Reduced repeat length of nascent nucleosomal DNA is generated by replicating chromatin in vivo

Karl M. Jakob*, Smadar Ben Yosef and Israel Tal

Department of Plant Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel

Received 8 May 1984; Accepted 29 May 1984

ABSTRACT

Micrococcal nuclease digestion of nuclei from sea urchin embryos revealed transient changes in chromatin structure which resulted in a reduction in the repeat length of nascent chromatin DNA as compared with bulk DNA. This was considered to be entirely the consequence of in vivo events at the replication fork (Cell 14, 259, 1978). However, a micrococcal nuclease-generated sliding of nucleosome cores relative to nascent DNA, which might account for the smaller DNA fragments, was not excluded.

In vivo [3H]thymidine pulse-labeled nuclei were fixed with a formaldehyde prior to micrococcal nuclease digestion. This linked chromatin proteins to DNA and thus prevented any in vitro sliding of histone cores. All the nascent DNAs exhibiting shorter repeat lengths after micrococcal nuclease digestion, were resolved at identical mobilities in polyacrylamide gels of DNA from fixed and unfixed nuclei. We conclude that these differences in repeat lengths between nascent and bulk DNA was generated in vivo by changes in chromatin structure during replication, rather than by micrococcal nuclease-induced sliding of histone cores in vitro.

INTRODUCTION

Transiently occurring reduced repeat sizes of nascent chromatin DNA were first suggested in our report of ~150 and 300 deoxyribonucleotide pairs (bp) as well as larger nascent DNAs which were obtained from micrococcal nuclease digests of nuclei of sea urchin blastulae (1). These micrococcal nuclease hyperresistant chromatin DNAs were shown to be protected by discrete deoxyribonucleoprotein structures (2). They were interpreted, though not finally proven, to be closely packed nucleosome cores (1,3) containing nascent DNAs which are integral multiples of ~150 bp, while bulk chromatin DNA had a repeat length of ~200 bp. Since then many cases of reduced repeat lengths of nascent chromatin DNA as compared with bulk DNA, have been reported in other systems (4-8).

Some time ago, Jackson et al (7) suggested that reduced repeat length of nascent chromatin DNA as well as its hyperresistance to micrococcal nu-
clease may be due to the sliding of nucleosome cores on nascent DNA. Such sliding could be effected by the micrococcal nuclease treatment, as histone H1 is being removed at higher levels of this enzyme. They reported that increasing time of micrococcal nuclease digestion led to a gradual decrease in the repeat length of pulse-labeled chromatin DNA as compared with bulk DNA of Hepatoma tissue culture (HTC) cells. Our nascent DNA gels from high and low digests did not indicate such a result (1). This effect could also not be confirmed by Annunziato and Seale (8) for newly replicated chromatin DNA of Hela cells.

An analogy of the reduced repeat size of nascent chromatin DNA with that observed in vitro as compact di-and-oligonucleosomes in H1 depleted chromatin (9, 10) has been proposed (1, 7, 11). It has also been demonstrated in vitro that in absence of histone H1, histone cores can slide along the DNA (12). We have interpreted the ~300 and 450 bp nascent DNAs of sea urchin embryos (1,3) as a consequence of in vivo changes in chromatin structure at the replication fork. However even if this is an in vitro event, it would reflect an interesting in vivo modification of chromatin with nascent DNA which, after micrococcal nuclease digestion, results in the compaction of certain nucleosome cores only.

It was suggested that the question of in vitro nucleosome core sliding during micrococcal nuclease treatment could be resolved by formaldehyde fixation prior to digestion (7), thus cross-linking the histone cores with the DNA. Such an experiment was described for CHO cells by Vaury et al (13) who report a shorter repeat length of DNA from a chromatin fraction enriched in replicating material, than the DNA repeat from a fraction enriched in parental chromatin. This difference disappeared when nuclei were fixed with formaldehyde before micrococcal nuclease digestion. They conclude that the actual repetitive unit of replicating cellular chromatin is not different from bulk chromatin.

In view of the importance of the above conclusion for future studies of chromatin structure during DNA replication, we wanted to determine whether or not our ~300 bp LN DNA and the ~450 bp nascent trimer DNA (1,3) are the consequence of micrococcal nuclease induced sliding of their nucleosome cores in vitro. In contrast to the report of Vaury et al (13), we find that the difference in the size between the micrococcal nuclease hyper-resistant nascent DNAs and the bulk nucleosomal DNAs into which these nascent DNAs eventually mature, are not eliminated by fixation of the nuclei before digestion. We conclude therefore that these transient changes in
repeat sizes reflect in vivo modifications of chromatin organization during DNA replication.

MATERIALS AND METHODS

Nuclei were isolated from pulse and long labeled sea urchin (Paracentrotus lividus) blastulae and nucleic acids and radioactive DNA were determined as described in refs. 1 and 2. Nuclei in 50% solution W, pH 7.4 (1) and 50% glycerol (2) were transferred by centrifugation (4080 x g) to solution WF (30 mM Tris-HCL, 2% formaldehyde, 60 mM KCl, 15mM NaCl, 0.05 mg/ml dithiothreitol, 0.32M sucrose; pH 7) or to solution W (1), but at a pH of 7. They were then treated as described in the legends of figures and the table.

RESULTS

Our approach to resolve the nascent and bulk DNA fragments, which are protected by chromatin structure against micrococcal nuclease digestion, was to directly extract total DNA from digested nuclei (1,3). A typical DNA fragment pattern of [14C]thymidine long and [3H]thymidine pulse labeled chromatin DNA from such a digest, separated in a polyacrylamide gel, is presented in Figure 1A.

As shown in previous publications (1,3) the bulk DNA fragments represent multiples of ~ 200 bp repeats with monomers of ~ 180 bp, reflecting a partial degradation of nucleosome linker regions. The [3H]thymidine pulse labeled, nascent monomer DNA fragments are slightly shorter than their bulk counterparts at low digestion and approach ~ 150 bp at relatively high levels of nascent DNA digestion (3). In addition, there are the ~ 300 bp large nascent (LN) DNA as well as ~ 450 bp nascent fragments which migrate between bulk monomers and dimers and trimers respectively. Both of these nascent fragments lack bulk equivalents (Fig. 1A and refs. 1-3). Variations in the size of the LN DNA ranging from ~ 270 to 320 bp have been observed (1). This heterogeneity may occur in discrete peaks in the same preparation as is the case in the present experiment. The nature of this variation is presently being studied.

Those nascent DNAs consisting of ~ 150 bp repeats, are hyperresistant to further cleavage by micrococcal nuclease. This results in a tail of nascent fragments which, at high levels of the enzyme, extends to the bulk tetramer (1,3).

Does fixation before micrococcal digestion eliminate the difference in size between some nascent and bulk chromatin DNAs?

Formaldehyde fixation crosslinks proteins as well as proteins and DNA,
Figure 1.- The nascent and bulk DNAs from a micrococcal nuclease digest of fixed nuclei. A mixture of nuclei from [methyl-14C]thymidine long (14 hr, 0.2 pCi/ml) and [methyl-3H]thymidine pulse (25 sec, 60 pCi/ml, 79.2 Ci/mmole) labeled embryos was fixed for 1/2 hr in 2% formaldehyde in solution W in ice (B), or kept on ice in solution W adjusted to pH 7 only (A). After 3 rinses with cold solution W, pH 7.4 (centrifug. 3300xg), the nuclei were re-suspended at 20 A260/ml in the same solution and treated for 45 sec with 100 U/ml micrococcal nuclease (Worthington) in presence of 1mM CaCl2 at 37°C. Digestion was terminated by rapid cooling in ice and addition of EDTA to make 10mM. After removal of samples to determine digestion percentages to acid soluble nucleotides as described in ref. 1, SDS and proteinase K (Boehringer) were added to make 1% and 50 μg/ml respectively. This was followed by incubation at 37°C overnight, after which 1.5 vol. of 1M NaCl was added for 5 min. at 39°C and DNA was extracted with chloroform: isoamyl alcohol as described in ref. 1. Fractionation in 1.5% Tris- Na Acetate polyacrylamide tube gels and counting was as described in ref. 1. I-IV: Bulk [14C]DNAs, monomer-tetramer (~200 bp repeats) LN DNA: Large Nascent DNA; Arrows: Nascent "trimer".
Table 1. The Effectiveness of Formaldehyde Fixation in Binding DNA and Proteins in Chromatin

| Treatments       | Yield of Nucleic Acids |         |         |
|------------------|------------------------|---------|---------|
|                  | A260 | Percent of | [3H]cpm | Percent of |
|                  |      | original A260 recovered |         | original [3H]cpm recovered |
| No. fixation     | 1,250 | 31.0       | 55,500  | 27.0       |
| 0.7% Formaldehyde| 0.206 | 5.0        | 8,426   | 4.1        |
| 2% Formaldehyde  | 0.027 | 0.7        | 800     | 0.4        |

Isolated nuclei from embryos labeled as in Fig. 1. contained 4 A260 of nucleic acids and 2.06 x 10^5 [3H]thymidine cpm per treatment. These were fixed as indicated and then digested for 60 sec with micrococcal nuclease as already described. DNA was extracted by the addition of 1.5 vol of 1.5% SDS and 1.5M NaCl for 5 min at 39°C and subsequent extraction with chloroform-isoamyl alcohol as described in ref. 1. The radioactivities in DNA were counted on GF/C glass filters, and the results were adjusted for the reduced efficiency of [3H]counting in double labeled material. The [14C] thymidine counts (not shown) corresponded to the A260 results. Thus making it impossible for nucleosomal histone cores to slide relative to the DNA. If the differential repeat length between some nascent and bulk DNA is the result of micrococcal nuclease-generated sliding of histone cores, we expect identical nascent and bulk fragment sizes when fixation precedes digestion. It can be seen that the difference in electrophoretic migration between bulk and nascent DNA characteristic of the control (Fig. 1A), is maintained in Fig. 1B, when nuclei had been fixed prior to enzyme treatment.

In order to ascertain that our formaldehyde fixation was really effective in linking DNA with proteins, we omitted the overnight sodium dodecyl sulfate (SDS)-proteinase K incubation used in the experiments in Figures 1 and 2. Instead, nucleic acid extraction was attempted directly by the usual SDS-NaCl-chloroform extraction procedure described in ref. 1. This direct extraction of DNA from a micrococcal nuclease digest of fixed nuclei resulted in an 0.7% yield of nucleic acids (Table 1). An identical digest of unfixed nuclei yielded a normal 31% of extracted nucleic acids and normal [3H]pulse labeled radioactivity. It is also clear from Table I that the [3H]thymidine pulse labeled DNA did in no way escape formaldehyde fixation. Therefore, the fixation used in Figure 1B was effective.

The foregoing results show that the micrococcal nuclease hyperresistant [3H]thymidine pulse-labeled nucleosomal DNAs of sea urchin embryos, have not
Figure 2. - The hyperresistance of the large nascent (LN) and ~450 bp nascent DNAs to cleavage by micrococcal nuclease, following fixation. - Labeling, fixation, extraction of DNA and gel electrophoresis were as described in Fig. 1B, but micrococcal nuclease treatment lasted for 5 min. Sensitivity of fixed and unfixed chromatin DNA to digestion and cleavage by micrococcal nuclease.

The usual higher digestibility of nascent than bulk chromatin DNA to acid soluble nucleotides by micrococcal nuclease (1,14), is also obtained in fixed nuclei (Fig. 1B; right top). Quite similar differences in digestion percentages are also observed when one compares bulk with nascent DNA from fixed or unfixed nuclei (Fig. 1).

In addition to the nucleosomes containing nascent DNAs of shorter repeat length, which are hyperresistant to micrococcal nuclease (1,3), there are also nucleosomes containing nascent DNA and linkers which are more sensitive to cleavage by micrococcal nuclease, than bulk DNA linkers (1, 2). This leads to a more rapid rate of cleavage of nascent than bulk DNA to smaller oligomers and monomers (1, 14). Formaldehyde fixation increases cleavability of both bulk and nascent chromatin DNA by micrococcal nuclease, as can be seen by the increase in the \(^{14}C\) and \(^{3}H\) radioactivities in smaller oligomers in Figure 1B over 1A. This indicates a general effect of fixation toward greater bulk as well as nascent linker sensitivity to micrococcal nuclease.

Although the fixation of nuclei results in increased linker sensitivity to micrococcal nuclease, the ~300 bp LN and the ~450 bp nascent DNAs
remain hyperresistant to extensive digestion by this enzyme in fixed nuclei (Fig. 2). This result is consistent with the hypothesis (1, 3) that these hyperresistant nascent DNA fragments, are derived in vivo from immature, closely packed nucleosome cores which lack intercore linkers and therefore are not affected by fixation.

**DISCUSSION**

This paper addresses itself to the question raised in the recent literature (7, 13, 15) whether the origin of reduced repeat lengths reported in nascent as compared with bulk chromatin DNA, may be an artifact of micrococcal nuclease digestion in vitro. The question is important because its answer may point to approaches to answer further questions on nucleosome structure near the center of the replication fork. It is also valid to ask if, why and how our result differ from those reported elsewhere.

Vaury et al (13) report a reduced repeat length (157 bp) of DNA from their unfixed S1 fraction, enriched in replicative chromatin (16), as compared to DNA from the S2 fraction (195 bp), which was enriched in parental chromatin. When nuclei were fixed prior to micrococcal nuclease digestion, DNA from both S1 and S2 showed ~190 bp repeats.

Our present finding that formaldehyde fixation increases the linker sensitivity of nucleosomes (Fig. 2) raises a question about the level of enrichment in replicative chromatin obtained by the above authors from fixed nuclei. Increased linker sensitivity results in the greater accumulation of mononucleosomes which would reduce this enrichment for replicative chromatin in the S1 from fixed nuclei. Such reduced enrichment for replicative chromatin was clearly shown by Worcel et al in their S1, under conditions of increasing levels of micrococcal nuclease digestion (see ref. 16, Fig. 2). Since bulk DNA fragments of normal size and their corresponding shorter nascent DNA fragments are not clearly separated from each other in gels (Fig. 1 and ref. 15), an average between them would reflect their relative proportion in the mixture. The sizes of DNA fragments in Vaury et al's gels (13) appear to represent an average of replicating and bulk DNA in each of the two fractions (S1 and S2) from fixed and unfixed nuclei. Therefore, reduced enrichment in nascent DNA of the S1 from fixed nuclei, could bring the average migration distances of the S1 DNA bands to a repeat length similar or identical to that of the S2 bulk DNA.

There is however a more general explanation for the disagreements be-
tween our present as well as our early reports (1-3, 11), and that of Vaury et al (13) as well as of all other accounts on the repeat lengths of nascent chromatin DNA (4-8). We are probably analyzing a somewhat earlier stage of chromatin replication and/or are using a very different (embryonic) system than the investigators of chromatin replication in mammalian tissue culture cells. It appears to be difficult or impossible to obtain sufficiently rapid incorporation of $[^3H]$thymidine pulses into DNA of mammalian tissue culture cells (6-8, 15) so as to label DNPs which contain the micrococcal nuclease hyperresistant DNAs before they begin to mature. The prompt and effective termination of incorporation (1) is critical, so that these "early" labeled DNAs do not become an unresolvable minority among the very rapidly appearing newly replicated DNAs from partially matured DNP structures (5-8, 13).

In sea urchin embryos, the presumably closely packed DNPs and their nascent DNAs (1-3) can be detected after very short pulses of $[^3H]$ thymidine. After longer $[^3H]$thymidine pulses, or pulse chases, these nascent DNAs mature to dimers, trimers etc (1). The loss of hyperresistance to micrococcal nuclease is one of the first events in the maturation of the ~300 bp LN DNA to a bulk dimer (ref. 17, and Ben Yosef and Jakob in prep). It seems likely therefore that 1) DNPs containing micrococcal nuclease hyperresistant nascent DNA escape detection in mammalian tissue culture cells (6-8, 13) possibly because of their more rapid loss of this hyperresistance than that observed in sea urchin embryos. Alternatively 2) these structures may not occur at all at the replication fork of mammalian tissue culture cells.

The greater plausibility of the first of the above interpretations has been strengthened by the account of Smith et al (15) whose very recent paper on maturation of newly replicated chromatin in HTC cells appeared only after preparation of this manuscript. They report a micrococcal nuclease-generated sliding of nucleosomes containing newly replicated DNA, which gives rise to reduced repeat lengths between between about 5 and 15 min of thymidine chases following a 2 min $[^3H]$thymidine label. These DNAs are replaced by normal bulk repeat lengths when formaldehyde fixation was performed before micrococcal nuclease digestion or after 30 min chase, by which time newly replicated chromatin had fully matured.

While we have no data on these interesting results of these stages of maturation of nucleosomes, they are consistent with our original proposal (1) in which we suggest the sliding of histone cores relative to newly
replicated DNA during maturation of the micrococcal nuclease hyperresistant closely packed nucleosome cores. Thus a capacity of maturing nucleosome cores to slide during micrococcal nuclease digestion in vitro, at least does not exclude such an event during maturation in vivo.

CONCLUSION

The shorter repeat length of micrococcal nuclease - hyperresistant, short pulse-labeled, chromatin DNA, as compared with that of bulk chromatin DNA, exists in sea urchin blastulae before micrococcal nuclease digestion of the isolated nuclei. Therefore the structural changes in chromatin containing these nascent DNAs as described in refs. 1-3, 11 and 17, were generated in vivo during DNA replication.

ACKNOWLEDGEMENTS

We are grateful to Gad Galili for advice and discussions during the preparation of this manuscript. This work was supported in part by the MINERVA Foundation, Munich, Germany.

*To whom correspondence should be addressed.

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