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DNA-histone interaction in the vicinity of replication points

Ernst-Jürgen Schlaeger and Rolf Knippers

Fachbereich Biologie, Universität Konstanz, D-7750 Konstanz, GFR

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ABSTRACT

Chromatin replication was studied in isolated nuclei from Concanavalin A activated lymphocytes. Digestion with micrococcal nuclease revealed that the resistant fraction of *in vitro* replicated DNA is associated with nucleosomes. Earlier experiments had shown that the nuclease resistant fraction of nascent DNA is composed of fragments which are shorter than the nuclease resistant fragments of bulk DNA. In this communication we demonstrate that the short fragments of nascent DNA are differently bound to nucleosome like structures compared to bulk DNA. At 0.5 M NaCl a fraction of pulse labeled DNA is released from these structures and appears as free double stranded DNA of about 140 base pair length (5S DNA) while the 185 pair fragments of mature replicated DNA remain attached to nucleosomes under these conditions. The experiments may indicate that the interaction of a fraction of replicating DNA with histones differs from that of bulk DNA.

INTRODUCTION

Replicating nuclear DNA is associated with nucleosomes (the chromatin repeating units composed of histone octamers and stretches of DNA of 180-200 base pair lengths) (1,2,3). Whether and how the structure of nucleosomes is affected when the replication fork moves along the DNA is still uncertain. Nucleosomes from the unreplicated "parental" DNA are transferred to nascent and, at later stages of the replication process, to replicated daughter DNA strands (4,5) suggesting that the histone octamers most probably remain associated with DNA during replication. This conclusion is also supported by the observation that the individual histone components of the parental nucleosomes remain associated during replication (6). The information of nucleosomes in the vicinity of replication forks appears to be different from that

of nucleosomes in bulk chromatin,
since nuclease resistant fragments of pulse labeled DNA are generally smaller (140-170 base pairs) than fragments from bulk DNA (185-200 base pairs) (7-9).

In this communication we show that a fraction of nuclease resistant nascent DNA appears to be differently bound to nucleosome like structures than mature DNA since the 140 base pair fragments of pulse labeled DNA can be released by 0.5 M NaCl as a free sedimenting 5S DNA fragment while fragments of mature DNA (185 base pairs) remain stably associated with nucleosomes.

The experiments presented here were performed with isolated nuclei from bovine lymphocytes activated by Concanavalin A (10). DNA replication in isolated nuclei from lymphocytes, as well as in nuclei from other mammalian cells (see review, in ref. 11), appears to proceed normally for a limited length of time (12,13). Moreover, nucleosomes in in vitro replicated DNA are organized like the nucleosomes in bulk DNA (9,14). The advantage of this experimental system is several-fold: pulse-chase experiments are possible without worrying about precursor pools of unknown or, at least, uncertain size; the rate of fork movement is slower than in intact cells, which facilitates the detection of intermediates in DNA replication and chromatin assembly; and histone synthesis does not occur in isolated nuclei (9), allowing an observation of the fate of parental nucleosomes.

MATERIALS AND METHODS

Preparation of nuclei

Preparation und cultivation of lymphocytes from bovine retropharyngeal lympfnodes have been described (15). Nuclei were prepared from Concanavalin A activated lymphocytes when the rate of (^{3}H)-thymidine incorporation was maximal. The preparation of nuclei and the condition for in vitro DNA replication have been described previously (13).

Nuclease treatment

Digestion of chromatin by micrococcal nuclease (E.C. 3.1.4.7) (Boehringer, Mannheim) was performed using intact nuclei as reported previously (9) and was terminated

by adding EDTA (final concentration: 5mM) or the nuclei were pelleted (360 xg, 2 min, 0°C) and resuspended for lysis in 10mM Tris-HCl, pH 7.5, and 5mM EDTA. Salt-treated nuclear lysates were prepared by adding 0.5 M NaCl (final concentration).

Centrifugation

The nuclear lysates were layered on top of a 5-25% sucrose gradient containing 2mM EDTA, pH 7.5, and 10mM 2-mercaptoethanol. The gradients contained 0.5 M NaCl when salt-treated nuclear lysates were investigated. Centrifugation was carried out in the Beckman SW27 rotor at 26,000 rpm and 4°C for 22-28 hrs. The gradients were collected from the bottom and pumped through a quartz cuvette to record the ultraviolet light absorbance at 260 nm as described (16).

Preparation of DNA fragments for electrophoresis

Nuclease resistant DNA fragments were deproteinized by protease K (Merck, Darmstadt) treatment and phenol-chloroform extraction and the extracted DNA was analysed by agarose gel electrophoresis as described (9).

RESULTS

DNA replicated *in vitro* is associated with chromatin subunits

Isolated nuclei were incubated under DNA replication conditions (13) for 15 min in the presence of (^{3}H)dTTP. The nuclei were then transferred to nuclease buffer and treated with micrococcal nuclease as described in the legend to Fig. 1. The nuclease resistant digestion products were analysed on sucrose gradients without salt and in 0.5 M NaCl (Fig. 1A and B). The nuclease resistant *in vitro* replicated DNA sedimented like the mono-, di-, trimeric etc. nucleosomes of bulk chromatin (9) when the sucrose gradient centrifugation was carried out in the absence of salt (Fig. 1A). When the same preparation was sedimented through sucrose gradients containing 0.5 M NaCl (Fig. 1B), however, a new slower sedimenting peak with an apparent sedimentation coefficient of about 5S (relative to monomeric nucleosomes and to bovine hemoglobin) appeared. This 5S peak contained a significant fraction of the newly

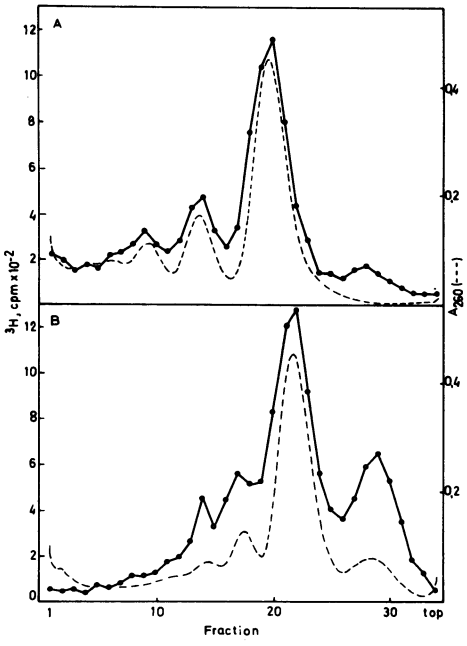

Figure 1. Sucrose gradient analysis of a nuclease digest of chromatin replicated in vitro.
Lymphocyte nuclei were incubated under DNA replication conditions (13) with (^{3}H)dTTP for 15 min. About 2.5 x 10^7 nuclei were transferred to 0.3 ml nuclease buffer (10mM Tris-HCl, pH 7.5, 10mM 2-mercaptoethanol, 1mM $CaCl_2$ and 1 mM phenylmethanesulfonyl fluoride) and treated for 10 min with 60 units micrococcal nuclease at 37°C. About 40% of the in vitro synthesized DNA remained in an acid precipitable form. (In other similar experiments, we have found that, under these conditions, 65% of bulk DNA is still acid precipitable (9)). The salt resistant material was centrifuged through a standard sucrose gradient without salt (A) or through a sucrose gradient containing 0.5 M NaCl (B).

replicated nuclease resistant (^{3}H) labeled DNA (Fig. 1B). The nucleosomes from bulk chromatin sedimented slightly slower in the presence than in the absence of salt probably due to a change of their frictional coefficient (17).

The results concerning the appearance of 5S DNA were similar under a variety of conditions for nuclease digestion; e.g. at 0 and 80mM NaCl(18), at 0°C and 37°C as well as after short and longer incubation times when less than 5 or about 50% of the newly synthesized DNA became acid soluble.

The appearance of the ^{3}H-labeled 5S peak in sucrose gradients with 0.5 M NaCl depended on the action of micrococcal nuclease. No 5S peak could be detected in chromatin that was not treated with the nuclease (data not shown).

5S DNA arises from monomeric nucleosomes upon salt treatment

Monomeric nucleosomes containing (^{3}H) labeled *in vitro* replicated DNA were recovered from a low salt sucrose gradient like the one shown in Fig. 1A, then recentrifuged under low and high salt conditions. As shown in Fig. 2A, the monomeric nucleosomes sedimented as a homogenous 11S peak through a gradient without salt while a significant portion of the ^{3}H-labeled DNA appeared as slower sedimenting 5S material when the centrifugation was carried out in the presence of 0.5 M NaCl (Fig. 2B). (This experiment does not exclude the possibility that 5S DNA may also be released from structures sedimenting faster than 11S). We shall demonstrate below that the ^{3}H-labeled 5S fraction of replicated DNA consists of nascent DNA. The appearance of a peak of uv light absorbing 5S material indicates, however, that another and possibly larger portion

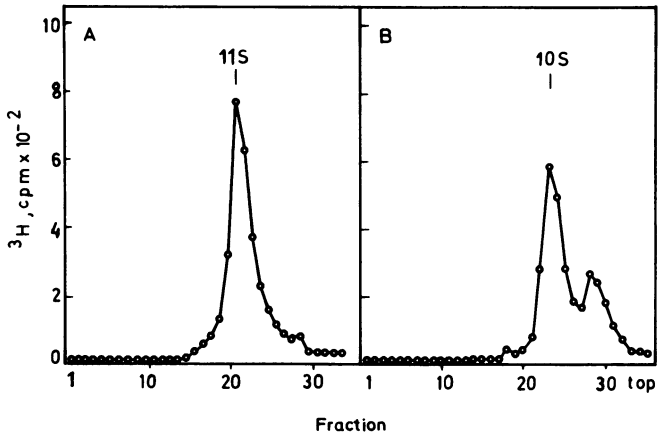

Figure 2. Resedimentation of monomeric nucleosomes.
Monomeric nucleosomes with (^{3}H) labeled DNA were recovered from a salt free sucrose gradient like the one shown in Fig. 1A. The sample was split into two parts and centrifuged through sucrose gradients without salt (A) and in the presence of 0.5 M NaCl (B).

of salt dissociable DNA fragments may originate from other sections of the chromatin (19) (see below).

DNA in 5S material is shorter in length than that in monomeric nucleosomes

DNA from salt treated monomeric nucleosomes (Fig. 1B, 2B) as well as from the fractions containing the 5S peak were deproteinized and analyzed by agarose gel electrophoresis. As a control the unfractionated nuclease digestion products were also investigated. The DNA present in the 5S peak migrated faster than that from monomeric nucleosomes (Fig. 3).

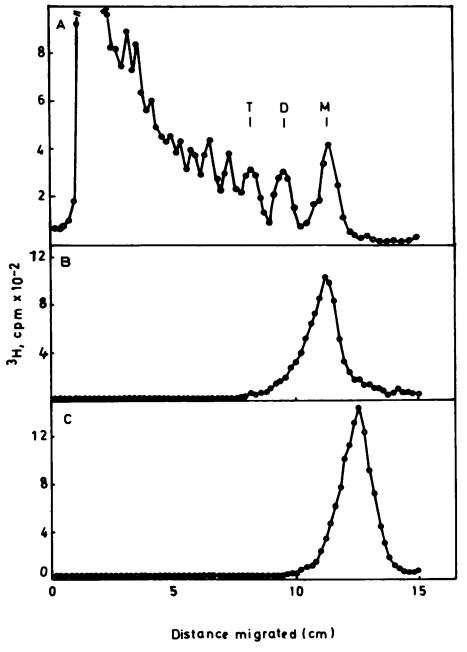

Figure 3. Migration of the ^{3}H-labeled 5S DNA in agarose gel electrophoresis.

Monomeric nucleosomes and 5S material were recovered from a sucrose gradient containing 0.5 M NaCl. The DNA was deproteinized (9) and analyzed by electrophoresis on 1.7% agarose gels. The distribution of radioactivity was measured in 2 mm gel slices (9).
A: Control: cleavage products of in vitro replicated chromatin; the resulting DNA fragments were extracted and prepared for agarose gel electrophoresis as described (9).
B: DNA from monomeric nucleosomes. C: DNA from the 5S region. The calibration of this gel with Hae II restriction fragments of Col EI was carried out as previously described (9).

Calibration with the Hae II restriction endonuclease fragments of the plasmid Col E1 (9) showed that the (^{3}H) DNA from salt resistant monomeric nucleosomes was 185±10 base pairs long, but the (^{3}H) DNA in the 5S peak was (under the nuclease degradation conditions used) only about 140 base pairs long.

The 5S DNA appears to be largely free of protein

The 5S (^{3}H) DNA from a sucrose gradient (Fig. 1B) was as rapidly degraded to acid soluble material by nucleases as a phenol extracted protein free control DNA though (^{3}H) DNA in nucleosomes was, of course, much more resistant (data not shown). Moreover, 5S (^{3}H) DNA taken directly from a sucrose gradient like the one shown in Fig. 1B migrated in agarose gel electrophoresis exactly like phenol-extracted 5S DNA (see Fig. 3), while monomeric nucleosomes with a full complement of core histones travelled much more slowly (Fig. 4).

The 5S (^{3}H) DNA appears to be largely double stranded because about 90% of it was resistant to nuclease S1 and because it did not bind to nitrocellulose filters at 0.9 M NaCl as expected for single stranded DNA (not shown).

The ^{3}H-labeled 5S peak contains nascent DNA

Lymphocyte nuclei were incubated under DNA replication conditions in the presence of deoxynucleoside triphosphates, including (^{3}H)dTTP, (13) for various time periods from 1 to 30 min. After the times indicated in Fig. 5, DNA replication was terminated and the nuclei were transferred to nuclease buffer (9). In this experiment, micrococcal nuclease digestion was carried out for 20 min at 0°C to avoid extensive exonucleolytic degradation of the cleavage products. Control experiments have shown that, in this experiment, less than 5% of bulk, as well as of (^{3}H) labeled, DNA became acid soluble under these incubation conditions. The cleavage products were analyzed on sucrose gradients containing 0.5 M NaCl. With increasing replication times, the fraction of radioactivity recovered in the 5S peak decreased (from about 45% of total recovered ^{3}H-cpm after 1 min to less than 10% after 30 min replication time, respectively) (Fig. 5).

In several other experiments, we used replication times of less than one minute and found a fraction of ^{3}H-labeled 5S DNA

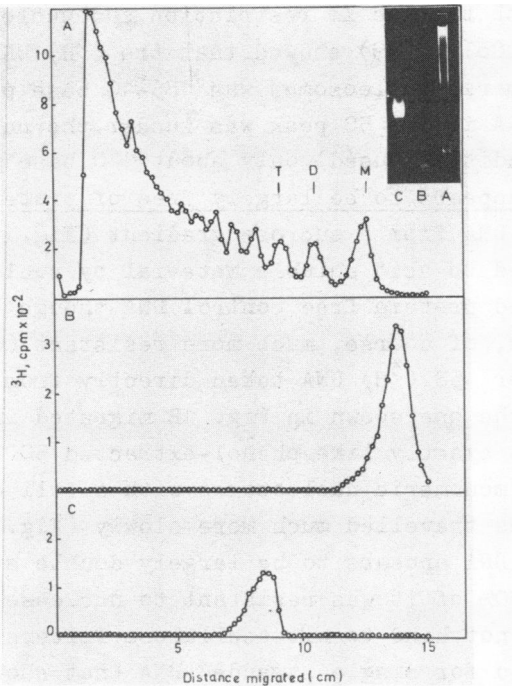

Figure 4. Electrophoresis of "intact" monomeric nucleosomes and non deproteinized 5S material.

DNA replication in isolated nuclei was allowed for 15 min in the presence of (^{3}H)dTTP. Nuclease digestion was carried out as described under Fig. 3. After nuclease treatment a control sample was removed and deproteinized. The remainder of the nuclease treated material was sedimented through sucrose gradients at 0.5 M NaCl as shown in Fig. 1B. The monomeric nucleosomes and the 5S material were recovered, concentrated by vacuum dialysis and directly applied to 1.7% agarose gels for electrophoresis.

A, control: cleavage products of nuclease digested chromatin; deproteinized DNA fragments: M, monomeric; D, dimers; T, trimers.

B, non deproteinized 5S DNA.

C, monomeric nucleosomes. Note that the salt treated nucleosomes migrate as one single peak while several subfractions of monomeric nucleosomes are detected by agarose gel electrophoresis when nucleosomes are taken from a low ionic strength sucrose gradient (20).

Insert: photograph of the gel stained with ethidium bromide

Graph : distribution of radioactivity as determined in 2 mm-slices of the gel (9).

of 50% and more of total recovered radioactivity in sucrose gradients at 0.5 M NaCl (not shown). The ^{3}H-labeled 5S peak

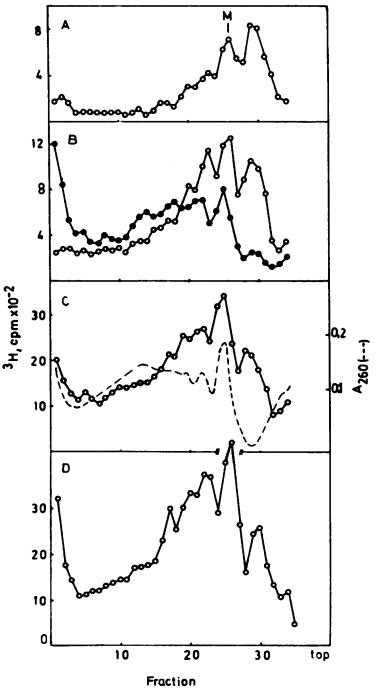

Figure 5. Nascent DNA on salt labile nucleosomes.
Lymphocyte nuclei were incubated with (^{3}H)dTTP under DNA replication conditions (13) for 1 (A), 2 (B), 10 (C), and 30 min (D) at 37°C. In (B) after 2 min of synthesis, a 100 fold excess of unlabeled dTTP was added to part of the reaction mixture and incubation was continued for 30 min. The nuclei (2.5×10^7) were then treated with 60 units of micrococcal nuclease in 0.3 ml nuclease buffer for 30 sec at 37°C and 20 min at 0°C. The cleavage products were centrifuged in the presence of 0.5 M NaCl as described under Fig. 1.
The optical density profile in each gradient was identical to that shown in (C).
M: Monomeric nucleosomes

disappeared when a 2 min replication period in the presence of (^{3}H)dTTP was followed by a 30 min incubation with an excess of unlabeled dTTP (Fig. 5B).

It should also be noted that after replicating times of 1-2 min (Fig. 5A, B) most of the recovered radioactivity appeared either in the 5S peak or in monomeric nucleosomes and little radioactivity was associated with larger nucleosome complexes. After longer replication times, the distribution of

nuclease resistant radioactivity resembled more that of the cleavage products of bulk chromatin. These results suggest that the linker regions between nucleosomes on nascent DNA are more exposed to nuclease attack than those between nucleosomes in bulk chromatin (9). Similar results were obtained with in vivo pulse labeled DNA by Hildebrand et al. (5) and in this laboratory by K.H.Klempnauer (unpubl.).

Thus, a fraction of detectable nascent DNA is released at 0.5 M NaCl from a structure that sediments at low ionic strengths like monomeric 11S nucleosomes. Nucleosomes on the completed replication products are resistant to salt, as are most of the nucleosomes in bulk chromatin.

DISCUSSION

Nascent DNA is associated with nucleosomes. This conclusion is based on the observation that the nuclease-resistant fraction of pulse labeled DNA is organized in structures which sediment through sucrose gradients at low ionic strength like the nucleosomes from bulk chromatin (5,9). The structure of nucleosomes on nascent DNA, however, differs from that of nucleosomes in bulk chromatin. This conclusion is obtained by micrococcal nuclease digestion of chromatin containing pulse labeled DNA. During incubation with nuclease, pulse labeled DNA appears in monomeric nucleosomes at a higher rate than bulk DNA (5,7-9) suggesting a more exposed linker region on nascent DNA. Furthermore, the cleavage products of pulse labeled DNA are shorter (165-170 base pairs in HeLa cell nuclei, 7, and about 140 base pairs in lymphocyte nuclei, 9) than the DNA in monomeric bulk nucleosomes (185 base pairs), which also suggest that DNA sections which are protected against nuclease attack in bulk chromatin are unprotected on nascent DNA.

In this communication, we show that the nascent DNA in these short fragments is at low ionic strength associated with structures that sediment through sucrose gradients like typical chromatin repeat units. Moreover, these structures band, after formaldehyde fixation, in CsCl equilibrium gradients like typical nucleosomes (not shown). These observations exclude a gross rearrangement of the histone octamer carrying nascent

DNA compared to those with mature DNA.

At the present time it is not known why nascent DNA is released from nucleosome like structures at high salt while mature DNA remains more stably bound. We have mentioned above that the 140 base pair long 5S DNA is largely double stranded. This does not exclude the possibility that the salt sensitivity of DNA histone interaction is due to a structural feature of nascent DNA (which includes Okazaki fragments, 13). It seems to be more likely, however, that alterations in the histone component of nucleosomes lead to the salt sensitivity of histone-DNA interaction. These alterations could include charge neutralizations of amino acid side chains, e.g. by acetylation or methylation.

The salt labile binding of the ^{3}H pulse labeled 5S DNA to nucleosome like structures is not a property of the *in vitro* system. A similar structure has been detected also among the micrococcal nuclease cleavage products of *in vivo* (^{3}H) thymidine pulse labeled chromatin (5; Klempnauer, unpubl.).

A salt sensitive binding of DNA fragments does most probably not only occur at nucleosomes around replication points but also in other regions of chromatin. We conclude this form the observation that, in the 5S region of high salt gradients, much more uv-light absorbing DNA appears than could be accounted for by the fraction of nascent DNA. Moreover, a peak of uv absorbing 5S DNA is also seen when nuclei from early G_1 phase lymphocytes (which do not replicate DNA) are treated with micrococcal nuclease and when the corresponding cleavage products are investigated by sucrose gradient centrifugation at 0.5 M salt (unpublished). Experiments of this type clearly show, that the nucleosomes in the vicinity of the replication point represent just part of a class of salt labile nucleosomes. It is conceivable that another and possibly larger portion of salt-sensitive nucleosomes originates from transcribed sections of the chromatin. In a recent report Johnson et al. (19) described the appearance of a similar 5S DNA among the nuclease cleavage products from chromatin of *Physarum*. Their 5S material was found to be enriched in sequences homologous to rRNA, when chromatin from cells actively transcribing rRNA

genes was investigated.

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