (33-37), and in developing sea urchin embryos (38). Although the association of DNA with the M-band is cell cycle dependent (34,36,38) a complete dissociation of DNA from the M-band does not occur (36,37). The presence of DNA in the M-band results from its attachment to the envelope since several authors (32,35,36,39) observed no association of exogenous DNA (single or double stranded) or chromatin with the M-band. Nevertheless, these results should be regarded with caution because the M-band material contains more than 50% of the cellular proteins and RNA (34) and was not subjected to biochemical identification of nuclear envelope proteins.

Newly synthesized DNA is often (40,41), but not always (42,43) localized at the interphase after phenol or chloroform-isoamyl alcohol extractions. However, Fakan et al. (44) suggested that artificial complexes between replicating DNA and denatured proteins can be formed, probably due to the partially single stranded nature of replicating DNA.

Mizuno et al. (40,45) and Cabadrilla and Toliver (46) isolated nuclear envelopes after a short pulse of radioactive thymidine and conclude a specific location of replication sites at the nuclear envelope. In contrast O'Brien et al. (47), Kay et al. (48) and Hyodo and Eberle (49) using the same approach conclude that DNA synthesis occurs at sites both close to and remove from the nuclear envelope. As pointed out by Fansler (50) the only apparent explanation for these disparities are the differences in preparing nuclear envelopes (see also section 1.4).

Convincing evidence that the nuclear envelope is not preferentially involved in the process of DNA replication has accumulated from electron microscope autoradiography, In mammalian cell lines (44,51-54) and plant cells (55,56) the results indicate that DNA is replicated throughout the nucleus. However, in nuclei of rapidly dividing sea urchin embryos Hobart

et al. (57) observed a preferental appearance of grains at the periphery of the nuclear envelope after a short exposure to radioactive thymidine in agreement with earlier biochemical findings (38). The observed preferential DNA replication at the nuclear envelope during late S-phase in both mammalian cells and plant cells is possibly due to DNA present in the constitutive heterochromatin (51,53,55). Thus, although from EM autoradiography it seems that the bulk of nuclear DNA is not replicated at the nuclear envelope in mammalian and plant cells, a major part of the biochemical data suggest the involvement of the nuclear envelope in DNA replication. A possible explanation for these differences is based on new insights concerning the structural organization of the nucleus.

1.4 The involvement of the nuclear protein matrix in DNA replication.

Several investigators have observed a fibrous network in nuclei after extraction of nuclei with high concentrations of salt. The network, called the ribonucleoprotein network, is attached to the nuclear envelope and extends throughout the nucleus (58-60). These nuclear structures are composed of intact nuclear envelopes, residual nucleoli and contain considerable amounts of ribonucleoprotein particles. Based on these observations Berezney and Coffey (61) isolated a three-dimensional structural network from rat liver nuclei termed the nuclear protein matrix and which is mainly composed of protein (61,62). Using the same or slightly modified procedures similar residual matrix structures were shown to be present in various organism (63-66).

According to Berezney et al. (62) the nuclear protein matrix consists of three main ultrastructural components:

a. a residual nuclear envelope, which forms a continuous structure surrounding the nuclear sphere. It should be noted that the residual envelope still contains nuclear pore complexes, although the nuclear membranes are removed (62,67,68).
b. residual nucleoli.
c. an internal fibrous matrix structure which extends from the residual nuclear envelope throughout the interior of the nuclear sphere.

Aaronson and Blobel (68) and Scheer et al. (69), using different procedures

isolated a nuclear fraction that mainly consists of the residual envelope. Possibly, depending on the procedure employed, the residual nuclear envelope becomes separated from the internal nuclear matrix (see also 67). The nuclear protein matrix prepared by Berezney et al. (62) contains a small amount of DNA, approximately 2-4% of the total nuclear DNA, associated with the residual envelope as well as the internal matrix structure (62,67). After a short pulse with radioactive thymidine newly synthesized DNA is preferentially recovered from the matrix, suggesting an important role of this residual structure in the replication of DNA (70,71,72). This finding is in agreement with the autoradiographical evidence demonstrating replication sites throughout the nuclear interior.

The aim of the present study was to further investigate the early observation that when nuclei isolated from in vitro cultured bovine liver cells (73) were subjected to conditions that dissociate chromatin into its constituents, nuclear DNA was found attached to a rapidly sedimenting structure. The attachment DNA to the rapidly sedimenting nuclear structure and the involvement of proteins in the stability of the structure are studied (chapter 3). The mode of attachment of in particular replicating DNA is investigated in detail (chapter 4). The whole mount structure and the polypeptide composition of the material are described in chapter 5. The fractionation of the proteins is investigated in chapter 6. In chapter 7 we describe the dissociation and purification of the proteins and their ability to bind to DNA. The last chapter (chapter 8) provides some aspects of the in vivo synthesis of the proteins.

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CHAPTER II

General methods and materials

2. General methods and materials

2.1 Cell culture and labelling procedures.

Monolayer cultures of bovine liver cells were grown in Carrel flasks or Roux bottles as described by Pieck (1), except that the serum concentration was reduced to 10%.

To pre-label the DNA cells were grown in medium containing 0.1 µCi/ml 2-(^{14}C) thymidine (52.8 mCi/mmole; NEN) for about 40 hr. After growing the cells for another hour in label free medium, 50 µCi/ml or 5 µCi/ml methyl-(^{3}H) thymidine (20 Ci/mmol; NEN) were added for 1 min, or 20 min, respectively. When Carrel flasks were used protein and DNA were labelled simultaneously by addition of 1.0 µCi/ml (^{14}C(U)) leucine and lysine (260 mCi/mmol; NEN) and 0.1 µCi/ml methyl-(^{3}H) thymidine to the medium for 40 hr. In Roux bottles, protein and DNA were labelled for 20 hr by adding 2 µCi/ml (^{3}H(N)) leucine (40-60 µCi/mmol; NEN) and 0.1 µCi/ml 2-(^{14}C) thymidine to a modified growth medium. In this medium a reduced lactalbumin hydrolysate concentration of 0.005% (w/vol) was used during the labelling period in order to improve the incorporation of radioactive amino acids.

2.2 Preparation of the nuclei.

After removal of the growth medium, monolayers were rinsed with a solution of Triton-Tris (0.1% Triton X-100 in 5 mM Tris/HCl pH 8). This and all subsequent steps were carried out at room temperature. Cells from one Carrel flask were washed off from the glass surface with 3 ml Triton-Tris and homogenized in a single step by vigorously forcing the suspension 5 times through a hypodermic needle (diameter 0.7 mm). The homogenate was then made up to a volume of 25 ml by addition of Triton-Tris and the nuclei were collected by centrifugation (2 min at 1000x g). For further purification the nuclear pellet was resuspended in 3 ml Triton-Tris and passed 3 times through a 0.2 mm needle. 20 ml of Triton-Tris were then added and the nuclear suspension was allowed to stand for 10 min. A final nuclear pellet was then obtained by centrifugation.

Cells from one Roux bottle were shaken off the glass surface with 20 ml

Triton-Tris and passed 5 times through a 0.7 mm needle. The crude nuclei were collected by centrifugation. The pellet was resuspended in 8 ml Triton-Tris and homogenized as described above. 17 ml of Triton-Tris was added and the nuclei were collected by centrifugation. The pellet after suspension in 8 ml Triton-Tris was carefully pressed 3 times through a 0.2 mm needle. The volume was brought to 25 ml with Triton-Tris and after 10 min the nuclei were collected by centrifugation. The final nuclear preparation was free of contamination as judged by phase contrast microscopy. The various fractions obtained during the course of isolation, were analysed by SDS-polyacrylamide gel electrophoresis. Fig. 1 shows that some proteins were removed by prolonged incubation in Triton-Tris, from nuclei which were obtained after the first homogenization.

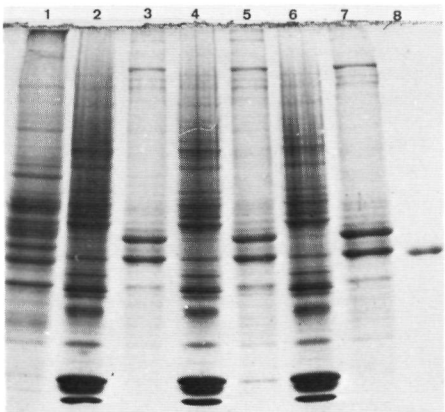

Fig.1:
Cells from one Roux bottle were shaken off the glass surface with 20 ml Triton-Tris. The suspension was passed 5 times through a 0.7 mm needle and divided in three equal parts. The crude nuclei were obtained by centrifugation (2 min at 1000 g). One third of the supernatant was used for electrophoresis.
The nuclear pellets were suspended in 5 ml Triton-Tris and treated as follows: A. suspension was kept at 25°C for 5 min. B. the suspension was homogenized 5 times through a 0.2 mm needle. C. the suspension was kept at 25°C for 20 min.
After termination of the various treatments the nuclei were collected immediately by centrifugation (5 min at 1000 g) and dissolved directly in 100 µl sample buffer. The proteins of the supernatants were precipitated by addition of TCA. The resulting pellets were prepared for electrophoresis and dissolved in 100 µl sample buffer. Slot 1: supernatant obtained after the first homogenation step. slot 2: nuclear pellet A. slot 3: supernatant A. slot 4: nuclear pellet B. slot 5: supernatant B. slot 6: nuclear pellet C. slot 7: supernatant C. slot 8: actin, prepared from bovine muscles according to Carsten et al. (8). Slot 1: 5 µl, slots 2 to 7: 7:15 µl.

The solubilized proteins mainly consist of two prominent bands. The electrophoretic pattern is distinctly different from those of the cytoplasm and whole nuclei.

Electrophoretic analysis of purified nuclei reveals up to 35 bands of different molecular weight including the various histones (Fig. 2).

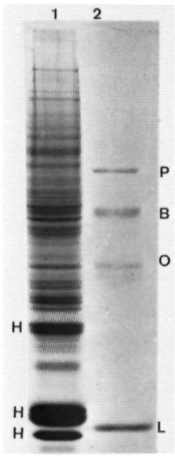

Fig. 2:
Polypeptide composition of purified nuclei. P: phosphorylase A. B: bovine serum albumin. O: ovalbumin. L: lysozyme. The bands indicated by H represent the various histones.

2.3 *Preparation of the nuclear lysate.*

The purified nuclear pellet isolated from one Carrel flask of cells was resuspended in 10 ml 50 mM Tris/HCl pH 8 by gentle passage through a 0.7 mm needle. 10 ml 2 M NaCl in 50 mM Tris/HCl pH 8 were added to the finely dispersed nuclear suspension. The lysate was homogenized by passing it 5 times through a 1 x 100 mm glass capillary at a pressure of 0.5 atm. The nuclear pellet from one Roux bottle was lysed in 60 ml 1 M NaCl, 50 mM Tris/HCl pH 8 and homogenized as described above.

In several experiments the resuspended nuclei were lysed by addition of 2 M NaCl, 50 mM Tris/HCl, pH 8 solution, pre-warmed to 60°C. The lysate was then placed in a 60°C water bath for 10 min and subsequently cooled with tap water.

2.4 Isolation of the rapidly sedimenting material.

Method 1.

Nuclear lysates from Carrel flasks were analysed by sucrose gradient centrifugation using the Spinco SW 27.2 rotor. 27 ml 15 - 40% sucrose gradients, containing 1 M NaCl, 50 mM Tris/HCl pH 8, were prepared on a 5 ml cushion of 65% sucrose, containing 0.4 g/ml CsCl. Samples of 4 ml were placed on the top and centrifuged for 1 hr at 20,000 r.p.m. at a temperature of 20°C. For larger quantitaties of rapidly sedimenting material, nuclear lysates prepared from Roux bottles were separated in slightly modified sucrose gradients: 22 ml 15 - 40% sucrose gradients, containing 1 M NaCl, 50 mM Tris/HCl pH 8 were layered on 5 ml of a 65% sucrose cushion containing 0.4 g/ml CsCl. Samples of 10 ml were placed on top of 6 identical gradients and centrifuged for 1 hr at 20,000 r.p.m. at a temperature of 20°C. Subsequently, the gradients were fractionated and the corresponding fractions of all 6 gradients were combined.

Method 2.

Large batches of rapidly sedimenting material were obtained in a more direct way as follows: the nuclear lysate (60 ml per Roux bottle) was centrifuged for 20 min in a Sorvall HB-4 rotor at 8,000 r.p.m. at a temperature of 20°C. The pellet was carefully resuspended in 30 ml 1 M NaCl, 50 mM Tris/HCl, pH 8. The rapidly sedimenting material was collected from this suspension by centrifugation for 20 min at 8,000 r.p.m.

2.5 Large scale preparation of the rapidly sedimenting protein structure.

Rapidly sedimenting material from 6 to 10 Roux bottles obtained by method 2 was gently resuspended in 30 ml 50 mM Tris/HCl pH 8 and 7.5 mM $MgCl_2$. DNase 1 (Sigma, electrophoretically purified) was added to a final concentration of 50 µg/ml and the sample was incubated for 16 hr at 25°C. 15 ml of 1 M $MgCl_2$ was then added and the rapidly sedimenting protein structures were purified by sucrose gradient centrifugation in a Spinco SW 27.2 rotor. 16 ml 15 - 40% sucrose gradients containing 50 mM Tris/HCl pH 8, were prepared on a 5 ml 60% sucrose cushion. Samples of 15 ml were placed on top of the gradient and centrifuged for 15 min at 5,000 r.p.m. at 20°C and the rapidly sedimenting

proteins were recovered as a white pellet at the bottom of the tubes.

At this stage of the isolation the material forms aggregates and adheres strongly to glass. The latter occurrence was largely prevented by use of silicone coated glassware. Aggregation could be effectively reduced by suspension of the pellet in glycerol containing buffer. Routinely, the pellet of one Roux bottle was resuspended by means of a Pasteur pipette in 1.5 ml of 40% (v/v) glycerol, 0.15 M NaCl and 50 mM Tris/HCl pH 8 and stored at 18^0C until use.

2.6 Isopycnic centrifugation in CsCl density gradients.

Samples of rapidly sedimenting material and free DNA from sucrose gradients were treated with 50 µg/ml pronase (Calbiochem) for 30 min at 37^oC. The incubation was terminated by addition of 2.5 vol of 96% ethanol, containing 1% K-Acetate. After storing overnight at -15^oC, the DNA was collected by centrifugation and was dissolved in 50 mM Tris/HCl pH 8. The volume was brought to 4.5 ml and 5.4 g CsCl dissolved in it. Centrifugation was performed in a Spinco 50 Ti rotor for 46 hr at 37,000 r.p.m. and at temperature of 4^oC. Spectrophotometric measurements of the fractions were made at 260 nm.

2.7 Sedimentation analysis in sucrose gradients.

Samples of fast sedimenting material from sucrose gradients were deproteinized as described in section 2.6. The DNA was purified, without alcohol precipitation, by chromatography on hydroxylapatite columns (2) or in CsCl density gradients. 1 ml samples of the purified DNA were layered on top of 16 ml of 15 - 20% or 15 - 40% sucrose gradients in 50 mM Tris/HCl pH 8 and centrifuged for 17 hr at a temperature of 20^oC in a Spinco SW 27-1 rotor at 17,000 r.p.m. or 20,000 r.p.m. respectively. Sedimentation coefficients were estimated according to McEwen (3).

2.8 Preparation of samples for electron microscopy.

Rapidly sedimenting material obtained by method 2 was gently resuspended in 50 mM Tris/HCl pH 8 and treated as specified under the figure legend.

Drops of 20 µl of the suspension were placed on a flat piece of Teflon and the material was picked up with Formvar-carbon coated copper grids. The excess fluid was removed from the grids by blotting with filter paper. The preparations were dehydrated in a graded ethanol series and air-dried from a final step in pentane. The material was stained with 1% phosphotungstic acid (PTA) during the 50% ethanol step for 5 to 10 min. Photomicrographs were taken with a Philips 201 electron microscope operating at 60 kV.

2.9 Preparation and electrophoresis of the proteins.

The proteins of suspensions of the rapidly sedimenting material were precipitated by adding an equal volume of cold 30% trichloroacetic acid (TCA). The mixture was kept at 0°C for 30 min and the precipitate was collected by centrifugation. The sediment was washed once with 5% TCA, twice with 5 ml cold acidified acetone and twice with cold acetone. The sediment was dried in the air and dissolved in sample buffer (5) containing 6 M urea.

Slab gel electrophoresis was carried out in the presence of SDS (sodium dodecyl sulfate) according to Laemmli (4) using a linear gradient of 6 - 18% polyacrylamide (5). Molecular weight determinations were performed in 10% SDS polyacrylamide gels with phosphorylase A, bovine serum albumin, ovalbumin, trypsin and lysozyme as molecular weight markers (6). The gels were stained with Coomassie blue and scanned at 580-650 nm.

2.10 Determination of radioactivities.

When DNA alone was labelled the procedure was as follows:
salmon sperm DNA (1 mg/ml) was added to each sample to a final concentration of 0.2 mg/ml. The DNA was precipitated by addition of 0.5 vol of ice-cold 20% trichloroacetic acid and the sample was kept at 0°C for 30 min. Subsequently, the precipitate was collected by centrifugation washed once with 70% ethanol, once with 0.5 N perchloric acid and then hydrolyzed for 1 hr at 80°C in 0.5 ml of 0.5 N perchloric acid.

When DNA and protein were labelled simultaneously salmon sperm DNA (1 mg/ml) and bovine serum albumin (2 mg/ml) were added to a final concentration of 0.2 and 0.4 mg/ml respectively. The samples were then mixed with

an equal volume of ice-cold 20% trichloroacetic acid and kept at 0°C for 30 min. The precipitates were collected by centrifugation, washed with 70% ethanol and dissolved in 0.9 ml 0.2 M NaOH. Each sample was prepared for counting by addition of 10 ml of a scintillation fluid which consisted of 4 g Omnifluor per liter of toluene-Triton X-100 (75 : 25 v/v) solution. The radioactivities of the samples were determined in a Philips liquid Scintillation Analyzer.

2.11 Determination of protein and DNA.

The protein content was determined according to Lowry et al. (7). The amount of DNA in purified fractions was determined from the optical density at 260 nm, assuming that 1 mg/ml DNA corresponds to 25 optical density units.

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Association of nuclear DNA with a rapidly sedimenting component

3. Association of nuclear DNA with a rapidly sedimenting component

3.1 Introduction.

An apparent association of replicating DNA with nuclear membranes has been reported on several occasions (1), but most of these findings may be disregarded as they are probably due to experimental artifacts (2,3). In this chapter results are described on the association of nuclear DNA with a rapidly sedimenting component.

3.2 Methods and Material

3.2.1 Preparation and denaturation of homologous ^{3}H-labelled DNA.

A monolayer culture was labelled for 40 hr with 0.1 µC/ml ^{3}H-thymidine. The cells were dissolved in 0.5% SDS and the DNA was isolated by zonal centrifugation (4) and chromatography on hydroxylapatite. Finally, it was dissolved at a concentration of 3.5 µg in 5 mM Tris/HCl pH 8 (spec. act. 8,000 DPM/µg) heated for 10 min at 100°C and cooled in an ice-bath.

3.3 Results

3.3.. 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 rapidly 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.

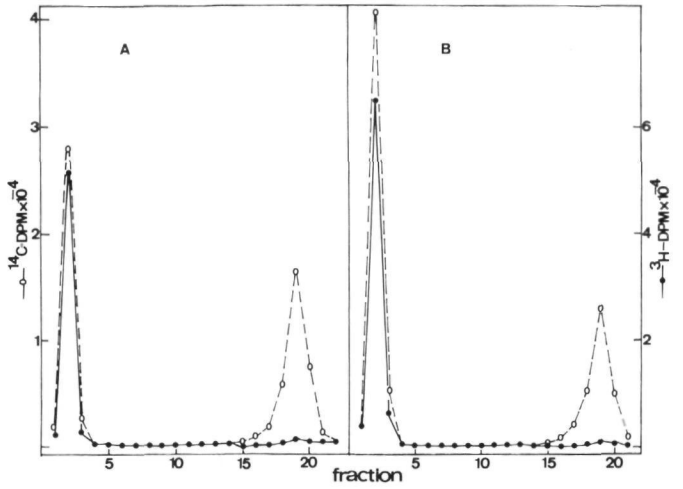

Fig. 1:

Association of nuclear DNA with rapidly sedimenting material. Cells were pre-labelled with ^{14}C-thymidine and pulse-labelled with ^{3}H-thymidine for 20 min. A nuclear suspension was prepared and half of it (A) was processed according to the standard procedure (chapter 2, method 1). 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 (3), 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-DPM 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.

3.3.2 Characterization of the fast sedimenting material by enzymatic 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

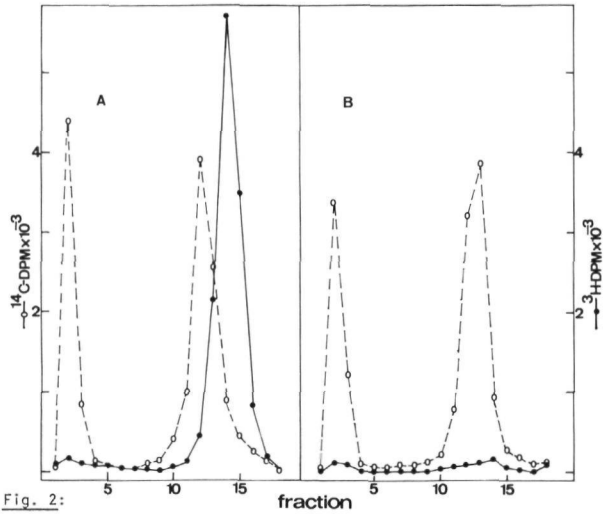

Fig. 2:

Failure of single stranded DNA to bind to the rapidly sedimenting material. A nuclear suspension was prepared from a monolayer culture pre-labelled with ^{14}C - thymidine only. Homologous ^{3}H - labelled DNA (15000 DPM), 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.

DNase I. Fig. 3 shows that with increasing DNase 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 detached 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 approximately 10^{8} for this fraction. A significant decrease of the sedimentation rate of the upper band was only observed at higher DNase 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 DNase resistent core from which at least 99% of the DNA can be detached without 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 DNase (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 to the slowly sedimenting band, but the degradation differed from that by DNase in the

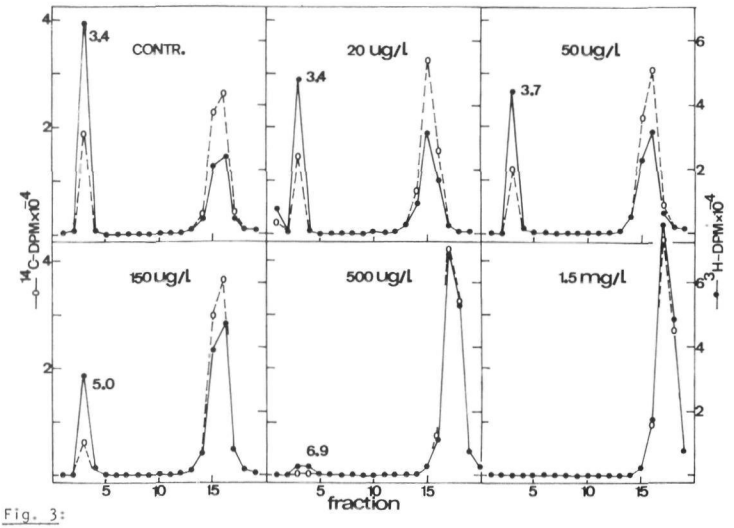

Fig. 3:

The release of DNA by DNase digestion. A lysate was prepared from cells, pre-labelled with ^{14}C-thymidine and pulse-labelled with ^{3}H-thymidine for 20 min 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 DNase was inactivated by heating for 10 min at 60°C. Sucrose gradient centrifugation was performed immediately afterwards. The numbers above the rapidly sedimenting peaks indicate the ^3H / ^{14}C ratios.

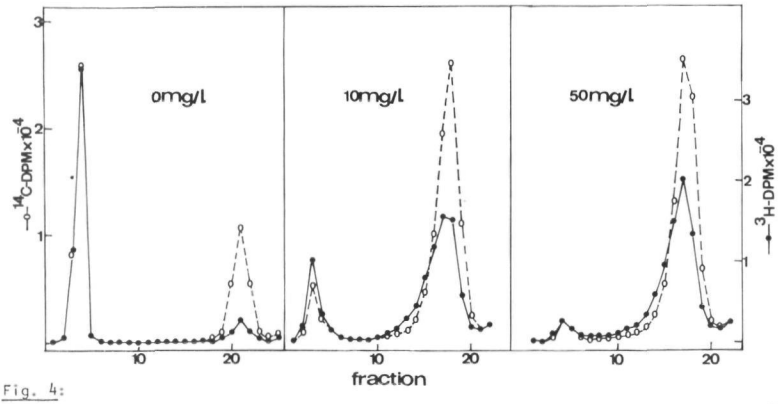

Fig. 4:

Degradation of the rapidly sedimenting complex by pronase. A lysate, prepared from cells pre-labelled with ^{14}C-thymidine and pulse-labelled with ^{3}H-thymidine for 20 min, 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 heated for 10 min at 60°C and analysed immediately by sucrose gradient centrifugation.

following ways (Fig. 4): (a) the $^3H/^{14}C$ 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.

3.3.3 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 hr

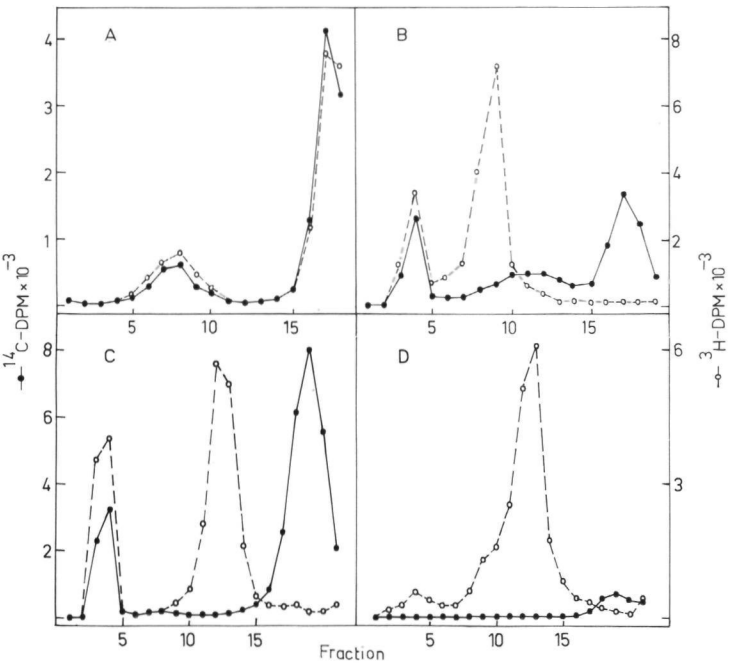

Fig. 5:

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 4000 r.p.m. (A), and for 15 hr at 25000 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 hr at 20000 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.

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 mean peak of labelled proteins after a limited centrifugation, could become completely separated from this peak by centrifugation for 15 hr 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% incompletely 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 sedimentation patterns of labelled DNA and proteins.

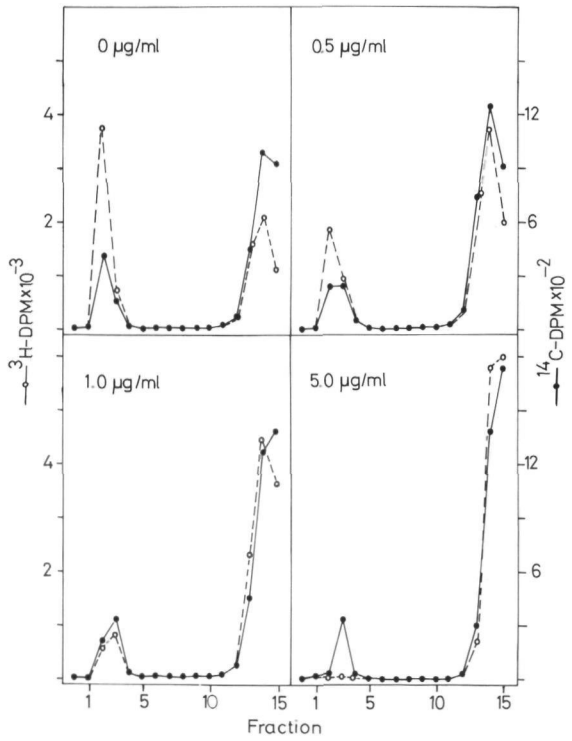

Fig. 6:

Evidence for a rapidly sedimenting protein structure. A nuclear lysate was prepared from cells, pre-labelled with radioactive aminoacids and ^{3}H-thymidine, and processed according to the standard procedure. The lysate was divided into 4 portions and Staphylococcus nuclease was added to the final concentrations, indicated in the figure.

To prove the presence of a rapidly sedimenting protein structure in the complex nuclear lysates were prepared from cells, pre-labelled with radioactive amino acids and thymidine; subsequently these lysates were incubated with Staphylococcus nuclease.

Fig. 6 shows the results of such incubations. In the original lysate the DNA-protein complex contains 17% of the total nuclear proteins and 45% of the total DNA. With increasing concentrations of the enzyme about 75% of the DNA is removed from the complex, but the proteins are fully retained. This indicates that the released DNA fragments are substantially free of proteins. The highest concentration of the enzyme causes a release of 99% of the DNA in the complex. Under these conditions about 70% of the proteins, originally found in the complex, are recovered from the underlay of the gradient. These results clearly prove the presence of large, nuclease resistent protein structures in the fast sedimenting complex. The protein label, released from the complex, appears at the top of the gradient and represents residual amounts of histones H_3 and H_4 (chapter 5). Similar results were obtained with DNase 1.

3.4 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 constituents. The data obtained with enzymatic degradation reveal that proteins are the main components which stabilize the complex structure. A part of the proteins in the complex constitutes rapidly sedimenting structures.

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 (5). 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 DNase activity during the isolation procedure. A limited DNase digestion of

the large complex detached fragments of roughly 10^8 molecular weight. Since a chromatid contains a single DNA molecule (6) 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 (7). 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 (8), 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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CHAPTER IV

Enzymatic analysis of the attachment of DNA to the protein structure

4. Enzymatic analysis of the attachment of DNA to the protein structure

4.1 Introduction.

In chapter 3 we described the isolation of a rapidly sedimenting DNA-protein complex from mammalian cell nuclei containing up to 90% of the nuclear DNA and 10 to 20% of the nuclear proteins. From enzymatic digestion data it was apparent that the proteins are the main components involved in the structural integrity of the complex. Digestion of nuclear lysates by increasing concentrations of DNase 1 indicated, that newly synthesized DNA is less susceptible to the enzyme than is continuously labelled DNA. From these data it was suggested that during replication additional binding takes place to the protein structure by DNA regions at the replication forks. In this chapter the association of replicating DNA with the protein structure was further investigated. To this purpose experiments were performed, in which nuclear lysates were prepared from cells either pulse-labelled for 1 or 20 minutes. The nuclear lysates were treated with various DNA-degrading enzymes and analysed by sucrose gradient centrifugation.

4.2 Methods and materials.

4.2.1 Enzymatic DNA digestions.

Nuclear lysates were incubated with DNase I (Sigma, electrophoretically purified) in the presence of 7.5 mM $MgCl_2$ for 30 min at 37°C. Incubations with Staphylococcus nuclease (Worthington) were carried out in the presence of 1 mM $CaCl_2$ for 10 min at 37°C. Incubations with Nuclease S_1 (Sigma) were done at pH 4.5. For this purpose the pH of the nuclear lysate was reduced to pH 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 performed in the presence of 1 mM $ZnSO_4$ for 30 min at 37°C. In all cases the incubations were stopped by addition of 0.25 M EDTA to a final concentration of 0.03 M.

When successive incubations were performed with Nuclease S_1 and DNase I the pH of the lysate was lowered to 4.5 as described. Nuclease S_1 was added to a final concentration of 3 µg/ml and the samples were incubated in the presence of $ZnSO_4$ as mentioned above. The incubation was terminated by

addition of 0.5 M Tris, pH 8 to a final concentration of 0.05 M. After EDTA (final concentration 2 mM) was added to the lysate the various samples were incubated in the presence of DNase 1 (final concentrations 0.3 - 1.5 µg/ml) and 10 mM $MgCl_2$ for 30 min at 37°C. The incubations were terminated as described.

4.2.2 Analysis of the size distribution of pulse-labelled nascent DNA chains.

In the various experiments aliquots of nuclei were dissolved in 0.5% SDS, 0.1 M NaOH, 10 mM EDTA. The lysates were layered on 5 - 20% sucrose gradients, prepared on a 65% sucrose cushion. Centrifugation were performed in a Spinco SW 27-1 rotor for 17 hr at 24,000 r.p.m. and 20°C.

4.3 Results.

4.3.1 Digestion of nuclear lysates by DNase I.

The DNA-protein complex, prepared from cells pulse-labelled with ^{3}H-thymidine for 20 min, showed a marked enrichment with pulse-label upon digestion with DNase I. Based on this observation it was suggested that in addition to the postulated binding of DNA at the origin of each replicon, replicating DNA is bound to the protein structure at the replication forks.

Preferential association of pulse-labelled DNA with the protein structure should be even more pronounced when cells are pulse-labelled for only short periods of time, since a much larger proportion of the pulse-label is then known to reside in Okazaki fragments in the replication forks. Figs. 1A through 1D show the results of DNase I-digestions of a nuclear lysate prepared from cells pulse-labelled for 1 minute. The $^3H/^{14}C$-ratio increased several fold when samples of the nuclear lysates were incubated with increasing concentrations of the enzyme.

In order to compare quantitatively the various experiments, in which different batches of enzymes were used, the $^3H/^{14}C$-ratios were plotted versus the corresponding percentages of pre-labelled DNA in the complex. As shown in Fig. 1E the ratio of the 1 minute pulse experiment increases more rapidly and reaches a 3 to 4 fold higher value compared to a 20 minute pulse experiment. This observation clearly indicates, that in particular the replicating DNA at the regions of the replication forks is less accessible to degradation by

DNase 1.

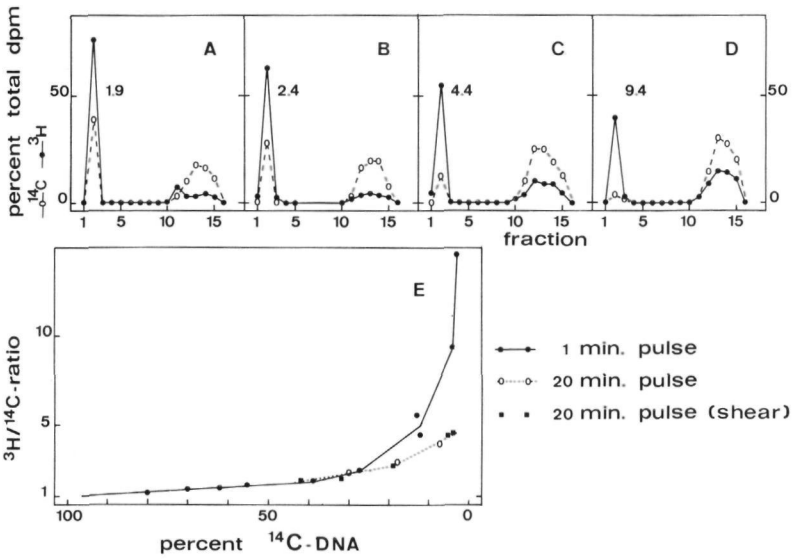

Fig. 1:

Digestion of nuclear lysates by DNase 1.
A-D: nuclear lysate was prepared from cells, pulse-labelled for 1 minute. The lysate was divided in 4 portions and DNase 1 was added to a final concentration of 0.0 (A), 0.3 (B), 0.6 (C) and 1.0 µg/ml (D). After termination of the incubations the various samples were analysed by sucrose gradient centrifugation (chapter 2, method 1). The numbers above the rapidly sedimenting peaks represent the ratios of the percentages of the ^3H- and ^{14}C-DPM, present in the complex. In all figures the direction of sedimentation is from right to left.
E: Plots of the $^3H/^{14}C$ ratios of nuclear lysates incubated with increasing concentrations of DNase 1 versus the percentage of ^{14}C-labelled DNA in the complex. Nuclear lysates were prepared from cells pulse-labelled for 1 and 20 min.
In one experiment DNA was removed from the complex by shear. Therefore samples of a nuclear lysate prepared from cells pulse-labelled for 20 min, were forced 3, 5 and 7 times through a glass capillary at 1 atm pressure. The average total DPM in gradients A-D were as follows: ^3H: 19000 DPM., ^{14}C: 11000 DPM.

4.3.2 Characterization of the DNA-protein complex by shear.

From the postulated mode of attachment of the DNA to the rapidly sedimenting structure (8) it can be inferred that the DNA should also be removable by shearing forces. Therefore samples of a nuclear lysate, prepared from cells pulse-labelled for 20 minutes, were sheared by passage through a cappillary of 0.7 mm diameter, at a pressure of 1,0 atm.

Fig. 2 shows that increasing amounts of DNA are detached from the complex under conditions of increasing shear. The released DNA appears at the top of the gradient. No DNA fragments of intermediate sedimentation rates are observed between the DNA-protein complex and the bulk DNA. In all gradients the DNA-protein complex is recovered from the top of the underlay. This

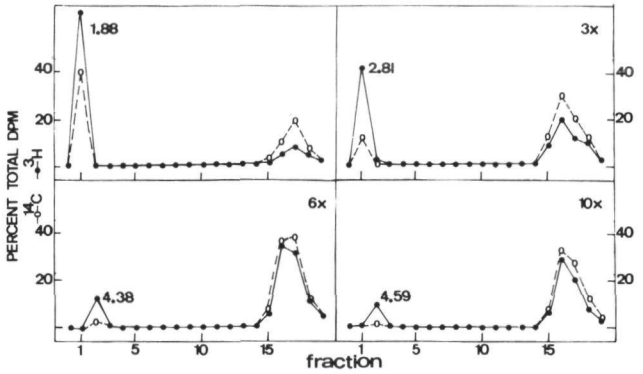

Fig. 2:

Release of DNA from the complex by shear. A nuclear lysate prepared from cells pulse-labelled for 20 min, was divided into 4 portions. The samples were pressed the indicated number of times through a glass capillary at 1.0 atm and analysed by sucrose gradient centrifugation.

indicates that under these conditions the protein structure is unaffected by shearing forces. The $^{3}H/^{14}C$-ratio of the DNA-protein complex increases 2 to 3 fold in the various shear experiments, indicating again the close association of replicating DNA with the protein structure. About 90% of the pre-labelled DNA in the complex can be easily removed by shear. The remaining DNA (3% of the total pre-labelled DNA) seems to be strongly shielded by the protein structure against shear. Obviously this DNA consists of fragments which are completely inside the protein structure or emerged from the structure to length, which are no longer subject to shear.

The results of the experiments are qualitatively similar to the DNase 1 digestion patterns of nuclear lysates, prepared from cells pulse-labelled for 20 min. Quantitative agreement is shown by plotting the $^{3}H/^{14}C$-ratios versus the corresponding percentages of pre-labelled DNA in the complex (Fig. 1E).

4.3.3 Digestion of nuclear lysates by Nuclease S_1.

In the replication models presented it is generally assumed that as a consequence of discontinuous DNA synthesis part of the parental DNA in the fork is single-stranded. To find out whether these single-stranded regions are accessible to single-strand specific nucleases, nuclear lysates were digested by Nuclease S_1 (1).

Incubation of lysates from cells pulse-labelled for 1 minute resulted in a significant reduction of the $^{3}H/^{14}C$-ratio of the DNA-protein complex

(Figs. 3A-D). This is the consequence of a very specific release of nascent DNA. At an enzyme concentration of 3 µg/ml about 30% of the pulse-labelled DNA can be detached, without detectable loss of continuously labelled DNA (Fig. 3C). In experiments using cells, pulse-labelled for 20 minutes, the reduction of the ratio of the DNA-protein complex was much smaller and corresponded to a release of 5 to 10% of the pulse-labelled DNA (Fig. 3E).

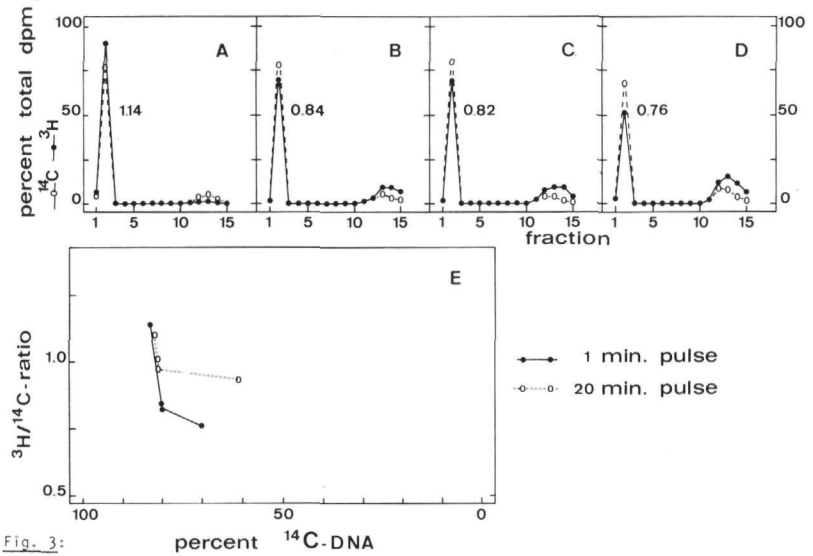

Fig. 3:

Digestion of nuclear lysates by Nuclease S_1:
A-D: A nuclear lysate was prepared from cells, pulse-labelled for 1 minute. The lysate was divided into 4 portions and Nuclease S_1 was added to a final concentration of 0.0 (A), 1.0 (B), 3.0 (C) and 10 µg/ml (D). After termination of the incubations the various samples were analysed by sucrose gradient centrifugation. E: Plots of the $^{3}H/^{14}C$ ratios of nuclear lysates incubated with increasing concentrations of Nuclease S_1. Nuclear lysates were prepared from cells pulse-labelled for 1 or 20 minutes. Each curve represents the values of a single experiment. The $^{3}H/^{14}C$ ratio of a non-incubated sample of the nuclear lysates is found at the highest percentage of ^{14}C-labelled DNA. The average total DPM in gradients A-D were as follows: ^{3}H: 12000 DPM, ^{14}C: 13000 DPM.

When the slowly sedimenting DNA fraction was further resolved, it was found, as shown in Fig. 4 that the released pulse-labelled DNA was significantly smaller than the pre-labelled material which is most probably detached from the complex by shear during the lysis procedures. These results suggest that a major part of the replicating DNA can be released from the complex probably by degradation of single-stranded gaps or nicks at the replication forks. The amount of pulse-labelled DNA released by nuclease S_1 in the various lysates, corresponds to the amount of replicative intermediates smaller than 10S, as observed after analysis of SDS-lysates on alkaline sucrose gradients (Fig. 5). To rule out a possible modification of the complex, caused

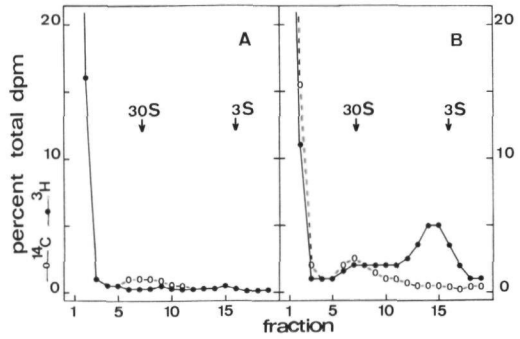

Fig. 4:

Analysis of the pulse-labelled DNA fragment released by Nuclease S_1:
A nuclear lysate was prepared from cells pulse-labelled for 1 minute. The lysate was divided into 2 samples. To one of the samples Nuclease S_1 was added to a final concentration of 3.0 µg/ml. The two samples were then analysed in sucrose gradients. A. Sample, incubated without enzyme. B. Sample, incubated in the presence of Nuclease S_1. The total DPM in gradients A and B were as follows:A: ^{3}H 57050 DPM, ^{14}C 5200 DPM. B: ^{3}H 9050 DPM, ^{14}C 5600 DPM.

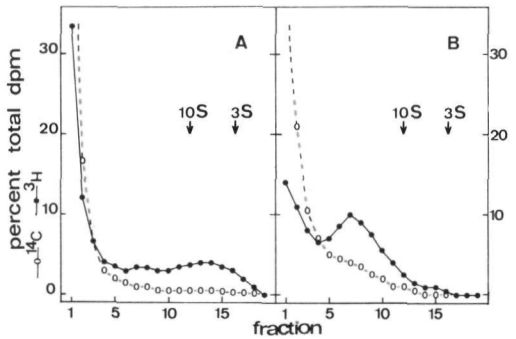

Fig. 5:

Analysis of the size distribution of pulse-labelled DNA, present in the various nuclear lysates.
Nuclei, isolated from cells pulse-labelled for 1 to 20 minutes, were lysed in alkaline SDS. The SDS lysates were analysed in alkaline sucrose gradients. A. 1 minute pulse, B. 20 minute pulse. The total DPM in the gradients were as follows: A: ^{3}H 26250 DPM, ^{14}C 20150 DPM. B: ^{3}H 2.075800 DPM, ^{14}C 14350 DPM.

by the lowering of the pH to 4.5, nuclear lysates were prepared at both pH's and analysed on sucrose gradients. It was found that the label distribution in both cases was the same, Moreover, DNase I digestion of the nuclear lysates at pH 8.0 was similar to digestion at pH 4.5 (data not shown).

4.3.4 Digestion of nuclear lysates by Staphylococcus nuclease.

In view of the differences observed between the digestion patterns of DNase I and Nuclease S_1, it seemed interesting to examine the digestion of

nuclear lysates by an enzyme containing both single- and double-strand activity. The use of this enzyme has the advantage over successive incubation with Nuclease S_1 and DNase I, in that several laborious treatments required for the sequential incubations, can be omitted.

Staphylococcus nuclease hydrolyses single-stranded DNA as well as double-stranded DNA (2). Although the enzyme has a much higher affinity for the single-stranded material (3,4), it possesses in a way the properties of both DNase I and Nuclease S_1.

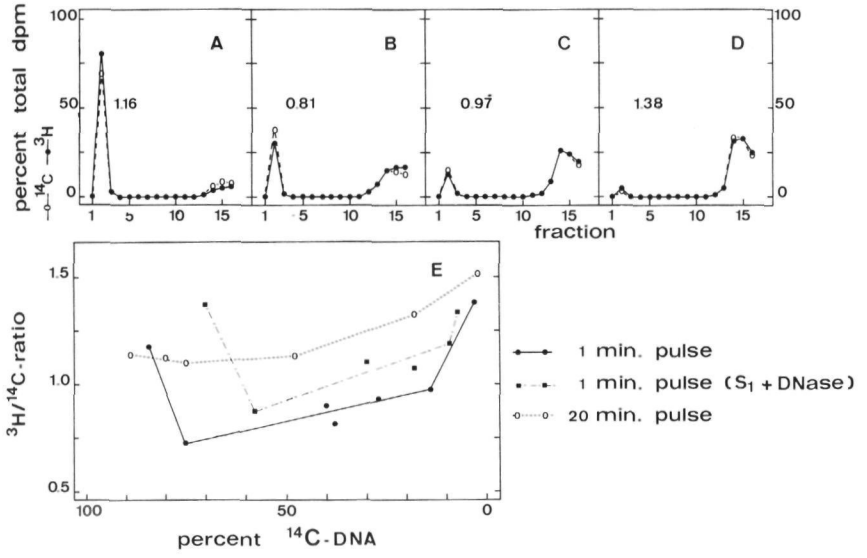

Fig. 6:

Digestion of nuclear lysates by Staphylococcus nuclease.
A-D: A nuclear lysate was prepared from cells, pulse-labelled for 1 minute. The lysate was divided into 4 portions and Staphylococcus nuclease was added to a final concentration of 0.0 (A), 0.5 (B), 1.0 (C) and 2 µg/ml (D). After termination of the incubations the various samples were analysed by sucrose gradient centrifugation.
E: Plots of the $^{3}H/^{14}C$ ratios of nuclear lysates, incubated with increasing concentration of Staphylococcus nuclease. The nuclear lysates were prepared from cells pulse-labelled for 1 and 20 minutes.
In one experiment a nuclear lysate, prepared from cells pulse-labelled for 1 minute was incubated with Nuclease S_1, and subsequently samples were digested by increasing concentrations of DNase 1. Each curve represents the values of a single experiment. The $^{3}H/^{14}C$ ratio of a non-incubated sample of the nuclear lysates is found at the highest percentage of ^{14}C-labelled DNA. The average total DPM in gradients A-D were as follows: ^{3}H 71500 DPM, C 11500 DPM.

Figs. 6A through D show the results of an incubation of a nuclear lysate prepared from cells pulse-labelled for 1 minute. With increasing concentrations, the enzyme caused the release of both pre- and pulse-labelled DNA. At the highest concentration used, more than 90% of the pre-labelled DNA was detached.

The $^{3}H/^{14}C$-ratio of the DNA-protein complex however does not vary uniformly during the course of digestion. As shown in Fig. 6E, in all digestions a reduction of the ratio was initially observed. Analogous to the digestions by Nuclease S_1, the amount of pulse-labelled DNA released, was related to the amount of small replication intermediates (data not shown). Hardly any pre-labelled DNA was released, when low enzyme concentrations were used. Obviously the detachment of the replicating DNA from the complex results from the preferential degradation of single-stranded regions.

At higher enzyme concentrations the $^{3}H/^{14}C$-ratio rose again in the complex isolated from cells pulsed for 1 minute and in the complex isolated from cells pulsed for 20 minutes. In the latter ratios distinctly higher than the initial ratio were repeatedly observed. The course of the ratio found in the experiment with lysates from cells, pulse-labelled for 1 minute, could be mimicked by a sequential treatment of the lysate with a low concentration of Nuclease S_1 and increasing concentrations of DNase I.

Thus after degradation of the single-stranded regions, Staphylococcus nuclease digests the remaining DNA in the complex similar to DNase I. As a consequence we have to assume, that the remaining replicating DNA is still more or less closely associated with the protein structure.

4.3.5 Some properties of the DNA in the complex.

In chapter 3 we proposed, that each replicon is attached to the protein structure at its origin. From this starting point it was investigated, whether the base composition of the DNA at the origins differs from the base composition of the bulk DNA. Therefore nuclear lysates were prepared from cells labelled for 40 hr with either ^{3}H-thymidine (0.1 µC/ml) or ^{14}C-thymidine. Subsequently the lysates were incubated with DNase I to reduce the amount of rapidly sedimenting DNA. Figs. 7A and B shows that in both lysates about 3% of the DNA label was recovered from the DNA-protein complex. A sample of purified ^{14}C-labelled rapidly sedimenting DNA was mixed with purified ^{3}H-labelled bulk DNA and analysed by isopycnic centrifugation in CsCl density gradients. From Fig. 7C it is clear, that the peaks of both rapidly sedimenting DNA and bulk DNA coincide at the buoyant density of calf thymus DNA. The same results were obtained when the DNA was obtained by shearing. Consequently the base composition of the DNA presumably containing the origins of replication, is

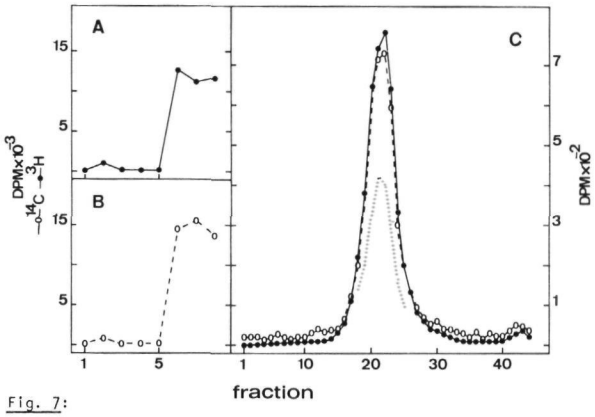

Fig. 7:

Isopycnic centrifugation in CsCl of DNA, obtained from the complex and free DNA. A and B: Nuclear lysates were prepared from cells pre-labelled with ^{3}H-thymidine (0.1 µC/ml) or ^{14}C-thymidine for 40 hr. DNase 1 and $MgCl_2$ were added to final concentrations of 1 µg/ml and 7.5 mM. The samples were incubated for 30 min at 37°C and DNase 1 was inactivated by heating for 10 min at 60°C. Both lysates were separated by sucrose gradient centrifugation. The complex in the two gradients contained respectively 2.9% (A) and 2.4% (B) of the labelled DNA. C: A sample of ^{14}C-labelled rapidly sedimenting DNA (fraction2) was mixed with ^{3}H-labelled free DNA (fractions 6-8). The DNA was purified (chapter 2, section 2.6) and banded in CsCl. ---- Calf thymus DNA.

very similar to the bulk DNA. An analogous observation was recently reported (5). From the buoyant density it follows, that rapidly sedimenting DNA as well as bulk DNA are double stranded. This was also confirmed by chromatography of purified DNA on hydroxylapatite columns. We analysed the length of the DNA in the complex by sucrose gradient centrifugation. For this purpose the amount of DNA in the complex was reduced by DNase 1 digestion or shear. Fig. 8 shows as an example the sedimentation of rapidly sedimenting DNA, representing 8.5% of

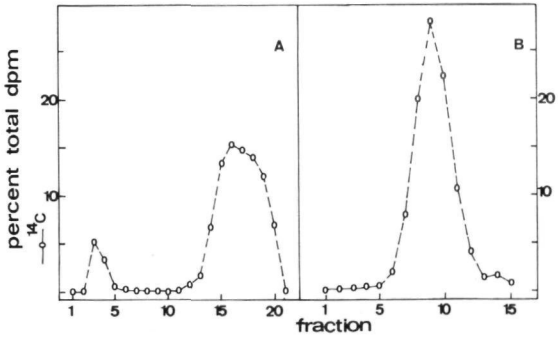

Fig. 8:

Sedimentation profile of purified rapidly sedimenting DNA. A: A nuclear lysate was prepared from cells pre-labelled with ^{14}C-thymidine. DNase 1 and $MgCl_2$ were added to final concentrations of 0.25 µg/ml and 7.5 mM. The lysate was incubated for 30 min at 37°C, subsequently heated for 10 min at 60°C and separated by sucrose gradient centrifugation. B: Purified DNA from the complex, containing 8.5% of the labelled DNA, was analysed in a 5-20% linear sucrose gradient. 70% of the label were recovered between 17 and 23S.

the total nuclear DNA. In general the average sedimentation constant of the rapidly sedimenting DNA decreases as more DNA is removed by DNase 1 digestion or shear (Table 1). Assuming the molecular weight of 1 µm of DNA to be 2.0×10^6, the length of the DNA, corresponding to the average sedimentation constant, varies from 2.0 µm (3.7% rapidly sedimenting DNA) to 13.7 µm (20% rapidly sedimenting DNA).

Table 1:

% labelled DNA in the complex (1)	Total number of base pairs $\times 10^{-8}$ (2)	Average sedimentation constant	Molecular weight $\times 10^{-6}$ D. (3)	Number of base pairs/ DNA molecule	Number of attachment sites (4)
3.7	6.29	17.0	3.98	5 940	106 000
8.4	14.28	20.0	6.44	9 612	148 000
21.7	36.89	33.0	27.40	40 918	90 000

Number of replicons/nucleus (5): 110 000

Table 1:

Sedimentation constants of purified rapidly sedimenting DNA and the calculated number of attachment sites/ nucleus.

(1). Determined from the label distribution in sucrose gradients.

(2). The total number of base pairs present in the rapidly sedimenting DNA was calculated from $18.3 \cdot 10^{-12}$ g DNA/nucleus (6).

(3). The molecular weight corresponding to the average sedimentation constant was calculated from the Studier equation (7). From this value the number of base pairs/DNA molecule was calculated.

(4). The number of attachment sites/nucleus is the coefficient of the total number of base pairs and the number of base pairs/DNA molecule, assuming one binding site per single DNA molecule.

(5). The number of replicons/nucleus was estimated from $18.3 \cdot 10^{-12}$ g DNA/nucleus and an average replicon length of 50 µM.

4.4 Discussion.

In order to explain the spatial and temporal organization of the DNA replication during the S-phase and the segregation of the daughter molecules during mitosis, models have been proposed which postulate scaffoldlike structures to which DNA is attached. According to the model proposed by Dingman (8), DNA is attached to a nuclear structure at the origin of each replicon. The DNA between successive anchoring points is thought to emanate from the structure as a loop (Fig. 9).

Recently E.M. studies presented firm evidence in support of this model. Residual metaphase chromosomes, which were histone-depleted either by treatment with heparin and dextran-sulphate or by extraction with 2 M NaCl,

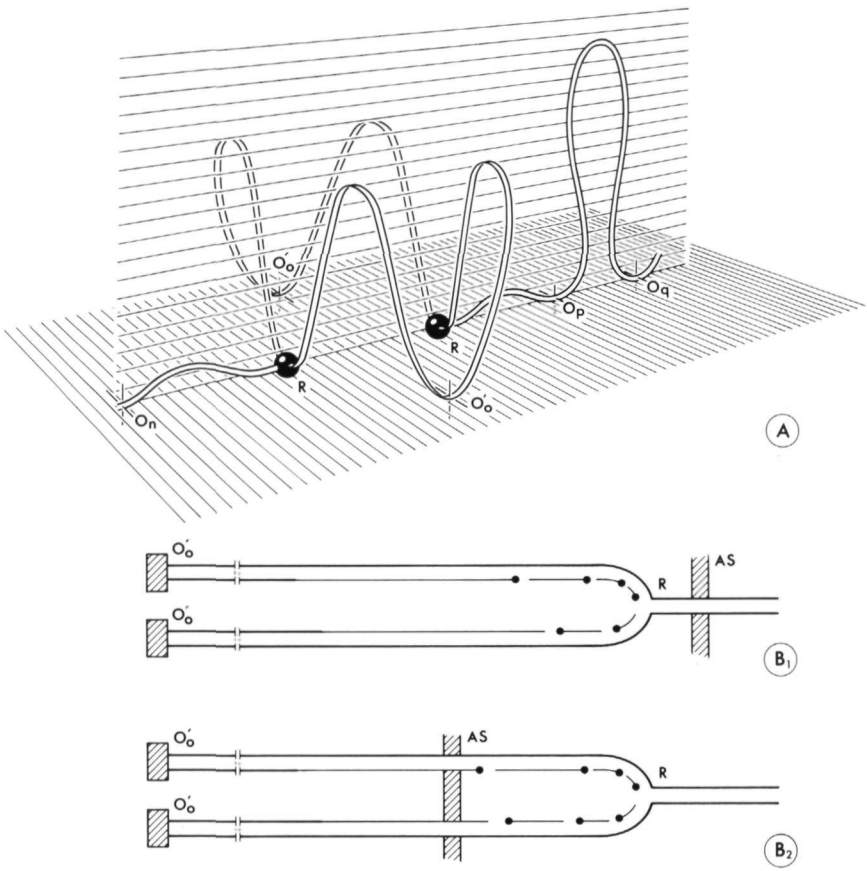

Fig. 9:

A. Model of the spatial organization of DNA in the nucleus according to Dingman (8). DNA is attached to the protein structure 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 protein structure takes place at the replication forks (R).
B. B_1 and B_2 represent two possible modes of attachment of the replication fork to the protein structure. The results presented above are compatible with the occurrence of an attachment site (AS) behind the branch point.

were shown to consist of a central non-histone protein core (scaffold") surrounded by DNA. The latter consisted of DNA-loops of roughly replicon-length, anchored to the scaffold at two points in the immediate vicinity of each other (9,10). Indirect evidence of a possible compatible binding of chromosomal DNA to a nuclear protein structure, resistent to high concentrations of NaCl (1-2 M), has also been obtained with interphase nuclei (11). It was found that replicative DNA was attached by additional binding sites close to the replication fork. This finding is important, because it

presents evidence for another postulate of the Dingman model, which is the attachment of the replication point. The aim of this study was to further characterize this binding by means of digestion of the attached DNA by various DNA degrading enzymes.

As can be deduced from Fig. 9A, digestion of the DNA-protein complex by an enzyme which induces random breaks in the DNA, will result in a preferential loss of continuously labelled DNA. Consequently it can be expected that the complex will become enriched with pulse-labelled DNA. This expectation was confirmed by DNase I digestion of nuclear lysates from cells pulse-labelled for different times. From the observed difference between the final ratios of lysates prepared from cells pulse-labelled for 1 and 20 minutes it can be concluded that the attachment of the replicating DNA to the matrix has to occur rather close to the replication fork.

Discontinuous DNA replication gives rise to transient single-stranded regions (11), as depicted in Figs. $9B_1$ and $9B_2$. It can be reasoned that the attachment of replicating DNA to the matrix takes place over the entire region actively engaged in the replicating process. In addition, binding could also occur at certain defined points, for instance behind or ahead of the branch point, or at certain defined regions such as the single-strand gaps.

To discriminate between these alternatives, nuclear lysates were digested by the single-strand specific nuclease S1. It was found that degradation of the single-stranded regions in the DNA-protein complex by low concentrations of this enzyme caused the release of part of the nascent label without detectable loss of pre-labelled DNA. From the incorporation data and the estimated number of active replicons, the expected amount of pre-labelled DNA, necessarily accompanying the loss of the nascent DNA, was estimated to be below the ^{14}C-background. The amount of pulse-label released was found to be quantitatively related to the amount of label present in the Okazaki fragments. Although these data do not rule out the possibility that DNA is bound to the structure over very short regions in the single-strand gaps. they certainly indicate that binding over the whole region actively replicating is highly improbable as this would prevent release of the Okazaki fragments from the structure.

To discriminate between the other alternatives mentioned, it was chosen to investigate whether the structural organization was retained in spite of

removal of the single-stranded DNA regions. In Figs. $9B_1$ and $9B_2$ two possible modes of attachment are shown. According to the first model, digestion of the single-strand gaps would result in detachment from the matrix of the two duplicated strands. Contrary to this, the binding of the two strands would be retained if the attachment would take place as shown in Fig. $9B_2$. To investigate which of the alternatives is most probable, nuclear lysates were digested by Staphylococcus nuclease. Digestion of the DNA-protein complex by low concentrations of this enzyme released Okazaki fragments as does S1. If the DNA would be attached to the structure as shown in Fig. 9B1, the remaining pulse-label would be situated at a free end. Digestion with higher concentrations of Staphylococcus nuclease should then cause a further rapid release of pulse-label. However, the results reported above show just the contrary: subsequent digestion causes preferential release of pre-labelled DNA as indicated by the increase of the $^{3}H/^{14}C$-ratio. This suggests that the remaining pulse-label is still attached to the nuclear structure after removal of the most recently replicated part of the fork (Fig. 9B2). These data, however, do not rule out the other possible modes of attachment mentioned above, i.e. additional binding slightly ahead of the fork or binding over short regions in the single-strand gaps.

Concerning the mode of binding of replicating DNA to the nuclear structure, one could ask why the binding sites should be situated behind the branch points. The reason for this mode of attachment might be understood from its consequence. It is obvious that replication of the DNA double helix requires unwinding of the two parental strands. This unwinding process has to occur ahead of the branch point. If, in addition to attachment at the origins, binding to the nuclear structure takes place behind the branch points, as indicated by our results, it is apparent that any reentanglement of the two daughter double helices will hereby have become impossible. As a consequence, at the end of S-phase each nucleus will contain two DNA-molecules, which can be separated easily during mitosis.

Nuclear DNA can be released from the protein structure by either enzymatic digestion or shear. Basing the results of the shear experiments about 3% of the nuclear DNA seem to be tightly bound to the protein structure. Comparable amounts of residual DNA have been reported in preparations of nuclear membranes (for review, see 12), the nuclear protein matrix (13) and

nuclear ghosts (14). The base composition of the residual DNA, presumably containing the origins of replications, is very similar to bulk DNA. Thus the specific binding of nuclear DNA to the protein structure does not result from a different base composition of the origins although it can not be excluded that the residual DNA fragments were still too large to show minor differences in CsCl density gradients. Alternatively the specific binding could reside in a unique nucleotide sequence contained by the tightly bound DNA.

From the estimated average molecular weights of the DNA fragments in the complex, it is possible to calculate the number of attachment sites per nucleus. Table 1 shows that the number of attachment sites corresponds to the calculated average number of replicons per nucleus. However it must be stated, that these estimations are only preliminary. An accurate calculation can be made by taking in account the number of molecules in the various size classes of DNA, present in the DNA-protein complex.

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Analysis of the whole mount structure and polypeptide composition of the DNA-protein complex

5. *Analysis of the whole mount structure and polypeptide composition of the DNA-protein complex*

5.1 *Introduction.*

In chapters 3 and 4 we described the isolation of a DNA-protein complex from mammalian cell nuclei by sedimentation of nuclear lysates in 1 M NaCl through sucrose gradients. The results suggested that nuclear DNA is attached to a structural component which largely consists of proteins. During replication additional binding to the protein structure seems to take place by DNA regions at the replication forks.

The complex contained between 10 and 20% of the nuclear proteins. In this chapter we present results of biochemical and ultrastructural studies on this DNA-protein complex. Also, evidence will be provided that the structure is related to the pore-complex lamina described by Blobel and co-workers (1,2,3) and to the nuclear matrix described by Berezney and Coffey (4,5).

5.2 *Results.*

5.2.1 *Electrophoretic analysis of the rapidly sedimenting complex.*

When nuclear lysates in 1 M NaCl are sedimented through sucrose gradients a rapidly sedimenting DNA-protein complex separates from the dissociated proteins and detached DNA (6). Since larger yields than those obtained originally were required for analysis of the protein components of the rapidly sedimenting structure, the isolation procedure had to be modified accordingly. Sedimentation patterns of the finally adopted procedure again showed a complete separation of the rapidly sedimenting complex from the soluble nuclear material (Fig. 1). The proportion of nuclear proteins present in the complex varied between 10 and 20% in similar experiments.

Separation patterns obtained by SDS gel electrophoresis of the proteins of this material are shown in Fig. 2. In the first experiment the rapidly sedimenting material was prepared according to the original standard procedure (6), i.e. by omitting the additional nuclear purification step but including the heat treatment. The electrophoretic pattern showed a number of prominent bands (Fig. 2A, slot 2), of which those numbered 1 through 9 were non-histone

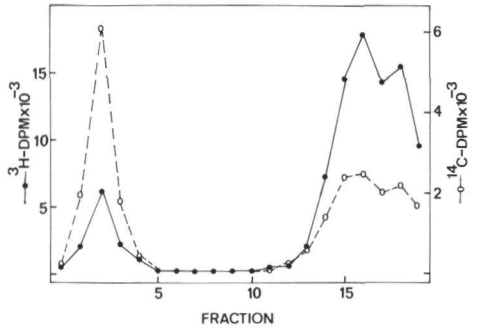

Fig. 1:

Preparation of the rapidly sedimenting complex by sucrose gradient centrifugation from 1 Roux bottle of cells labelled with ^{3}H-leucine and ^{14}C-thymidine. Samples of 10 ml were layered on top of 6 gradients. After centrifugation, samples of 0.5 ml were taken from the combined fractions of 6 identical gradient for the determination of the radio-activities.

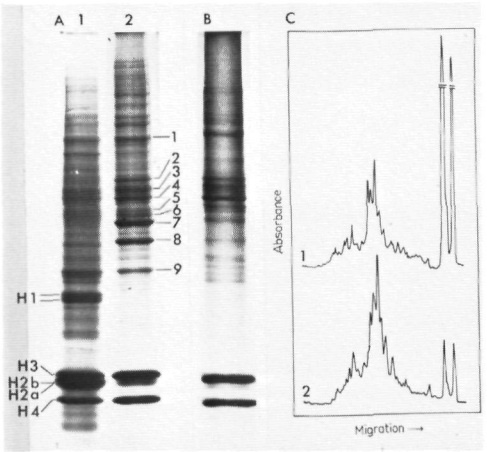

Fig. 2:

SDS-polyacrylamide electrophoresis of pooled protein fractions of sucrose gradients. A. Electrophoretic patterns of free proteins (slot 1) of the upper zone (corresponding to fraction 15 to 19 of fig. 1), and of the rapidly sedimenting complex (slot 2). Only 1/3 of the sample of the free proteins was used. Lysates were prepared according to the original standard procedure (6). B. Electrophoretic pattern of the rapidly sedimenting complex of an unheated lysate which was prepared from nuclei after an additional purification stap. C. Scanning patterns of gels of rapidly sedimenting complexes prepared in 1 M NaCl (1) and 2 M NaCl (2) respectively.

polypeptides. Some of these bands were absent and others were less pronounced in the pattern of the slowly sedimenting protein fraction (Fig. 2A, slot 1). Histones H3 and H4 were present in both fractions but other histones were practically absent from the rapidly sedimenting complex. Other differences in band patterns were poorly reproducible. The overall electrophoretic pattern of the slowly sedimenting protein fraction was distinctly different

from that of the rapidly sedimenting material.

When lysates were prepared by the modified procedure, i.e. including an additional purification of the nuclei but omitting the heat-treatment, the electrophoretic pattern were largely devoid of the polypeptide band 7,8 and 9 (Figs. 2B and 2C). Experiments, not reported here, showed that bands 7 and 8 were lost from the nuclei by the extended extraction with Triton-Tris, which is part of the additional purification step. Band 9 represents a polypeptide which appeared to become associated with the DNA-protein complex by the heat-treatment. Obviously none of them form a structurally important part of the rapidly sedimenting complex. Figs. 2B and 2C shows that the other 6 non-histone bands were reproducibly present, even when lysates were prepared in 2 M NaCl. The molecular weights of the polypeptides 1 to 6, as determined according to Weber and Osborn (7) were respectively: 105, 75, 70, 64 and 61.10^3 Daltons (bands 5 and 6). Minor changes in the stain intensity of the various polypeptide bands were usually observed. The amounts of histones H3 and H4 in the protein-DNA complex were extremely variable. In general they were much less at higher NaCl concentrations.

5.3.2 The effect of DNase and $MgCl_2$ on the complex.

It has been shown previously that DNA can be removed from the rapidly sedimenting complex by DNase treatment without loss of the structure that confers to it the high rate of sedimentation (6). We have, therefore, studied the effect of such treatment on the protein composition. To this aim the rapidly sedimenting material was obtained from the lysate by high speed centrifugation according to method 2 (chapter 2). When the washed sediment was resuspended in 1 M NaCl and centrifuged through a sucrose gradient, more than 70% of the label appeared as a rapidly sedimenting complex (Fig. 3B). The remainder is probably contaminating, and includes, among other proteins, histones H2a and H2b (see Fig. 4 slots 1 and 2) which are removed from the complex in the presence of NaCl (Fig. 3A and 3B). The $^{14}C/^{3}H$ ratio of the washed sediment was 4.72, as compared to a ratio of 4.6 for the rapidly sedimenting material obtained from the same lysate, by centrifugation through a sucrose gradient according to method 1 (Fig. 3F). In addition, the protein composition of the washed sediment (Fig. 4, slot 1) was the same as that of the rapidly sedimenting complex obtained by method 1 (Fig. 2B), except for the slight

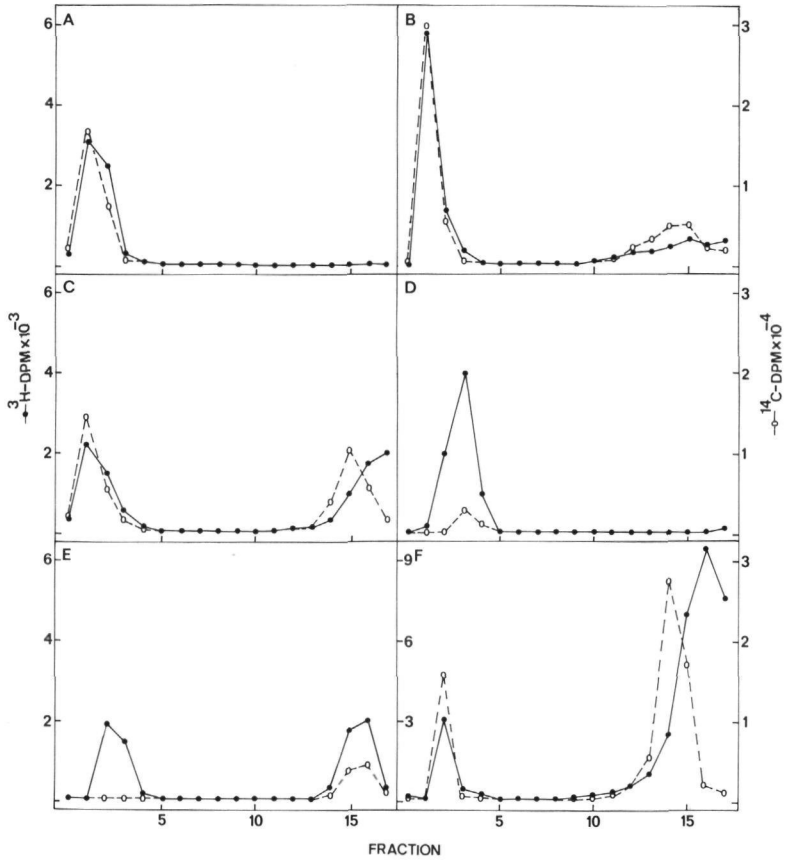

Fig. 3:

Effects of various treatments on the DNA-protein complex. Rapidly sedimenting material was prepared from cells of 3 unlabelled and 1 labelled Roux bottle according to method 2. The final pellet was suspended in 50 ml 50 mM Tris buffer pH 8 and divided into 5 equal parts, which were treated as follows: A. untreated. B. 5 ml 3 M NaCl were added. C. 5 ml 1 M $MgCl_2$ were added. D. DNase I and $MgCl_2$ were added to final concentrations of 50 µg/ml and 7.5 mM respectively, and the sample was incubated for 30 min at 25°C. E. The same treatment as D, 5 ml 1 M $MgCl_2$ were added after the incubation.
Samples A and D were brought to 15 ml by adding 5 ml Tris buffer. 5 ml of each sample were centrifuged in sucrose gradients. F is a 5 ml sample of the original lysate. Symbols are the same as in fig. 1.

contamination mentioned above. Thus, for the most purposes method 2 provides equally useful material.

Digestion of the washed sediment by DNase I made almost 90% of the DNA acid soluble without removing any protein label from the rapidly sedimenting structure (Fig. 3D). In addition electrophoretic analysis showed that the protein composition of the sediment was not affected by the treatment

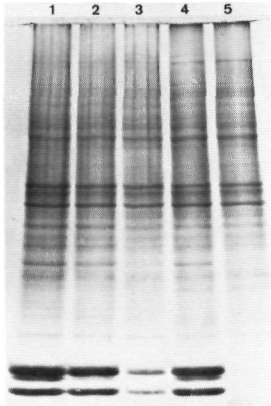

Fig. 4:
Effects of various treatments on the polypeptide composition of the DNA-protein complex. The remaining 10 ml of each sample of fig. 3 were centrifuged for 30 min at 8 000 r.p.m. and 20°C. Slots 1 to 5 are electrophoretic patterns of the pellets obtained from the samples A through E respectively.

(Fig. 4, slots 1 and 4).

At a concentration of 0.33 M $MgCl_2$ about 50% of the protein label dissociated from the rapidly sedimenting complex. This was independent of whether the material had been digested with DNase prior to the addition of $MgCl_2$, or not (Fig. 3C and 3E). Analysis of the protein composition by gel electrophoresis revealed that $MgCl_2$ selectively removed the residual amount of the histones H3 and H4 (Fig. 4, slots 3 and 5). The final preparation contained 5% of the total nuclear proteins. $MgCl_2$ also displaced a part of the DNA from the rapidly sedimenting complex (Fig. 3C), and less than 0.1% of the total DNA label was left when the $MgCl_2$ was added after a DNase treatment (Fig. 3E). This is a similar effect as obtained by direct DNase digestion of the original NaCl lysate (6). Treatment with RNase neither affected the protein composition nor altered the sedimentation pattern of the rapidly sedimenting material.

5.2.3 Association of additional proteins with the residual protein structure.

As described in section 1 the rapidly sedimenting complex can be isolated with additional polypeptides, depending on the isolation of the nuclei. We investigated whether these proteins are bound to the structure or mainly to DNA. Therefore the rapidly sedimenting material was prepared in 2 M NaCl from nuclei, which were not subject to additional purification in Triton-Tris, and digested by DNase 1. The electrophoretic pattern of the pellet, obtained by

centrifugation of the digested material, contained three additional bands (Fig. 5, slots 1 and 2). Two distinct polypeptides of 45 and 55000 Daltons, which correspond to bands 7 and 8 in Fig. 2, were observed together with a high molecular weight polypeptide of moderate staining intensity. Although some minor bands are removed the three major polypeptides are not released from the protein structure by an additional wash of the rapidly sedimenting material in Triton-Tris (Fig. 5, slot 2). Fig. 5, slot 3 shows the polypeptide composition of the residual protein structure, obtained from purified nuclei.

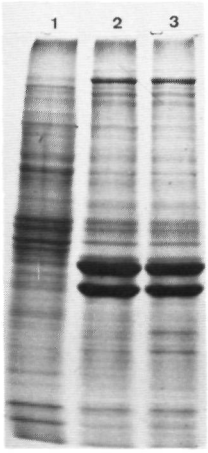

Fig. 5:

The polypeptide composition of the residual protein structure obtained from unpurified nuclei. The DNA-protein complex was prepared in 2 M NaCl from either rapidly isolated nuclei or highly purified nuclei. The complex was resuspended in 10 ml 50 mM Tris-buffer pH 8 per Roux bottle and incubated at 25°C for 60 min with 50 µg/ml DNase 1 and 7.5 mM $MgCl_2$. The digested material prepared from unpurified nuclei, was divided in two equal parts and 10% Triton was added at a final concentration of 0.1% to one portion. Incubation was then continued for 30 min at 25°C. After termination of the incubations each sample was centrifuged for 30 min at 8 000 r.p.m. and 20°C and the pellets prepared for electrophoresis. Slot 1: Pellet from purified nuclei. Slot 2: Pellet treated with an additional Triton-Triswash. Slot 3: Pellet from unpurified nuclei.

5.2.4 Whole mount structure of the rapidly sedimenting complex.

The washed sediment as obtained by method 2 contained ghost-like remnants of the nuclei and fragments of these. They were recognized as such by the presence of annular structures (outer diameter 80 - 120 nm) which are part of the nuclear pore complex (8). The annuli were still associated with

a less regular, two-dimensional structure identified as lamina of the nuclear envelope (3). Large masses of DNA fibres were spreading out from the fragments. Individual strands, which could be removed by digestion with DNase, could be traced over distances of several µm. They appeared to emerge in greater numbers from the annular structures (14). Electronmicroscopic examination of the residual protein structures, obtained from the complex by DNase treatment and high salt extraction, revealed two different structures. Among the material, sheats of interconnected annuli very similar to those reported for the complex-lamina (3,10) were observed (Fig. 6).

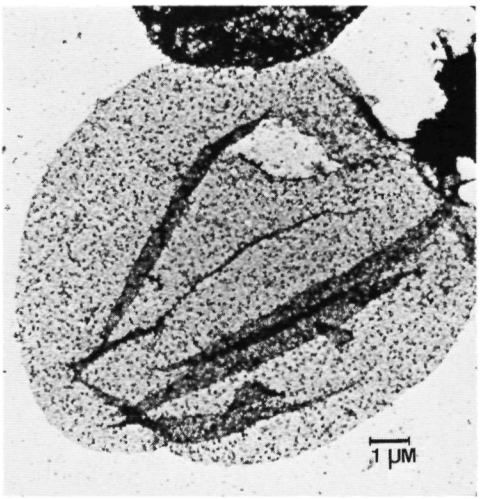

Fig. 6:
Whole mount preparation of the rapidly sedimenting complex digested with DNase and extracted with 0.33 M $MgCl_2$ (chapter 2). PTA staining. Courtesy of A.G.M. Bekers.

However, more frequently the residual protein structures were constituted of a pore-complex lamina associated with strongly stained structures (Fig. 7).

It is difficult to conclude from the two-dimensional images of whole mount preparations whether a three-dimensional nuclear matrix as demonstrated in sections (4) is present. However, Comings and Okada (10) have shown in a comparative study that strongly stained structures such as those in undisrupted or only partially disrupted nuclear ghosts (Fig. 7) represent intra-nuclear matrix material. It should be noted that the diameters of the nuclear ghosts were 2 to 3 times larger than those of whole mount preparations of nuclei (8). This is due to the sudden increase of the nuclear volume upon

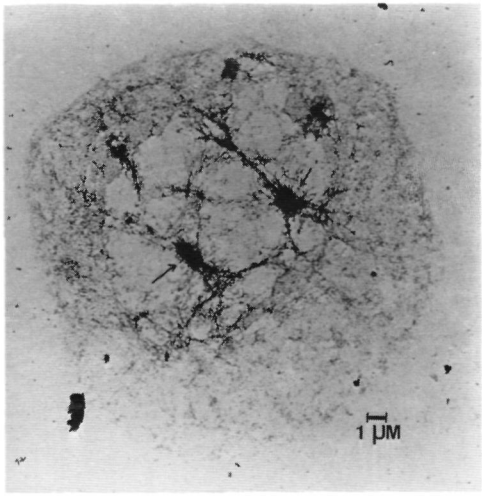

Fig. 7:
Whole mount preparation of the rapidly sedimenting complex digested with DNase and extracted with 0.33 M $MgCl_2$ (chapter 2). The electron dense structures (arrows) probably represent the intra-nuclear matrix. The pore complex-lamina is not significantly affected by the treatment. PTA staining. Courtesy of A.G.M. Bekers.

addition of the concentrated NaCl solution, which usually causes the varying degrees of disruption (6).

It should be kept in mind that, due to this expansion, the residual structures in the electron micrographs appear less compact than they would be in the nuclei in vivo.

When residual protein structures were prepared from nuclei, which were not subject to additional purification in Triton-Tris, the nuclear ghosts were surrounded by an irrigular fibrous network (Fig. 8). The diameter of the smallest fibers observed was about 5 nm. The electronmicrographs of this network resembled the cytoskeleton of tissue culture cells, which includes the nucleus and microfilament bundles (11,12,13).

It should be noted that the diameter of the nuclear ghosts inside the fibrous network corresponded to the diameter of whole mount preparations of nuclei (8). Obviously the cytoskeleton limits the increase of the nuclear volume upon addition of concentrated NaCl.

5.2.5 *Comparison of the rapidly sedimenting complex with other preparations of residual nuclear structures.*

As already mentioned, our ultrastructural data suggest that the rapidly sedimenting complex consists of fragments of the pore complex-lamina (1,2,3)

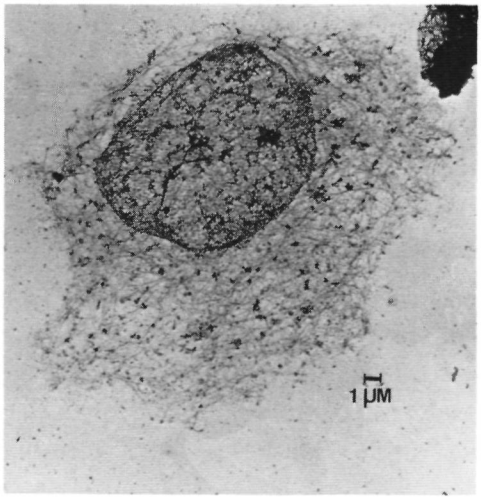

Fig. 8:

Whole mount preparation of the rapidly sedimenting complex, prepared from unpurified nuclei in 2 M NaCl and digested by DNase 1. PTA staining. Courtesy of A.G.M. Bekers.

and parts of the intra-nuclear matrix (4,5,10). Electrophoretic patterns showed a clustering of polypeptide bands in the 60 to 75 000 Daltons range as also reported for the latter structures. However, the molecular weights determined by us did not correspond fully with those reported by the other authors. We have therefore made nuclear matrix and pore complex-lamina preparations according to the methods described originally (2,4). Nuclear spheres (4) and ghosts (2) were identified in the preparations by phase contrast and electron microscopy (14). The various electrophoretic protein pattern and a pattern of the total nuclear proteins are shown in Fig. 9. All 3 preparations exhibit a selective enrichment of the bands 2,3 and 4. There is also a good correspondence of several minor bands, in particular between the patterns of the rapidly sedimenting complex and the structural protein matrix. The pore complex-lamina lacks polypeptide 1 but shows an additional band at a higher molecular weight. The differences between the molecular weights of the polypeptide 2,3 and 4 and those reported by other investigators might therefore be due to species specificity as suggested by Berezney and Coffey (5).

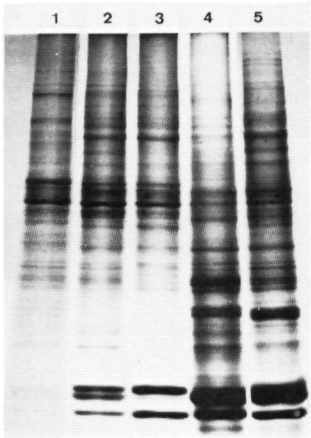

Fig. 9:
Comparison of electrophoretic patterns of various nuclear preparations.
Slot 1: pore complex-lamina prepared according to Aaronson and Blobel (2).
Slot 2: structural protein matrix of the nucleus prepared according to Berezney and Coffey (4).
Slot 3: the rapidly sedimenting DNA-protein complex.
Slot 4: free polypeptides from the upper zone of the sucrose gradientshown in fig. 1.
Slot 5: total polypeptides of nuclei isolated in Triton-Tris.

5.3 Discussion

Nuclear DNA appears to be attached to a nuclear component which retains its structural integrity at NaCl concentrations that dissociate chromatin into DNA and histones. The complex can be isolated by sucrose gradient centrifugation with up to almost 90% of the nuclear DNA remaining attached to it if the lysate is prepared very gently (6).

The findings reported here confirm that the structure consists, at least to a substantial part, of protein. Electrophoretic analysis shows basicly 3 major and 3 minor bands of non-histone polypeptides. In addition 2 polypeptides of 45 and 55 000 Daltons are present when lysates are prepared very rapidly. They can be extracted, however, by washing the nuclei thoroughly with 0.1% Triton before the NaCl lysate is prepared.

Varying amounts of histones H3 and H4, accounting usually for about 50% of the total protein content of the rapidly sedimenting structure are also present. They are not bound to the DNA which remains attached to the rapidly sedimenting structure. This follows from the observation that they are not released from the latter when the DNA is removed for more than 99% by

DNase digestion. The histones can be extracted from the isolated complex with 0.33 M $MgCl_2$. It seems possible that their binding occurs artificially when they are dissociated from the chromatin in the 1 M NaCl concentration. This could explain why the amounts of histones bound to the complex vary so greatly. The constant H3/H4 ratio may be due to the formation of strong dimers or tetramers at a 1:1 ratio of the two histones (15). In contrast to Berezney and Coffey (4) we find also a marked contamination by the 2 histones in the nuclear matrix preparations. A similar observation was already reported by Hodge et al. (16). The reason for this discrepancy is not clear.

The ultrastructural data show that the rapidly sedimenting complex consists mainly of the pore complex-lamina, in association with the intra-nuclear matrix. Preparations of both structures have been reported to contain 3 dominant polypeptides in the 60 to 70 000 Daltons range (1-5, 10). It is possible, therefore, that preparations of the 2 components are usually cross-contaminated with each other, indicating a rigid association between the pore complex-lamina and the intra-nuclear matrix. A similar protein composition has also been found in so-called nuclear ghosts (17,18). In our preparations the molecular weights of the 3 prominent non-histone polypeptides are 64, 70 and 75.10^3 Daltons. Bornens and Kasper have shown that 3 polypeptides with the same molecular weight are present in nuclear membranes of rat liver, but not in microsomal membranes (19). It is known that nuclear membranes and the pore complex-lamina form the nuclear envelope and are not separated from each other by the usual isolation procedures.

Our results indicate, that the nuclear matrix is associated with a fibrous network, probably corresponding to the cytoskeleton of the cell. According to Osborn and Weber (12) the cytoskeleton of HeLa cells consists of two major polypeptides of 43 000 (corresponding to actin) and 58 000 Daltons together with a high molecular weight polypeptide. These polypeptides correspond very well to the three additional polypeptides in nuclear ghost preparations of unpurified nuclei. However in contrast to their findings the network was soluble in Triton and the 45 000 Daltons polypeptide did not comigrate with actin from bovine muscles (chapter 2) nor with actin from rabbit muscles (F. Ramakers, personal communication), although 5 nm filaments are observed in the fibrous network.

Recently it was shown by Letko et al. (20) that a polypeptide of 58 000

Daltons, consisting of 10 nm filaments, was associated with the major polypeptides of the nuclear matrix of human fibroblasts. They suggest, that these filaments are the major components which anchor the nucleus in nuclear monolayers. It is possible that, despite the observed differences in molecular weight, this polypeptide is also present in our preparations, thus contributing to the structural integrity of nuclear matrix and cytoskeleton. However it is important to note, that high salt treatment probably results in a irreversible modification of the proteins of the fibrous network, since the proteins are not removed by a subsequent Triton-Tris wash.

Preliminary evidence suggests that the nuclear DNA molecules, regardless whether they are replicating or not, are attached to the rapidly sedimenting structure by one binding site per replicon (6). Replicating DNA, in addition might be bound to it via regions at the replication forks. Autoradiographic studies have revealed that DNA replication occurs throughout the nucleus (21,22,23). Replicative DNA, therefore, should be attached to the intra-nuclear matrix rather than the nuclear envelope. This is consistent with results of labelling experiments (5). Biochemical data on this subject are inconsistent in that some authors find a preferential association of newly synthesized DNA with the nuclear envelope (24-27) while others do not (28,29). A possible explanation might be that, depending on the isolation procedure, a variable proportion of the matrix material carrying the rapidly labelled (i.e. replicating) DNA might remain attached to the nuclear envelope. This is supported by the observation that DNA fibers are attached to the intra-nuclear matrix (10). Our own electron micrographs show an abundant association of DNA with the pore complex-lamina. The latter might therefore contain the putative attachment sites for the origins of replicons, as suggested by Dingman (30).

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CHAPTER VI

Fractionation of the DNA-protein complex in SDS-sucrose gradients

6. *Fractionation of the DNA-protein complex in SDS-sucrose gradients*

6.1 Introduction.

As described in the previous chapters the DNA-protein complex appears to be resistent to high ionic strength (1-2 M NaCl). In order to dissociate the complex completely, centrifugation of the nuclear lysates were performed in the presence of sodium dodecylsulphate (SDS), since it was shown by Wanka (1) that in vitro cultures of bovine liver cells can be easily dissolved in SDS.

In preliminary experiments we observed that low concentrations of SDS only solubilized a fraction of the proteins of the DNA-protein complex. No release of DNA from the complex was observed. Since it has been shown that the rapidly sedimenting protein structure consists of several ultrastructural components, we investigated the possibility that SDS preferentially solubilizes proteins which correspond to a particular substructure.

In this chapter we describe the separation of nuclear lysates through SDS-sucrose gradients and the composition of the SDS treated DNA-protein complex and the dissociated proteins.

6.2 Methods and materials.

SDS-sucrose gradient centrifugation.
All gradients were prepared in nitrocellulose tubes. All solutions used contained 50 mM Tris/HCl buffer pH 8. Centrifugations were performed in a Spinco SW 27-2 rotor.

6.2.1 Analytical gradients.

A 27 ml 15 - 40% sucrose gradient, containing a linear gradient of 0.0 - 0.1% (w/v) SDS, was prepared from equal volumes of 15% sucrose and 40% sucrose, 0.1% SDS. This gradient was layered over a 5 ml cushion of 70% sucrose, 0.1% SDS. Before addition of the sample (4 ml) a spacer containing 1.5 ml 15% sucrose was placed at the top of the gradient to prevent precipitation of SDS in the presence of NaCl. Samples were centrifuged at 5000 r.p.m. and a temperature of 25°C for different periods of time. The times are

indicated in the legends of the figures. Similar gradients containing 1 M NaCl were used in control experiments.

6.2.2 Preparative gradients.

7 ml 40% sucrose, 0.5% SDS was layered over a 4 ml cushion of 70% sucrose, 0.5% SDS. Subsequently a spacer of 4 ml 35% sucrose was carefully placed on top of the SDS-sucrose layer, followed by 14 ml of a 15 - 25% sucrose gradient containing 1 M NaCl. 6 Samples of 10 ml each were centrifuged simultanously for 3 hr at 8000 r.p.m. and a temperature of 25°C and the corresponding fractions were collected for electrophoretic analysis. In control experiments similar gradients containing 1 M NaCl were prepared on 70% sucrose shelfs containing 0.4 g/ml CsCl.

6.3 Results.

6.3.1 Sedimentation analysis of nuclear lysates in SDS-sucrose gradients.

Since bovine liver cells are easily solubilized by the detergent sodium dodecylsulfate (1), we investigated the effect of SDS on the structural stability of the DNA-protein complex. Samples of nuclear lysates were therefore sedimented at a moderate rotor speed for different times through sucrose gradients, containing a linear gradient of SDS.

Fig. 1 shows the results of such an experiment, in which a nuclear lysate was prepared from cells labelled for 40 hr with both radioactive thymidine and with amino acids. After centrifugation for 20 min at 5000 r.p.m. through a sucrose gradient containing 1 M NaCl, the DNA-protein complex was accumulated on the sucrose shelf and consisted of 64% of the labelled nuclear DNA and 35% of the labelled nuclear proteins (Fig. 1A). A sample of the lysate centrifuged under the same conditions through a sucrose gradient, containing 0.0 - 0.1% SDS, showed marked differences (Fig. 1B). No material was found at the concentrated sucrose shelf. Instead both the DNA and proteins appeared entirely in the upper half of the gradient, indicating a considerable decrease of the rate of sedimentation of the DNA-protein complex.

Upon continued centrifugation (Fig. 1C and 1D) the nuclear lysate was separated in three distinct fractions. Besides the free DNA and proteins at

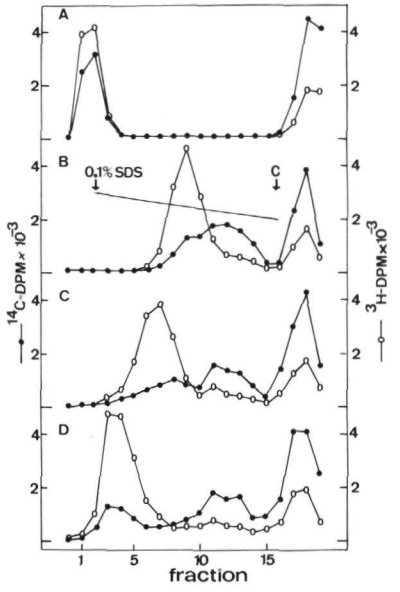

Fig. 1:

Separation of nuclear lysates through SDS-sucrose gradients. A nuclear lysate was prepared from cells pre-labelled with ^{14}C-leucine, ^{14}C-lysine and ^{3}H-thymidine. The lysate was divided in 4 equal portions and the various samples were centrifuged through sucrose gradients, containing either 1 M NaCl (A) or 0 - 0.1% SDS (B-D).
A. 20 min, 5 000 r.p.m. B. 20 min, 5 000 r.p.m. C. 40 min, 5 000 r.p.m. D. 120 min, 5 000 r.p.m.

the top of the gradient about 55% of the DNA and 12% of the proteins sedimented as a broad peak into the lower half of the gradient. The $^{3}H/^{14}C$ ratio was constant over the top fractions of the peak (Fig. 1D, fractions 3,4 and 5), suggesting that DNA and proteins were still bound to each other. Between these two fractions about 10% of the DNA and 27% of the proteins (Fig. 1D, fractions 9-14) were recovered as a broad peak, which did not sediment significantly under these conditions. The amount of DNA dissociated by the detergent, was somewhat variable, but did not exceed more than 10% of the total labelled DNA. However, in most experiments no DNA at all was detached.

Thus under the conditions described SDS dissociates a major part of the proteins but only a minor part of the DNA from the DNA-protein complex.

6.3.2 *Electrophoretic analysis of the polypeptide composition of the SDS-treated DNA-protein complex and the dissociated proteins.*

Since more material was required for electrophoretic analysis, nuclear lysates were prepared from Roux bottles and separated through adapted SDS-

sucrose gradients. Instead of centrifugation through a linear SDS-sucrose gradient, samples were centrifuged through a 7 ml sucrose layer, containing a constant SDS concentration of 0.5% as described in section 6.2.2.

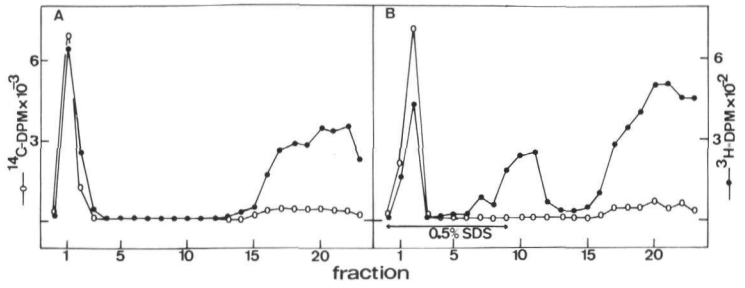

Fig. 2:

Preparative SDS-sucrose gradient centrifugation.
60 ml nuclear lysate was prepared from cells, pre-labelled with ^{3}H-leucine and ^{14}C-thymidine and separated by centrifugation through a 40% sucrose layer, containing either 1 M NaCl (A) or 0.5% SDS (B).

Fig. 2A shows the sedimentation pattern of a nuclear lysate, obtained from cells pre-labelled with thymidine and amino acids, through a sucrose gradient containing 1 M NaCl. The DNA-protein complex was composed of 65% of the labelled nuclear DNA and 25% of the labelled proteins. No material was found between the complex and the free DNA and proteins at the top of the gradient. In the corresponding gradients containing SDS, likewise a rapidly sedimenting DNA-protein complex was recovered on the concentrated sucrose shelf (Fig. 2B). This complex consisted of 65% of the labelled DNA and 12% of the labelled proteins. Thus under these conditions none of the DNA was detached from the complex by SDS. The dissociated proteins were found in a broad peak at the boundary of the SDS-sucrose layer and represented 60% of the labelled proteins originally associated with the complex. The dissociation of the proteins appeared to be complete since the detached proteins were found in a discrete peak, entirely separated from the free DNA and bulk proteins and the remaining complex. Fig. 3, slot 1 shows the polypeptide composition of the untreated DNA-protein complex, which was composed of the nuclear matrix polypeptides (bands 1-5), the two polypeptides of 45 000 and 55 000 Daltons (bands 6 and 7) and histones H_3 and H_4. After centrifugation through SDS a number of polypeptides were selectively removed from the DNA-protein complex Fig. 3, slots 2 and 3). The bands 2,4,6 and 7 and the histones were either completely or almost completely dissociated from the complex (slot 3). The

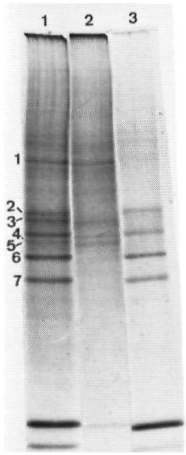

Fig. 3:
SDS-polyacrylamide electrophoresis of pooled fractions of SDS-sucrose gradients obtained as described in Fig. 2.
Slot 1: DNA-protein complex, from gradients containing NaCl.
Slot 2: DNA-protein complex, from gradients containing SDS.
Slot 3: dissociated proteins.

depleted complex still contained the nuclear matrix polypeptides 1,3 and 5. In Fig. 4 it is demonstrated that polypeptides 2 and 4 represent the major polypeptides of the pore complex-lamina (2).

It should be noted that the remaining DNA-protein complex is not stable in the presence of SDS. After storage of 48 hr at -20°C and subsequent dialysis, the DNA of the SDS treated complex appeared to sediment as protein free DNA (Fig. 5A and 5B) and 75% of the proteins appeared at the top of the gradient (Fig. 5A, fractions 11-18). About 14% of the proteins were recovered as a protein peak at the concentrated sucrose shelf.

Incubation of nuclear lysates with 100 µg/ml RNase A for 2 hr at 37°C did not change the sedimentation pattern in SDS-sucrose gradients nor the polypeptide composition of the various fractions.

6.3.3 Sedimentation pattern of replicative DNA in SDS-sucrose gradients.

In chapter 5 it was suggested, that the attachment sites of the nuclear DNA and its replication points are situated at different morphological structures, i.e. the pore complex-lamina and the intra-nuclear matrix respectively. Since SDS preferentially released some proteins from the DNA-protein complex which might correspond to one of the distinct substructures, we investigated

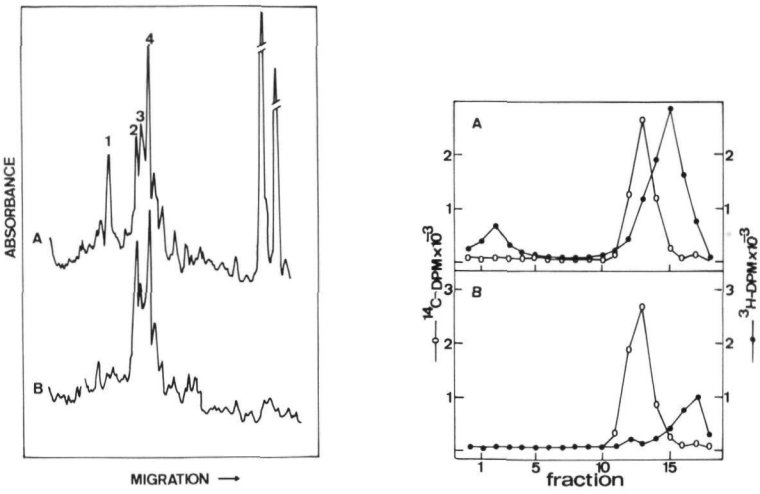

Fig. 4:

Densitometer tracings of the polypeptides of the DNA-protein complex and the lamina-pore complex. The DNA-protein complex (A) was isolated from purified nuclei by sucrose gradient centrifugation. The pore complex-lamina (B) was obtained according to Aaronson and Blobel (2).

Fig. 5:

Recentrifugation of the SDS treated DNA-protein complex.
Samples from the concentrated sucrose shelf of preparative SDS-sucrose gradients (fig. 2) were kept at -20°C for 48 hr and dialysed against 2 vol. of 500 ml. 50 mM Tris pH 8, 0.15 M NaCl and divided in 2 equal portions. A. 5 ml dialysate, B. 5 ml dialysate, incubated with 100 µg/ml pronase for 2 hr at 25°C. The various incubations were terminated by addition of 10% SDS to a concentration of 0.5% and the samples were centrifuged in a SW 27-2 rotor, containing a 15-40% sucrose gradient for 15 hr at 15 000 r.p.m. and at a temperature of 18°C.

the binding of replicative DNA in the presence of SDS. Therefore a nuclear lysate was prepared from cells pulse-labelled for 1 min and analysed through SDS-sucrose gradients. Fig. 6A shows the separation of a sample of the nuclear lysate through a sucrose gradient containing 1 M NaCl. The $^{3}H/^{14}C$ ratio (see chapter 4) of the DNA-protein complex was about 5.88, indicating a preferential association of nascent DNA with the complex. No label was found between the DNA-protein complex and the free DNA at the top of the gradient. When a sample of the lysate was centrifuged through a SDS-sucrose gradient the remaining DNA-protein complex was recovered in the lower half of the gradient indicating a reduction of its rate of sedimentation (Fig. 6B). No detachment of nascent DNA was observed since the $^{3}H/^{14}C$ ratio of this complex (5.63) was very similar to the control experiment and no label appeared between the two peaks in the gradient.

In further control experiments, samples of nuclear lysate prepared from cells pre-labelled with amino acids, were centrifuged either through a

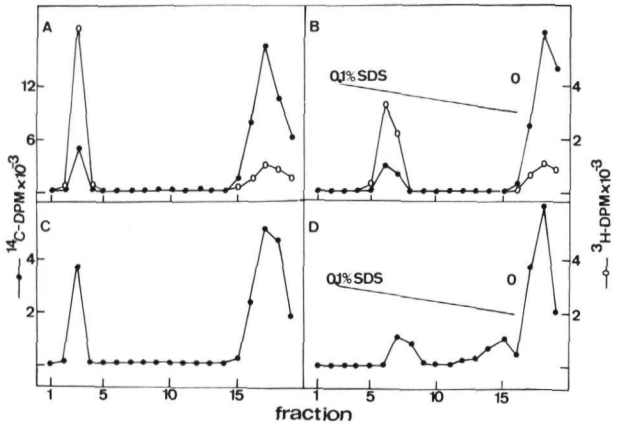

Fig. 6:

Separation of replicating DNA through SDS-sucrose gradients. ^{14}C-thymidine and pulse-labelled for 1 min with ^{3}H-thymidine (A and B). In a control experiment a nuclear lysate was prepared from cells, pre-labelled with ^{14}C-leucine and ^{14}C-lysine (C and D).
Samples were centrifuged through sucrose gradients, containing either 1 M NaCl or 0 - 0.1% SDS.
A and C: centrifugation through a sucrose gradient containing NaCl, for 120 min and 5 000 r.p.m.
B and D: centrifugation through a sucrose gradient containing SDS, for 120 min and 5 000 r.p.m.

gradient containing 1 M NaCl or a SDS-sucrose gradient (Fig. 6C and 6D). In the SDS-sucrose gradient about half the labelled proteins originally present in the complex, appeared as dissociated proteins in the upper portion of the gradient (Fig. 6D, fractions 14 and 15). The latter observation indicates that the failure to remove pre-labelled or pulse-labelled DNA was not due to an insufficient dissociation of proteins by SDS.

6.4 Discussion.

From the results presented in this chapter it is apparent that SDS under the conditions used, does not completely solubilize the DNA-protein complex immediately. Instead a DNA-protein complex which sedimented at a very reduced rate compared to the original complex, was still observed in SDS-sucrose gradients. In most experiments no DNA was detached from the complex although SDS removed a major part of the proteins. However the association of DNA and protein after treatment with SDS is not stable since the residual complex dissociates upon storage at -20^{o}C and subsequent dialysis. Electrophoretic analysis of the dissociated proteins showed that the residual histones of the complex are almost completely removed from the complex by SDS. Since none of DNA was detached, we conclude that histones are not involved in the binding

of DNA to the complex. Because a major proportion of the amino acid label is incorporated in histones, the concentration range of SDS necessary to dissociate the proteins, is very similar to that found to remove histones from calf thymus chromatin (3).

In addition the polypeptides of 45 000 and 55 000 Daltons are fully dissociated from the complex. It was demonstrated in chapter 5 that these two polypeptides are not important for the structural integrity of the complex. Thus it is perhaps not supprising that removal of these proteins could occur without disruption of the DNA-protein complex. Treatment with SDS also solubilized two polypeptides of 75 000 and 64 000 Daltons from the complex. These two polypeptides have been shown to be major polypeptides occuring in preparations of the pore complex-lamina, the protein matrix and the DNA-protein complex. Obviously the presence of these polypeptides in the complex is not required for the binding of the DNA to the complex although their involvement in additional arrangement of DNA is not excluded. The proteins of the residual complex are polypeptides which correspond to major constituents of the protein matrix and the DNA-protein complex. These polypeptides are found in only small amounts in the pore complex-lamina. Thus these biochemical data suggest that at least some constituents corresponding to the pore complex-lamina are removed from the complex by SDS. Moreover the remaining structures still contain the attachment sites for newly synthesized as well as continuously labelled DNA.

Preliminary electron microscopic observations have indicated that structures probably corresponding to the dense lamina and nuclear pores are removed by the SDS treatment. (A. Bekers, personal communication). Interestingly Kirschner et al. (4) investigating the structure of nuclei by high resolution scanning microscopy, noted the disappearance of nuclear pores and dense lamina from the nuclear surface after treatment with 1% DOC. Yet the treated nuclei retained their integrity despite the absence of any limiting peripherical structures. As pointed out by these authors, the integrity of the DOC treated nuclei could be possibly due to the intranuclear protein matrix.

If SDS preferentially removes the nuclear pore complexes it is supprisingly that no DNA or only a small amount of the total nuclear DNA is detached since an attachment of chromatin to nuclear pores is frequently observed (5,6 and 7). Possibly DNA molecules are also bound to other nuclear structures

such as the intranuclear matrix described by Berezney et al. (8) and only a minor fraction of the DNA is bound to nuclear pore complexes. The reduced rate of sedimentation of the remaining complex may indicate a drastic alteration of the matrix structure. In particular removal of the dense lamina in the absence of possible physical interdigitations (4) of the chromatin is expected to result in a more or less desintergration of the nuclear shape and a possible expansion of the intranuclear matrix. Alternatively SDS affects the conformation of the DNA in the complex. It has been shown in nuclear structures called nucleotides (9), which are comparable to the rapidly sedimenting DNA-protein complex, that the attached DNA is supercoiled (10). It is possible that the removal of certain proteins by SDS results in a relaxation of the DNA and consequently in a strong reduction of the sedimentation rate of the remaining complex (10).

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Dissociation of the DNA-protein complex and DNA binding experiments

7. Dissociation of the DNA-protein complex and DNA binding experiments

7.1 Introduction.

As described in the previous chapters it is assumed that the nuclear DNA is bound to the matrix at the origins of replication. From this assumption we investigated whether the matrix proteins, free of DNA, could be preferentially reassociated with the DNA originally present in the DNA-protein complex. In order to obtain matrix proteins essentially free of DNA we attempted to dissociate the DNA-protein complex without the use of SDS. The dissociation of the DNA-protein complex was obtained using 5 M urea and 2 M NaCl, a medium which has been widely used to dissociate and solubilize chromatin (1-4). The dissociated matrix proteins could be separated from histones and DNA by high speed centrifugation and chromatography on hydroxylapatite. The binding of these proteins to purified DNA was studied in detail.

7.2 Methods and material.

7.2.1 Purification of the matrix proteins.

Samples of DNA-protein complex were obtained from 10-to 20 Roux bottles of cells using high speed centrifugation (chapter 2, method 2). Per Roux bottle 10 ml of 5 M urea, 2 M NaCl and 1 mM phosphate pH 6.8, were added and the pellet was homogenized by forcing it through a hypodermic needle. The suspension was kept at 4°C for 24 hr and stirred continuously. The suspension was subsequently centrifuged for 16 hr at 40000 r.p.m. and at 15°C in a Spinco SW-40 rotor. The supernatant was bound to a column which contained 5 g hydroxylapatite (Biorad, DNA-grade) prepared in 5 M urea, 2 M NaCl, 1 mM phosphate. Histones and other basic proteins were eluted with 50 ml of 5 M urea, 2 M NaCl, 1 mM phosphate and acidic non-histone proteins with 50 ml of 5 M urea, 2 M NaCl, 0.2 M phosphate (4).

7.2.2 Purification of DNA.

2 Roux bottles of cells were labelled for 40 hr with 0.04 μC/ml ^{14}C-thymidine. After removal of the medium the cells were grown in label free

medium for another 2 hr. The nuclear lysate was prepared and incubated with 0.25 µg/ml DNase 1 in the presence of 7.5 mM $MgCl_2$ for 30 min and 37°C. The incubation was terminated by addition of EDTA to 30 mM and heating for 10 min at 60°C. The DNA-protein complex was isolated according to method 2 (chapter 2). The pellet was resuspended in 5 ml of 50 mM Tris pH 8 and successively incubated with 50 µg/ml RNase A for 30 min at 37°C and 50 µg/ml Pronase for 90 min at 37°C. The DNA was further purified by isopycnic centrifugation in CsCl-gradients. The free DNA was obtained from the supernatant of the nuclear lysate by alcohol precipitation. The pellet obtained after centrifugation, was treated by the enzymes and prepared for isopycnic centrifugation similar to the DNA-protein complex.

7.2.3 DNA binding experiments.

Routinely, 1 ml of the 0.2 M phosphate fraction, containing 20 to 40 µg of protein/ml was mixed with 1.5 to 2.5 µg of DNA obtained from the complex. The sample was dialysed for 6 hr at 4°C against 2 vol of 500 ml of 0.15 M NaCl, 50 mM Tris pH 8. Unless stated otherwise the dialysed material was centrifuged in 15 - 40% sucrose gradients containing 0.15 M NaCl, 50 mM Tris pH 8. Centrifugations were performed in a Spinco SW 27-1 rotor for 16 hr at 20 000 r.p.m. and a temperature of 15°C.

7.3 Results.

7.3.1 Dissociation of the DNA-protein complex by urea.

We investigated whether a medium containing urea and high salt dissociated the complex. Therefore urea was added to a final concentration of 5 M to a nuclear lysate prepared in 2 M NaCl.

As shown in Fig. 1 addition of urea dissociated the DNA-protein complex. In a control sample of the nuclear lysate the DNA-protein complex consisted of 20% of the labelled proteins and 45% of the labelled DNA. In contrast after addition of urea to the lysate less than 3% of the total labelled proteins and DNA were found at the concentrated sucrose layer (Fig. 1A and 1B). After 1 hr of centrifugation both DNA and protein which were initially associated in the DNA-protein complex, were recovered, almost entirely, at the top of the gradient. Centrifugation of the untreated lysate for 16 hr showed,

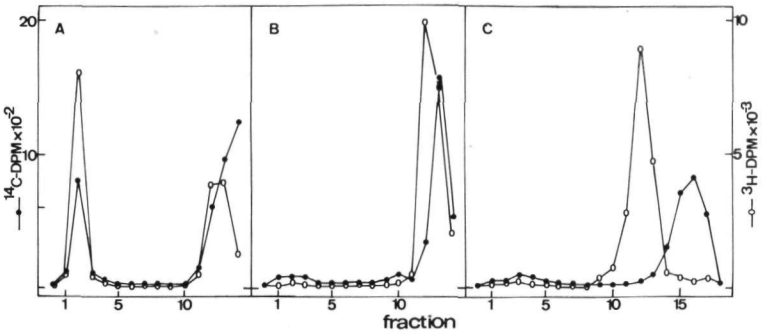


Fig. 1:

Dissociation of the DNA-protein complex by urea and NaCl.
The nuclear lysate was prepared in 2 M NaCl from cells grown in a Carrel flask and labelled for 40 hr with ^{14}C-lysine and ^{14}C-leucine and ^{3}H-thymidine. To a portion of the lysate solid urea was added to a final concentration of 5 M and the samples were kept at room temperature for 30 min. The various samples were analysed in 30 - 40% sucrose gradients, containing 2 M NaCl.
A: Sample in 2 M NaCl. B and C: Samples in 2 M NaCl and 5 M urea.
Centrifugations were performed either in a Spinco SW 27-2 rotor for 1 hr at 25 000 r.p.m. (A and B) or 16 hr at 25 000 r.p.m. (C) and a temperature of 18°C.

that the DNA-protein complex was dissociated into free DNA and free proteins (Fig. 1C).

Analogous results were obtained when 5 M urea and 2 M NaCl was added to the DNA-protein complex isolated by high speed centrifugation (chapter 2, method 2). With material isolated under these conditions over 80% of the DNA and protein label was dissociated and recovered as two separate peaks in the upper part of the sucrose gradient (Fig. 2).

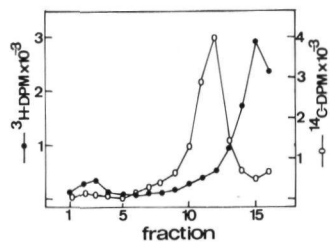


Fig. 2:

Dissociation of the DNA-protein complex by urea and NaCl.
One Roux bottle of cells was labelled for 20 hr with ^{14}C-thymidine and ^{3}H-leucine and the nuclear lysate was prepared in 2 M NaCl. The DNA-protein complex was isolated and dissolved in 20 ml 5 M urea, 2 M NaCl, 1 mM phosphate, pH 6.8. Undissolved material was removed by centrifugation for 20 min at 10 000 r.p.m. in a Sorvall HB-4 rotor and the supernatant was analysed in a 30 - 65% sucrose gradient, containing 2 M NaCl. Centrifugation was performed in a Spinco SW 27-1 rotor for 16 hr at 26 000 r.p.m. and 18°C.

For further purification, the majority of the dissociated DNA was separated from the proteins of the complex by centrifugation for 16 hr at

40 000 r.p.m. in a Spinco SW-40 rotor. By this method more than 90% of the DNA originally associated with the complex was sedimented, together with small variable amounts of protein. The polypeptide composition of the supernatant was very similar to the composition of the dissociated complex before centrifugation (Fig. 3).

7.3.2 Separation of the dissociated proteins by hydroxylapatite chromatography.

In order to separate the non-histone proteins from histones and residual DNA we employed chromatography on hydroxylapatite columns, in the presence of urea and NaCl, as described by McGillivray et al. (4). As shown in Fig. 4 a major fraction of the labelled proteins of the supernatant was not retained by the column. In agreement with previous reports (4,5) this fraction contained solely histones (Fig. 5, slot 2). The non-histone proteins were eluted from the column with 5 M urea, 2 M NaCl and 0.2 M phosphate. In this step at least 95% of the labelled proteins retained by hydroxylapatite, were recovered (Fig. 4). Electrophoretic analysis indicated that this fraction consisted of the matrix polypeptides originally present in the complex (Fig. 5, slots 1 and 3).

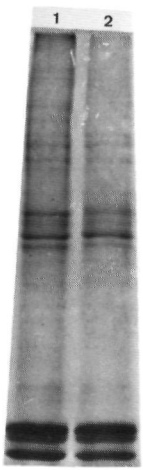


Fig. 3:

Electrophoretic patterns of the dissociated complex before and after centrifugation in the SW 40-rotor. The DNA-protein complex obtained from 5 Roux bottles according to method 2 (chapter 2) was dissolved in 60 ml 5 M urea, 2 M NaCl, 1 mM phosphate pH 6.8 and centrifuged for 17 hr at 40 000 r.p.m. in a Spinco SW 40-rotor. 3 ml Samples were taken before and after centrifugation, dialysed against 2 vol of 750 ml distilled water, and prepared for electrophoresis.
Slot 1: Samples before centrifugation.
Slot 2: Samples after centrifugation.

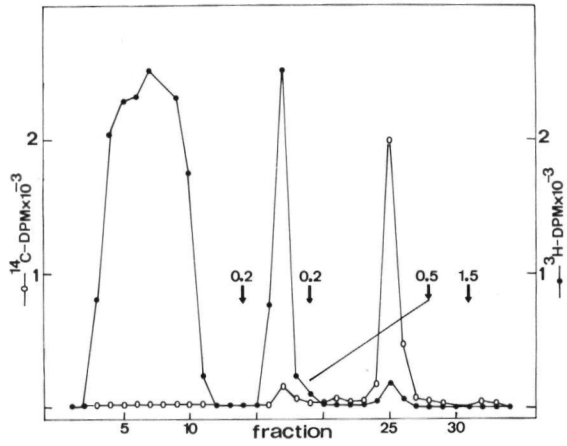


Fig. 4:

Hydroxylapatite chromatography.
The dissociated DNA-protein complex was obtained by method 2 from 5 Roux bottles of cells pre-labelled with ^{3}H-leucine and ^{14}C-thymidine for 20 hr. After centrifugation in the SW 40-rotor the supernatant was added to a hydroxylapatite column, which was eluted with urea/NaCl buffers, containing successively 1 mM phosphate (50 ml), 0.2 M phosphate (25 ml), a linear gradient of 0.2 - 0.5 M phosphate (50 ml), 0.5 M phosphate (25 ml) and 1.5 M phosphate (1.5 M).

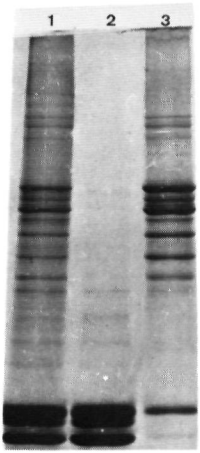


Fig. 5:

Electrophoretic patterns of the major fractions, obtained by chromatography on hydroxylapatite.
Samples were dialysed against 2 vol of 750 ml distilled water and prepared for electrophoresis.
Slot 1: The dissociated DNA-protein complex, obtained according to method 2, before centrifugation in a SW 40-rotor.
Slot 2: Protein fractions not retained by hydroxylapatite.
Slot 3: 0.2 M phosphate fraction.

A low molecular weight polypeptide normally masked by histones was observed at the position of histone H_4. The overall polypeptide composition of the 0.2 M phosphate fraction, including the low molecular weight polypeptide was similar to the composition of purified nuclear matrices (Fig. 6).

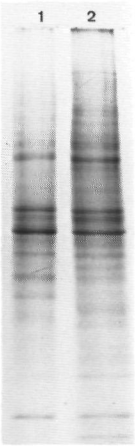


Fig. 6:
Comparison of the polypeptide composition of the 0.2 M phosphate fraction and intact nuclear matrix.
Slot 1: 0.2 M phosphate fraction.
Slot 2: Purified nuclear matrix, isolated by DNase treatment and $MgCl_2$ extraction as described in chapter 2.

The residual DNA present in the supernatant was completely retained by the column. None, or very small amounts of ^{14}C-label representing not more than 0.05% of the DNA present in the complex, were recovered in the 0.2 M phosphate fraction. This DNA was not bound to the eluted proteins because similar amounts of DNA were eluted at 0.2 M phosphate after enzymatic digestion of the proteins. The DNA retained on the column could be eluted from the hydroxylapatite at 0.4 M phosphate. Despite the presence of some protein label in this fraction no polypeptide bands were detected on polyacrylamide gels. Since the 0.2 M phosphate fraction contained the dissociated matrix proteins, free of associated DNA and histones, we examined the DNA-binding properties of this fraction.

7.3.3 DNA-binding properties of the matrix proteins.

We investigated whether the dissociated matrix proteins reassociated specifically with purified DNA after removal of urea and salt. Moreover, the

stability of the reconstituted complexes were tested under conditions which were used to isolate the DNA-protein complex.

Therefore, the DNA associated with the complex, and the free DNA were both purified by enzymatic digestion of RNA and proteins and isopycnic centrifugation in CsCl gradients. The DNA originating from the complex (called c-DNA) banded in the middle of the gradient at a density of 1.7 (Fig. 7). DNA binding experiments were performed by addition of purified c-DNA to the dissociated purified matrix proteins and dialysis of this material against 0.15 M NaCl, 50 mM Tris-HCl, pH 8.

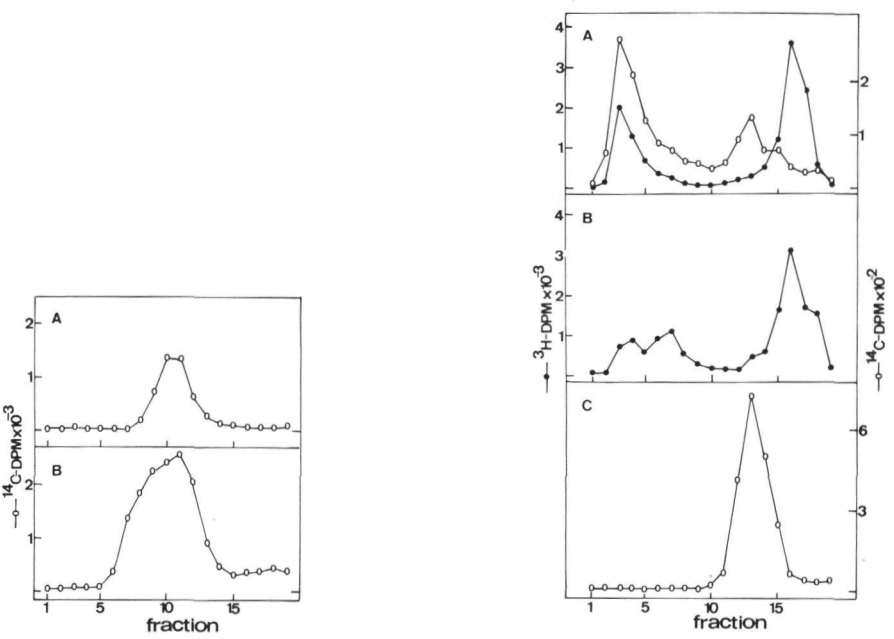


Fig. 7:

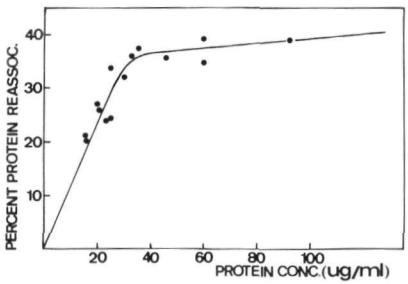
Purification of DNA in CsCl-density gradients representing 10% of the total nuclear DNA originally associated with the complex.
A: DNA isolated from the DNA-protein complex. B: free DNA.

Fig. 8:

Analysis of the reassociation of DNA with matrix proteins.
In a final volume of 1.5 ml 35 µg protein of the 0.2 M phosphate fraction was combined with 2.5 µg c-DNA (originally 10% of the nuclear DNA) and dialysed. Equal amounts of either protein or DNA were dialysed simultaneously with the joined fraction. The various samples were analysed in sucrose gradients.
A: Proteins and c-DNA. B: proteins only. C: c-DNA only.

Fig. 8 shows the results of sucrose gradient centrifugation of such dialysed material. A major fraction of the c-DNA (about 65%) was recovered in the lower half of the gradient in a broad peak associated with 34% of the proteins (Fig. 8A, fraction 1-8). When purified DNA alone was centrifuged no label was present in this region of the gradient (Fig. 8C). Centrifugation analysis of the proteins, dialysed in the absence of DNA, showed again that 34% of the proteins were found in complexes in the lower half of the gradient (Fig. 8B). The presence of DNA during dialysis caused a shift of a major portion of the reassociated proteins to the position of the reconstituted DNA-protein complex (Fig. 8A and 8B). Obviously the c-DNA can only be bound to the reassociated proteins.

In spite of the at least 10-fold excess of protein over DNA, the amount of DNA bound to the proteins varied in several experiments between 30 and 90% of the total. Similar results were obtained when the proteins were dialysed before addition of DNA. The relative amount of reassociated rapidly sedimenting protein which resulted from dialysis depended only partly on the initial concentration of the dissociated proteins (Fig. 9). At a concentration range of 30 to 100 µg/ml the percentage of reassociated proteins was almost constant



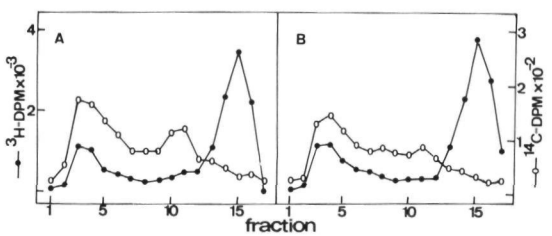


Fig. 9:

The dependence of the reassociation on the protein concentration.
Samples of 1.5 ml of 0.2 M phosphate fractions, containing different concentrations of protein were dialysed in the presence of 2.5 - 5 µg c-DNA (originally 10 - 20% of the nuclear DNA) and analysed in sucrose gradients. The percentage of the labelled proteins which reassociated (ordinate) are plotted against the protein concentration (abcissa).

Fig. 10:

DNA binding properties of the proteins of the 0.2 M phosphate fraction to c-DNA and to free DNA.
30 µg protein of the 0.2 M phosphate fraction was dialysed in the presence of 3.2 µg of either c-DNA or free DNA. The c-DNA was obtained from a DNA-protein complex, which contained 20% of the nuclear DNA. The dialysed samples were analysed in sucrose gradients.
A: c-DNA. B: free DNA.

and amounted to 30 - 40% of the dialysed proteins, indicating a possible specific association of the proteins. Fig. 10A and 10B demonstrate that no difference were observed when the dissociated proteins were dialysed in the presence of DNA obtained from the DNA-protein complex, or free DNA. In both cases 60% of the DNA was associated with the rapidly sedimenting proteins (fractions 1-8).

We further investigated the stability of the complex under conditions of high ionic strength. Since no association of DNA and protein was found when dialysis was performed against 1 M NaCl, 50 mM Tris pH 8 complexes were constituted in 0.15 M NaCl, 50 mM Tris pH 8 and centrifuged in the presence of 1 M NaCl.

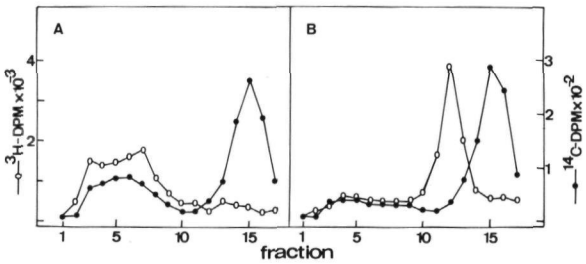


Fig. 11:
The stability of the reconstituted complex in 1 M NaCl.
35 µg protein of the 0.2 M phosphate fraction was dialysed in the presence of 2.0 µg c-DNA (originally 10% of the nuclear DNA) and the dialysed sample was analysed in sucrose gradient containing either 0.15 M NaCl or 1 M NaCl.
A: 0.15 M NaCl. B: 1 M NaCl.

From Fig. 11A and 11B it is apparent that 1 M NaCl dissociated the major part of the DNA from the reconstituted complex and decreased the amount of reassociated proteins. Nevertheless in 1 M NaCl 21% of the labelled proteins were found in the lower half of the gradient compared to 34% in 0.15 M NaCl. No differences were found when the DNA binding experiments were performed in the presence of β-mercaptoethanol.

The stability of the complex in 1 M NaCl was markedly enhanced when the proteins and DNA were combined and heated for 10 min at 60°C. In gradients containing either 1 M NaCl or 0.15 NaCl about 32% of the DNA was bound to 55% of the proteins (Fig. 12A and 12B, fractions 1-5). Obviously

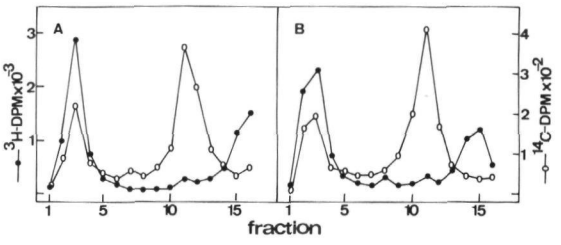


Fig. 12:

Stability of the DNA-protein complex, reconstituted at high temperature.
30 µg protein of the 0.2 M phosphate fraction was dialysed and heated to 60°C. 3.2 µg c-DNA (originally 20% of the nuclear DNA) was added at the same temperature to the proteins and the incubation was continued for 10 min at 60°C. The sample was slowly cooled to room temperature and analysed in sucrose gradients containing either 0.15 M NaCl (A) or 1 M NaCl (B).

the heating step stabilized not only the reconstituted complex, but stimulated also the reassociation of the proteins.

7.3.4 DNA binding experiments with intact matrices.

In order to investigate the DNA properties of intact nuclear structures, matrices were prepared as described in Chapters 2 and 5, except that the DNase digestion was performed for 20 hr at room temperature. Under these conditions no pre-labelled DNA was detectable in preparations of purified matrices. For binding experiments, 5 µg of c-DNA or free DNA was added to a 1 ml suspension of purified nuclear matrices (100 µg protein/ml) in 0.15 M NaCl, 50 mM Tris pH 8, 20% glycerol. The samples were kept at 25°C for 1 hr and then centrifuged in a 15-40% sucrose gradient in a Spinco SW-27.1 rotor for 1 hr at 25 000 r.p.m. Under these conditions no significant binding of DNA purified from the complex, or free DNA to the nuclear matrix was observed.

7.4 Discussion.

In the presence of 1 to 2 M NaCl the major portion of the nuclear DNA is still bound to the nuclear matrix. Thus this association is apparently not primarily stabilized by electrostatic interactions, but might be mainly based on hydrophobic interactions and hydrogen bonds. Evidence for this assumption was obtained by the dissociation of the complex in urea and NaCl. Addition of urea and NaCl to the nuclear lysate or isolated complex dissociated

the DNA and the matrix proteins. Since solutions of 5 M urea containing 1 to 2 M NaCl are often used to dissociate chromatin (1-4), it is very likely that certain preparations of chromatin proteins include matrix proteins. This suggestion is advanced by the finding that preparations of erythrocyte chromatin (6) and mouse liver chromatin (7) contained nuclear matrix proteins.

McGillivray et al. (4) have reported that histones, acidic proteins and nucleic acids can be separated in a single step by column chromatography on hydroxylapatite in the presence of urea and NaCl. In agreement with their observations, the histones present in the complex were not bound to the column. Since acidic proteins are retained (4) the matrix proteins must be considered to belong to the nuclear acidic proteins. This assumption is supported by the amino acid analysis of the rat liver matrix, which showed an acidic/basic ratio of 1.46 (8). The presence of a low molecular weight polypeptide in the electrophoretic pattern of 0.2 M phosphate fraction may indicate the presence of residual amounts of specifically modified histone H_4, histone non-histone complexes or low molecular weight acidic proteins. The available data do not support the existence of a specifically modified form of H_4 nor the occurrence of stable histone non-histone complexes in urea/NaCl (9,10). However low molecular weight nuclear acidic proteins are well known (10). It must be noticed that the low molecular weight polypeptide forms an integral component of the residual nuclear structure, since it is found in both the DNA-protein complex isolated in 2 M NaCl and in preparations of purified nuclear matrix and even after centrifugation through SDS (chapter 6).

We also investigated whether DNA-protein complexes could be reconstituted from the dissociated purified matrix proteins and purified DNA. Such reconstituted complexes should meet certain requirements. First of all the renatured proteins should bind to DNA. Since it is supposed that DNA is bound to the matrix via the origins of replication, only pieces of DNA containing such origins should bind. Analogous to the original complex a reconstituted complex should consist of DNA bound to a reassociated protein structure. Moreover the reconstituted complexes should be stable in 1 M NaCl.

From the DNA binding experiments it is apparent that the dissociated and dialysed matrix proteins only partially meet the criteria mentioned above. After dialysis in 0.15 M NaCl at least 40% of the dissociated proteins reassociated into complexes. Since it has been shown that chromatin proteins,

dissociated in urea/NaCl, can be effectively renaturated (11), specific proteins may be involved in the reassociated complexes although this has to be confirmed by electrophoresis. Purified DNA originating from the rapidly sedimenting comples as well as free DNA bind to these reassociated protein complexes. The binding of DNA may be regarded to be electrostatically as the majority of the bound DNA is redetached in the presence of 1 M NaCl. Indeed such electrostatic interactions may cause the observed non-specific binding of DNA. Instead of the assumed origins of replication non-specific regions of the DNA molecules might be involved in the binding to the reassociated matrix proteins. In addition the structural form of DNA depending on the presence of nucleosomes, might be necessary for correct association. It should be noticed that the extent of binding of DNA to the reassociated proteins is not great, as a more than 10-fold excess of protein over DNA is necessary to bind appreciable amounts of DNA. Possibly, only a minor component of the reassociated proteins is involved in the binding of DNA although other explanations are throughout acceptable.

After heating, the DNA seems to be bound to the reassociated proteins by interactions other than electrostatic, although it is unknown whether such an interaction represents the in vivo binding of DNA and the matrix proteins. At the moment it still has to be found out whether such binding is specific or not. Possibly protein to protein associations are disrupted which make otherwise certain regions of the proteins inaccessibly for DNA.

It is apparent that compared to the reassociated protein complex an intact matrix may represent much more the in vivo situation of the cell. In contrast no binding at all was observed when DNA was added to purified matrices. This could be a result of the presence of some nucleotides at the DNA binding sites which can not be removed by enzymatic digestion. Alternatively, it is possible that removal of DNA allows protein to protein interactions to occur which are not found in vivo. The preliminary observation that purified matrices are not soluble in urea/NaCl suggests that the latter may occur to some extent. In agreement with the results presented above Comings et al. (12) reported that only the proteins of the dissociated rat liver matrix binds to DNA under physiological conditions. Henry et al. (13) found that only a very small percentage of matrix proteins of HeLa cells (1 - 4%) could associate with calf thymus DNA. It is clear however that intensive investigations are needed

to resolve the nature of the binding of DNA to the nuclear protein matrix as data on the DNA binding properties of the matrix proteins are presently insufficient to allow conclusions to be drawn. Nevertheless, it is important to note that the results described in this chapter confirm the previous conclusion that the attachment of DNA to the matrix is not due to an artificial attachment occuring during the lysis of the nuclei.

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CHAPTER VIII

Some aspects of the synthesis of the proteins of the DNA-protein complex

8. Some aspects of the synthesis of the proteins of the DNA-protein complex

8.1 Introduction.

From the data presented in chapters 3 and 4, it is apparent that the protein matrix provides sites for attachment of the nuclear DNA and plays an important role in the process of DNA replication.

When, as a result of DNA synthesis, origins of replication are duplicated the number of attachment sites should increase. The required new attachment sites could be accomplished by de novo synthesis of various proteins of the complex during the period of DNA replication. Such a synthesis could also function of course as a regulatory device for the start of S-phase. Alternatively the binding of new origins could occur on pre-existing attachment sites. Such a process could be regulated by a cell cycle dependent modification of the matrix proteins for example by phosphorylation. In this regard Berezney and Coffey recently reported that phosphorylation of the matrix proteins in regenerating rat liver occurs to a maximal level at the time just preceeding the onset of DNA replication (1).

From the ultrastructure of the DNA-protein complex it is apparent, that nuclear pore complexes are major components of the protein structure. Moreover it has been suggested that nuclear pore complexes may play a role in the spatial organization of chromatin (2-4). In both mammalian cells (5) and in Physarum Polycephalum (4), but not in yeast (6), the number of pore complexes increases at the beginning of the S-phase. It is to be expected that such a change would be reflected in a cell cycle dependent synthesis of the proteins of the complex. In this chapter, we investigated the synthesis of the proteins of the complex by pulse-chase experiments. The temporal relationship between the synthesis of the proteins and DNA synthesis was also examined.

8.2 Methods and materials.

8.2.1 Gel autoradiography.

Samples of the DNA-protein complex were analysed in SDS polyacrylamide gels as described in chapter 2. The stained gels were dried on Whatman 3 MM paper and covered with X-ray film (Kodak, RP Royal X Omat). Films were

processed in a Kodak developer.

8.3 Results.

8.3.1 Incorporation of radioactive leucine and thymidine during cell growth in modified growth media.

In order to investigate the synthesis of the proteins of the complex, radioactive amino acids were added to growing monolayers of cells for short periods of time. When radioactive aminoacids were added to cells growing in normal medium the incorporation into protein was insufficient for gel autoradiography or for other investigations. The incorporation was improved when cells were grown during the period of pulse-labelling in either leucine free MEM-medium or in a medium which contained a reduced concentration of lactalbumine hydrolysate (L.A.H.) (0.005% (w/v)) (Fig. 1). We examined whether such modified media affected the growth of the cells.

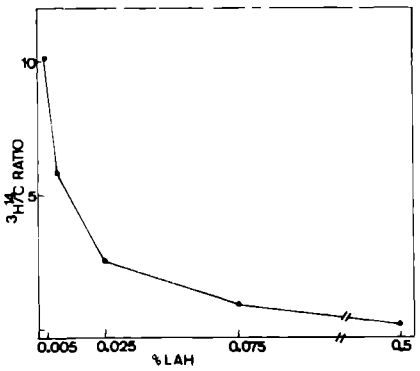


Fig 1

Cells were grown as monolayers on glass coverslips placed in a Petri dish DNA was pre-labelled by addition of 0 04 µC/ml ^{14}C-thymidine for 40 hr. The medium of various coverslip cultures was replaced by media, containing different LAH concentrations and 2 µC/ml ^{3}H-leucine was added for 1 hr The cells were rinsed with 0 9% NaCl and dissolved in 2 ml 0 5% SDS, 50 mM Tris-HCl, pH 8 per coverslip

As shown in Fig. 2, the incorporation of radioactive leucine into acid

precipitable counts was linear in both media for at least 8 hours, indicating no detectable effect of the medium during that period of growth. Upon continued growth, the rate of incorporation leveled off. This is probably due to a decrease of the growth rate since only a small percentage of the added radioactivity (about 10%) has been incorporated by the cells by that time. During the first 5 hours after addition of 0.005% L.A.H. containing medium the incorporation of thymidine was only slightly affected (Fig. 2). Thus within 5 hours of pulse-labelling, experiments can be performed without a detectable effect of the modified media.

8.3.2 Pulse-chase experiments: evidence for the presence of precursors.

Generally it was observed that the electrophoretic pattern of labelled polypeptides of the DNA-protein complex, prepared from cells pulse-labelled with leucine for 2 hr differed from the pattern of the stained polypeptides (Fig. 3). The high molecular weight polypeptides (fractions 1-20) contained

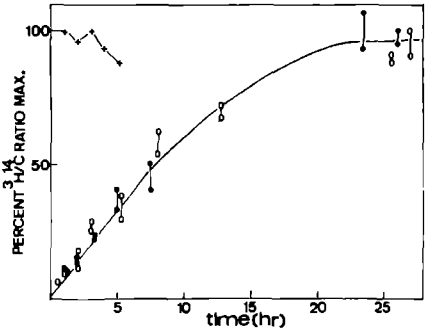
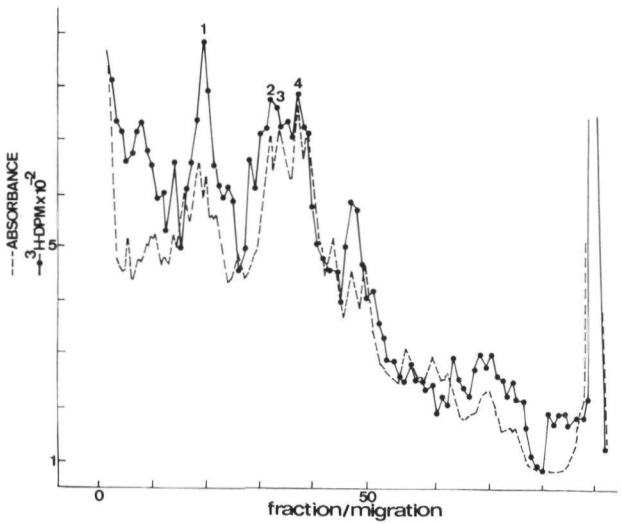


Fig 2

Incorporation of radioactive leucine and thymidine in leucine free MEM medium and in 0 005% LAH medium Coverslips were pre-labelled by addition of 0 04 µC/ml ^{14}C-thymidine for 40 hr The medium was then replaced by leucine free MEM medium or 0 005% LAH medium and ^{3}H-leucine (2 µC/ml) was added Coverslip cultures were removed at the times indicated in the figure, and treated as described in the legend of fig 1
-o- 0 005% LAH medium, -●- leucine free MEM medium
In some experiments cells were pre-labelled with ^{14}C-thymidine After replacement of the radioactive growth medium by 0 005 LAH medium coverslips were pulse-labelled for 1 hr with ^{3}H-thymidine (5 µC/ml), starting at the times indicated in the figure (-+-) The coverslips were treated as described above The ordinate represents the percentages of the maximal ^{3}H/^{14}C ratios

a relatively large amount of radioactivity compared to the amount of protein present in the stained pattern. In the region of the major matrix polypeptides (fractions 30-40) the label distribution did not coincide with the densito-

meter scanning of the stained polypeptides such that there appeared to be a distinct shoulder of radioactivity on the left side of the 75 000 Dalton polypeptide (band 2). In addition the lower part of the gel (fractions 60-80) contained relatively large amounts of label. Several, but not all of these features are also apparent when the labelled polypeptides are studied by gel autoradiography. As shown in Fig. 4, slots 1 and 2, the 75 000 Dalton polypeptide (P 75) was hardly labelled at all after a pulse-label of 2 hr. Instead



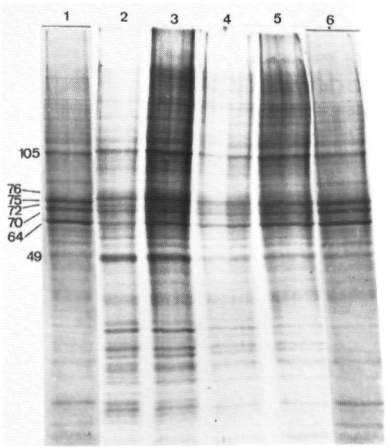


Fig. 3:

Distribution of pulse-labelled polypeptides in SDS polyacrylamide gels.
A Carrel flask was pulse-labelled for 2 hr with 5 µC/ml ^{3}H-leucine in leucine free MEM medium. The DNA-protein complex was isolated and analysed in a 6 - 18% SDS polyacrylamide gel. The stained gel was scanned and cut in 1 mm pieces, which were each incubated in 1.0 ml 30% H_2O_2 for 16 hr at 37°C. Subsequently 10 ml counting solution was added and radioactivities were determined as described.

Fig. 4:

Pulse-chase experiments. Analysis of the pulse-labelled polypeptides of the complex.
Carrel flasks were pulse-labelled for 2 hr with 2 µC/ml ^{14}C-leucine in leucine free MEM medium. After removal of the radioactive medium the cells were rinsed once with normal medium and growth was continued for different times in normal medium. The DNA-protein complex of the various samples was isolated by high speed centrifugation (method 2, chapter 2) and analysed in 16 - 18% SDS polyacrylamide gel. The stained gel was dried and processed for autoradiography.
Slot 1: 2 hr pulse-label, stained gel.
Slot 2: 2 hr pulse-label, autoradiogram.
Slot 3: 2 hr pulse-label and 1 hr chase, autoradiogram.
Slot 4: 2 hr pulse-label and 2 hr chase, autoradiogram.
Slot 5: 2 hr pulse-label and 4 hr chase, autoradiogram.
Slot 6: 2 hr pulse-label and 4 hr chase, stained gel.

there appeared a band on the autoradiogram of slightly higher molecular weight of approximately 76 000 D which is not visible in the stained electro-

pherogram. An additional minor band of approximately 72 000 D is only visible on the autoradiogram. In the lower region of the gel a distinct labelled polypeptide of 49 000 D is observed together with smaller polypeptides which do not correspond in intensity with the staining pattern. From the autoradiograms it is apparent, that the radioactivity in the upper portion of the sliced gel probably resulted from a variable background of label (Fig. 4, slots 2-5) which is not detectable in the various stained gel patterns (Fig. 4, slots 1 and 6). At the moment we do not know the origin and identity of this material.

We further investigated by pulse-chase experiments the turnover of the pulse-labelled polypeptides of the complex. After cells were pulse-labelled for 2 hr followed by a 4 hr chase in normal growth medium the autoradiogram correspond very well to the staining pattern (Fig. 4, slots 5 and 6). As shown in Fig 4, slots 2 through 5, the amounts of label incorporated in the polypeptides smaller than 50 000 D decreased during a 4 hr chase. In the region of the major matrix polypeptides, P76 was reduced. Simultaneous with this decrease label was observed to increase in a polypeptide, comigrating with P75. On the autoradiogram bands at the positions of P76 and P75 are observed 2 hr after chase (Fig. 4, slot 4). P72 disappeared almost completely after 4 hr of chase. Densitometer tracings of the stained gel and the autoradiogram indicated, that the reduction of label in P72 was accompanied by an increase of label in P64 (Fig. 5A and 5B). Thus there appears to be clear differences

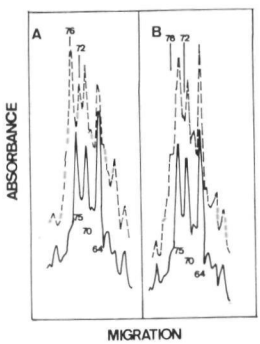


Fig. 5:
Scanning patterns of autoradiogram and stained gel.
A: 2 hr pulse-label. B: 2 hr pulse-label and 4 hr chase.
------ autoradiogram. ——— stained gel
Only the polypeptides of 50 to 80000 Dalton molecular weight are shown.

between the synthesis of the various matrix polypeptides. In particular P75 seemed to be synthesized in the form of a precursor.

8.3.3 Synthesis of the proteins of the complex in the presence of AdR.

In order to determine whether the synthesis of the proteins of the DNA-protein complex was coupled to DNA synthesis, cells were labelled for 16 hr in the presence of 2 mM deoxyadenosine (AdR). This component inhibits DNA synthesis and blocks cells at the G_1-S boundary (7,8).

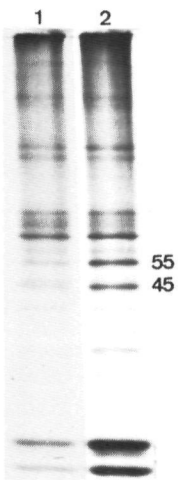


Fig. 6:
Synthesis of the proteins of the complex in the presence of AdR.
Cells were grown for 2 hr in 0.005% LAH medium or in 0.005% LAH medium containing 2 mM AdR. Subsequently ^{14}C-leucine (1 µC/ml) was added and the cells were grown for 15 hr. The DNA-protein complex was isolated (method 2, chapter 2) and analysed in a 6 - 18% SDS polyacrylamide gel. The stained gel was dried and processed for autoradiography.
Slot 1: labelled in the presence of AdR.
Slot 2: labelled without AdR.

As shown in Fig. 6 the pattern of the labelled polypeptides of the DNA protein complex prepared from cells treated with AdR differed markedly from those of untreated cells. It is apparent, that the incorporation of amino acids into residual histones associated with the complex and into polypeptides

P45 and P55 was strongly reduced in the presence of AdR. However in the presence of AdR, the synthesis of all major matrix polypeptides was very similar to the control. Obviously the synthesis of the major matrix polypeptides does not require the simultaneous occurrence of DNA synthesis.

8.3.4 Composition and synthesis of the proteins of the complex during the cell cycle.

In preliminary experiments the composition of the DNA-protein complex and the synthesis of the proteins at various stages of the cell cycle were investigated. Monolayers were synchronized by growing cells for 24 hr in the presence of AdR. After removal of the medium, growth was continued for 30 hr in medium without AdR followed by a second blockade for 24 hr. Cells were pulse-labelled for 2 hr at different times after removal of the second AdR block and the DNA-protein complex was prepared. No differences in polypeptide composition were observed during the cell cycle. Moreover the patterns of pulse-labelled polypeptides were very similar at each stage of the cell cycle.

8.4 Discussion.

Autoradiographic patterns of pulse-labelled polypeptides of the complex showed that there are clear differences in the rates of turnover and possibly in the manner of synthesis of the various proteins of the complex. During a 2 hr pulse-labelling the major matrix polypeptides P64, P70 and P105 appeared to be synthesized and processed to their final molecular weights. However P75 is hardly labelled at all and two additional polypeptides (P76 and P72) which are not found in the stained gel, are visible on the autoradiogram only.

There is evidence to suggest that P76 may be the precursor form of P75 as a reduction of label in P76 was accompanied by the simultaneous appearance of label in P75 during a 4 hr chase. Further, there is some evidence from the patterns of the stained gel and the autoradiogram that P72 may be the precursor polypeptide of P64.

When synthesized, P76 seems to be conserved for a rather long period of time since within 2 hours of pulse-labelling the polypeptide was seen as a very distinct band, while no label appeared in P75. In contrast P64, the assumed final product of P72 was clearly observed indicating that the

modification of both precursor polypeptides may not related to the same cellular process. An important question is whether the synthesis of the matrix polypeptides as well as the assumed precursor polypeptides is coupled to DNA synthesis or to any specific stage of the cell cycle. When DNA synthesis was blocked by deoxyadenosine as indicated by the very reduced amount of labelled histones (10), the major matrix polypeptides of the complex were still synthesized. Obviously the synthesis of these polypeptides is not restricted to DNA synthesis only. Preliminary pulse-chase experiments with synchronized cells revealed no evidence for cell cycle specific synthesis of the matrix polypeptides and P76 and P72. With regard to the formation of nuclear pores these results indicate that de novo synthesis of polypeptides of the pore complexex is not restricted to S-phase, or alternatively that only minor polypeptides are involved in nuclear pore formation.

Recently it has been reported that the polypeptide composition of nuclear ghosts (12) as well as nuclear matrices (13) prepared from HeLa cells, synchronized by double thymidine blockade or mitotic selection, did not change during the cell cycle. In addition no differences were observed in the composition and the mode of labelling of the nuclear envelope polypeptides of CHO-cells (14) synchronized by thymidine blockade at the various stages of the cell cycle. Thus the reported data as well as the data presented in this chapter suggest that the composition and synthesis of the major matrix polypeptides is fairly constant throughout the cell cycle.

Surprisingly the synthesis of the two major polypeptides P45 and P55 is reduced in the presence of AdR. We do not know whether this indicates a secondary effect of the presence of AdR. However it has been suggested that the cytoskeleton is directly associated with the nucleus by its micro filaments (10) and may have important functional roles related to nuclear division and linkage of the nucleus to other cell organelles (11). Possibly it may be involved in DNA replication in an unknown way.

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Summary

This thesis presents a study of a nuclear DNA-protein complex isolated from monolayer cultures of bovine liver cells.

In the first chapter a survey is given of the investigations to elucidate the possible involvement of nuclear membrane or related structures in eukaryotic DNA-replication. Moreover, the present knowledge of the structural arrangement of chromatin is briefly described.

In the third chapter the isolation and partial characterization of the DNA-protein complex is described. Nuclei were isolated from monolayer cultures of bovine liver cells by use of a Triton containing Tris buffer, and dissolved in 1-2 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 DNase digestion without causing a significant change of the sedimentation rate of the supporting structure. A limited digestion showed that newly synthesized DNA was more resistent 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 attachment of DNA and in particular replicating DNA to the protein structure was investigated in more detail by means of various DNA degrading enzymes and different pulse-label times (chapter 4).

Pulse-labelled DNA in the complex appeared to be less accessible to DNase 1 as the time of pulse-labelling was decreased. This observation clearly indicated that in particular the replicating DNA at the regions of the replication forks was most resistent to the enzyme. After incubation with Nuclease S_1 a major part of the pulse-labelled DNA was detached from the complex by specific hydrolysis of single-stranded gaps or nicks. The nascent DNA released by Nuclease S_1 appeared to originate from the replication forks since it consisted of single and multiple Okazaki-fragments up to 10S. Accordingly the amount of pulse-labelled DNA released by the enzyme, corre-

sponded to the relative amount of single and multiple Okazaki-fragments pulse-labelled in the various experiments. However, hydrolysis of the single-stranded regions of the replication forks did not result in a loss of the binding sites at the matrix, as could be shown by digestion of nuclear lysates with Staphylococcus nuclease or a sequential treatment with Nuclease S_1 and DNase 1. These data suggest that binding sites of replicating DNA at the protein structure are situated behind the replication forks, although other possible binding sites were not ruled out.

A biochemical and ultrastructural analysis of the complex was presented in chapter 5. In 1 M NaCl the complex basicly consisted of 4 major non-histone polypeptides (molecular weights: 105, 75, 70, 64 KDaltons) and residual amounts of histones H_3 and H_4. The rapidly sedimenting complex consisted of ghost-like material which contained interconnected annular structures. Biochemically and ultrastructurally the protein structures were related to the pore-complex lamina and nuclear protein matrix. These nuclear structures were often observed together with thin fibrils which were removed by DNase treatment, indicating an association of DNA and the nuclear structures. The involvement of these structures in DNA replication was discussed in relation to previous autoradiographical and biochemical studies.

Chapter 6 described the fractionation of the DNA-protein complex in SDS-sucrose-gradients. Under certain conditions 2 (molecular weights: 75 and 64 KDaltons) of the 4 major non-histone polypeptides could be extracted from the complex without any detachment of replicating or non-replicating DNA. Obviously the presence of the two polypeptides in the complex was not necessarily required for the binding of DNA. As a result of the release of the two polypeptides the rate of sedimentation of the remaining complex was markedly decreased. Based on these data it was assumed that extraction of the two polypeptides caused a pronounced alteration of the complex.

The DNA-protein complex could be completely dissociated in 5 M urea-2 M NaCl (chapter 7). After removal of the bulk of the DNA by centrifugation the histones, matrix proteins and residual DNA could be separated from each other on hydroxylapatite columns. The DNA-binding properties of the dissociated matrix proteins were investigated. In 0.15 M NaCl DNA formed complexes with reassociated matrix proteins. Since the binding was non-specific with regard to the added DNA and unstable in 1 M NaCl these complexes must be considered

to differ from the original DNA-protein complex. Binding of DNA did not occur when DNA was added to purified nuclear ghosts in the presence of 0.15 M NaCl.

In chapter 8 some aspects of the synthesis of the matrix polypeptides were described. Comparison of the patterns of pulse-labelled polypeptides obtained after different periods of chase and the stained polypeptides revealed that there were clear differences between the rate of appearance of the various matrix polypeptides. In addition the major matrix polypeptide of 75 000 Daltons appeared to be synthesized in the form of slightly larger precursor molecules. Also some evidence was presented for a precursor form (P72) of the polypeptide of 64 000 Daltons. The synthesis of the major matrix polypeptides proceeded when DNA synthesis was blocked. Under these conditions the synthesis of the histones and the two major polypeptides of the cytoskeleton was strongly reduced.

Samenvatting

In dit proefschrift worden de resultaten beschreven van een onderzoek naar een DNA-eiwit komplex, geïsoleerd uit kernen van in vitro gekweekte runderlevercellen.

Het eerste hoofdstuk geeft een overzicht van de onderzoekingen om de mogelijke funkties van kernmembraan en verwante strukturen bij de eukaryotische DNA-synthese op te helderen. Tevens is de huidige stand van zaken in het chromatine onderzoek kort vermeld.

Het derde hoofdstuk beschrijft de isolatie en gedeeltelijke karakterisering van het DNA-eiwit komplex. Kernen werden geïsoleerd uit runderlevercellen in Triton-Tris buffer en gelyseerd in 1-2 M NaCl. Sedimentatie analyse van het lysaat in sucrose gradienten toonden aan, dat een variabele hoeveelheid DNA verbonden was met een snel sedimenterende struktuur. Eiwitten vormen de basis voor de stabiliteit van het komplex. Het aangehechte DNA kon volledig uit het komplex verwijderd worden door digestie met DNase 1. Door gebruik te maken van lage concentraties DNase 1 kon worden aangetoond, dat nieuw gesynthetiseerd DNA minder snel verwijderd wordt door het enzym. De resultaten suggereerden, dat DNA-molekulen met één bindingsplaats per replicatie eenheid ('replicon') aangehecht zijn aan de eiwitstruktuur en dat gedurende de replikatie additionele binding plaats vindt nabij de replikatievorken.

De aanhechting van DNA en in het bijzonder replicerend DNA werd nauwkeuriger onderzocht door gebruik te maken van een aantal DNA-digesterende enzymen en verschillende radioaktieve pulse-markering tijden (hoofdstuk 4). Pulse-gemerkt DNA in het komplex bleek minder goed toegankelijk voor DNase 1 naarmate de tijd van pulse-markering korter was. De waarneming toonde aan, dat in het bijzonder het replicerend DNA in de replikatievorken het meest resistent was voor het enzym. Een belangrijk gedeelte van het nieuw gesynthetiseerd DNA kon worden verwijderd uit het komplex door digestie met het enkelstrengs specifieke enzym Nuclease S_1. Dit nieuw gesynthetiseerd DNA bleek afkomstig te zijn uit de replikatievorken, daar het was samengesteld uit Okazaki fragmenten of multipele hiervan tot 10S. Bovendien bleek in de verschillende experimenten de hoeveelheid pulse-gemerkt DNA, losgemaakt door Nuclease S_1 uit het komplex, overeen te komen met de relatieve hoeveelheid radioaktiviteit in Okazaki fragmenten tot 10S. Door gebruik te maken van Staphylococcus nuclease of een combinatie van Nuclease S_1 en DNase 1 kon worden aangetoond, dat ook na hydrolyse van enkelstrengs DNA uit de replikatievorken het replicerend DNA nog nauw verbonden was met de eiwitstruktuur. Hieruit werd gekonkludeerd, dat er zeker bindingsplaatsen van replicerend DNA met de eiwitstruktuur gelegen zijn achter de replikatievorken.

Hoofdstuk 5 bevat een biochemische analyse en de whole-mount struktuur van het komplex. In 1 M NaCl bestond het komplex in hoofdzaak uit 4 non-histone polypeptiden (molekuulgewichten: 105, 75, 70 en 64 KDaltons) en histonen H_3 en H_4. De non-histon eiwitten vormen snel sedimenterende strukturen, welke onderling verbonden kern porieën bevatten. Biochemisch en ultrastruktureel waren de eiwitstrukturen te relateren aan de pore komplex-lamina en de kern matrix. Aan deze kernstrukturen werden vaak dunnen draden waargenomen welke verwijderd konden worden door DNase 1 hetgeen wees op een associatie van DNA en de eiwitstrukturen. De mogelijke rol van dergelijke eiwitstrukturen bij de DNA replikatie werd besproken met betrekking tot eerder gepubliceerde autoradiografische en biochemische studies.

Hoofdstuk 6 beschrijft de fractionering van het komplex in SDS-sucrose gradienten. Onder bepaalde kondities werden 2 van de 4 belangrijke non-histone polypeptiden (molekuulgewichten: 75 en 64 KDaltons) uit het komplex verwijderd zonder dissociatie van replicerend of niet-replicerend DNA. De sedimentatie snelheid van het residue komplex nam echter aanzienlijk af.

Blijkbaar is de aanwezigheid van deze twee polypeptiden niet noodzakerlijkerwijs vereist voor de binding van DNA, maar resulteert de verwijdering van de polypeptiden uit het komplex mogelijk tot een drastische verandering van de eiwit strukturen.

Het DNA-eiwit komplex kon volledig worden gedissocieerd in 5 M ureum 2 M NaCl (hoofdstuk 7). Het bleek mogelijk te zijn de histonen, de matrix eiwitten en het residue DNA van elkaar te scheiden door hydroxylapatiet-chromatografie. De DNA bindende eigenschappen van de gedissocieerde matrix eiwitten werden onderzocht. In 0.15 M NaCl vormden de gereassocieerde matrix eiwitten komplexen met DNA. Daar de binding niet specifiek was met betrekking tot het toegevoegde DNA noch stabiel in 1 M NaCl verschilden deze komplexen van het oorspronkelijk DNA-eiwit komplex. Geen binding werd waargenomen wanneer DNA werd toegevoegd aan gezuiverde intakte eiwit strukturen.

Hoofdstuk 8 bevat enkele aspekten van de synthese van de matrix polypeptiden. Vergelijking van de pulse-gemerkte polypeptiden, geïsoleerd na verschillende chase tijden, en de gekleurde polypeptiden toonden aan, dat er duidelijke verschillen zijn tussen de synthese van de belangrijke matrix polypeptiden. De polypeptide van 75 000 Daltons bleek mogelijk te worden gesynthetiseerd in de vorm van een precursor, terwijl er enige aanwijzingen verkregen werden voor een precursor-produkt relatie tussen een polypeptide van 72 000 Daltons en de polypeptide van 64 000 Daltons. De synthese van de matrix polypeptiden werd niet merkbaar beïnvloed, wanneer de DNA synthese werd geblokkeerd. Onder die condities werd de synthese van histonen en de twee belangrijke polypeptiden van het cytoskeleton in belangrijke mate gereduceerd.

CURRICULUM VITAE

Leon Mullenders werd op 29 januari 1949 te Heerlen geboren. Zijn HBS-B opleiding volgde hij aan het Eyckhagencollege te Schaesberg. Nadat hij in 1967 zijn diploma had behaald, begon hij in datzelfde jaar zijn studie Scheikunde aan de Katholieke Universiteit te Nijmegen. In juli 1970 legde hij zijn kandidaatsexamen af. Na zijn doctoraalstudie begonnen te zijn met een bijvak op de afdeling Organische Chemie, richtte hij zijn belangstelling meer op de Biochemie en volgde op de afdeling Biofysische Chemie zijn hoofdvak. Vervolgens deed hij zijn tweede bijvak op de afdeling Moleculaire Biologie. Dit alles werd afgesloten met zijn doctoraalexamen in oktober 1973.

Vanaf oktober 1973 tot augustus 1978 was Leon werkzaam als wetenschappelijk medewerker op de afdeling Chemische Cytologie van Prof. dr. Ch.M.A. Kuyper. Gedurende het laatste jaar van deze periode heeft hij zich intensief bezig-gehouden met het schrijven van dit proefschrift. Deze drukke periode werd onderbroken door de geboorte van zijn zoon Jasper.

Vanaf augustus 1978 is hij docent aan de H.B.O.-V. te Zwolle.

STELLINGEN

I

De snel sedimenterende DNA-eiwit complexen, geïsoleerd in SDS, kunnen verklaard worden door aanwezigheid van residuen van kern matrix eiwitten in de complexen.

T. Andow en T. Ide, Exptl. Cell Res. *73*, 122-128 (1972).
Dit proefschrift, hfst. 6.

II

De conclusie betreffende de polypeptide samenstelling van verschillende kernstructuren door Riley en Keller zijn, gezien de getoonde patronen, dubieus.

D.E. Riley en J.M. Keller, Biochim. Biophys. Acta *444*, 899-911 (1976).

III

Bij het onderzoek door Seale van replicerend DNA in chromatine met behulp van Staphylococcus nuclease is ten onrechte geen rekening gehouden met de enkelstrengs specificiteit van het gebruikte enzym.

R.L. Seale, Cell *9*, 423-429 (1976).

IV

Tengevolge van het uitgebreid net van autosnelwegen en de bestaande en voorgenomen afgravingen van zand en mergel wordt in het limburgs volkslied de vraag "Waar in 't bronsgroen eikenhout, het nachtegaaltje zingt" steeds moeilijker te beantwoorden.

V

Het toenemend aanbod van elektronisch te besturen speelgoed lijkt geen goede voorbereiding te zijn op een zinvolle besteding van de later te verwachten toegenomen vrije tijd van de jongste generatie.

VI

Indien het verplicht stellen van een fietsreflector een reflectie is van de gedachten van de overheid met betrekking tot de veiligheid van de fietsers, dan ziet de toekomst van deze groep weggebruikers er somber uit.

VII

Bij de discussie over te verwachten olietekorten in de nabije toekomst wordt er te weinig nadruk gelegd op het belang van olie als grondstof voor de chemische industrie en teveel op het belang ervan als energiedrager.

Nijmegen, januari 1979

L.H.F. Mullenders