Vol. 110, No. 3, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 10, 1983 Pages 811-818

ASSEMBLY KINETICS OF REPLICATING CHROMATIN : ISOLATION AND CHARACTERIZATION OF PRENUCLEOSOMAL AND NUCLEOSOMAL DNA

C. VAURY, C. GILLY, D. ALIX, J.J. LAWRENCE*

Laboratoire de Biologie Moléculaire et Cellulaire,
Département de Recherche Fondamentale,
Centre d'Etudes Nucléaires de Grenoble,
85X,
F 38041 GRENOBLE
FRANCE

Received December 13, 1982

SUMMARY : Replicating chromatin is known to be more sensitive to micrococcal nuclease than bulk chromatin. We have used this property and a fractionation procedure based on the specific release of replicating material under mild micrococcal nuclease digestion, in order to analyse both the kinetics of maturation of newly replicated DNA into nucleosomes and the structure of the replicating material. As other authors, we initially observed that repetitive unit of newly replicated chromatin was shorter than that of bulk chromatin, however this result appears to be due to sliding of nucleosomes along the chromatin fibers close to the replicating fork. Replicative chromatin was fractionated and analysed. A prenucleosomal peak was observed and preliminary characterized.

INTRODUCTION : The replication of chromatin involves DNA and protein synthetic processes and assembly of these constituents at/or near the replication fork. The kinetics of histone-DNA assembly into nucleosomes have been studied in various systems (1-6). In combination with pulse-charge experiments we have used the hypersensitivity of newly replicated chromatin to estimate its kinetics of maturation.

The observed difference between the sensitivity of newly replicated and bulk chromatin is probably related to a difference in their structures. Indeed the repetitive unit has been reported to increase during maturation (3,7-12). However other authors have not observed this difference (5,13). Since nucleosomes sliding after micrococcal nuclease digestion has been reported (14), and this effect would be more apparent in newly replicated chromatin than on bulk chromatin due to its greater degree of digestion, we have measured the size of the repetitive unit before and after formaldehyde fixation. Another reason for the hypersensitivity of newly replicated chromatin might be because it is not yet structured into nucleosome (13,15,16). To test this hypothesis, a chromatin fraction enriched in replicative material was isolated according to Worcel's method (4). This

*Member of INSERM, to whom reprint requests should be addressed.

0006-291X/83/030811-08$01.50/0

method was prefered rather than a stepwise salt elution which may induce some protein rearrangement (15,16). Fractionation of this material shows a peak corresponding to replicative material lighter than mononucleosomes. It is demonstrated by studies on cells arrested in Go phase and chase experiments on cells in exponential phase of growth, that this peak is made of prenucleosomal replicative material.

METHODS : CHO cells were grown at 37°C in a 5 % CO2 atmosphere in α Minimum Essential Medium supplemented with 7 % foetal calf serum.

1. Labelling of DNA : Parental chromatin was labelled during 24 h with [^{14}C]-thymidine (0.02 µCi/ml, specific activity : 55 mCi/mMole). After this period, the [^{14}C]-thymidine was removed and the culture continued for a further 17 h. Newly replicated chromatin was labelled with [^{3}H]-thymidine (specific activity 50 Ci/mMole) for 20 or 30 seconds. The labelling was stopped by washing with cold isotonic saline solution (Tris-Dulbecco) and the cells were harvested.
In chase experiments, [^{3}H]-thymidine was diluted by the addition of a 100 to 1000 fold excess of unlabelled thymidine and the cells were incubated in fresh culture medium during an additional time at 37°C.

2. Nuclei isolation : Cells were pelleted and resuspended on ice, in 10 mM Tris-HCl (pH = 7.5), 2 mM $CaCl_2$ at a concentration of 10 cells per ml. After 10 min, the cells were lysed with a Dounce homogenizer. The lysate was layered over 10 ml of 1.7 M sucrose in 100 mM KCl, 5 mM PIPES (pH = 7.0), and centrifuged (18000xg; 1 h) (17). The nuclei were washed twice in the same buffer with 0.15 M sucrose and resuspended at the desired concentration.

3. Micrococcal nuclease digestion : The nuclei were resuspended at 2.10^7 nuclei/ml at 37°C in 60 mM KCl, 15 mM NaCl, 0.15 mM Spermine, 0.50 mM Spermidine, 15 mM 2-β mercaptoethanol, 15 mM Tris HCl, (pH = 7.4) (1). 1 mM PMSF was added to the nuclei suspension. 0.3, 3, 30 units of micrococcal nuclease (Worthington) were added to 2.10^6 nuclei with 1 mM $CaCl_2$.
The reaction was stopped at given times, by addition of 2 mM EDTA (final concentration) and acid-soluble material was measured after TCA precipitation.

4. Formaldehyde fixation of nuclei : Extracted nuclei were resuspended in 100 mM KCl, 1 mM $CaCl_2$, 0.15 M Sucrose, 5mM PIPES, (pH = 7.0), at 10^8 nuclei per ml and incubated with 2 % formaldehyde for 1 hour on ice. Nuclei were then centrifuged at 1400xg for 5 min and washed twice in the same buffer before digestion.

5. Fractionation of replicative chromatin : Fixed or unfixed nuclei were resuspended in the buffer (10^8 nuclei/ml)-Micrococcal nuclease (60 units/ml) was added to the suspension and digestion allowed to proceed at 37°C for 4 min for fixed nuclei and 6 min for unfixed nuclei. For these digestion times, the acid-soluble material was the same in both samples (≃10 % of [^{14}C] DNA acid-soluble). The digestion was stopped by the addition of EGTA (final concentration 2 mM) and chilling on ice. Digested nuclei were then gently mixed and centrifuged at 1400xg for 5 min as described by Worcel et al (4) who showed that this supernatant (S1) was enriched in replicative chromatin.
The bulk chromatin was obtained in a second supernatant (S2) after lysing the preceeding nuclei pellet in 0.2 mM EDTA (pH = 7.0), at 10 nuclei per ml, for 30 min on ice. This suspension was centrifuged at 1400xg for 5 min. The S1 and S2 supernatants were layered on the top of an isokinetic sucrose gradient (5-60 % w/v) (18) containing 5 mM PIPES (pH = 7.0), 100 mM KCl, 0.2 mM EGTA and centrifuged in a Beckman rotor SW 41 Ti 130000xg for 20 h at 4°C. Absorbance at 258 nm and radioactivity measurements were made for each fraction.

6. Electrophoretic analysis : DNA was extracted by the addition of 1 % SDS and 50 µg/ml proteinase K at 37°C overnight and was run on 2.5 % agarose slab gel as described (19).

RESULTS

1. Maturation of newly replicated chromatin : The kinetics of maturation were investigated by very short pulse labelling of newly replicated chromatin (20 s) followed by a chase. Figure 1 (insert) shows the kinetics of digestion of newly replicated material ([^{3}H] counts) as compared to bulk chromatin ([^{14}C] counts). As previously described by others (3,6,7,20,21), newly replicated chromatin was found to be more accessible to micrococcal nuclease than parental chromatin. Figure 1 shows the difference curve between these two curves and its variation as a function of the chase time. A maximum difference of 15 % between the amount of acid-soluble DNA released is observed for no chase, this difference is reduced to 5 % after a 1 minute chase and 2 % after only a 5 minutes chase. A similar difference, but of

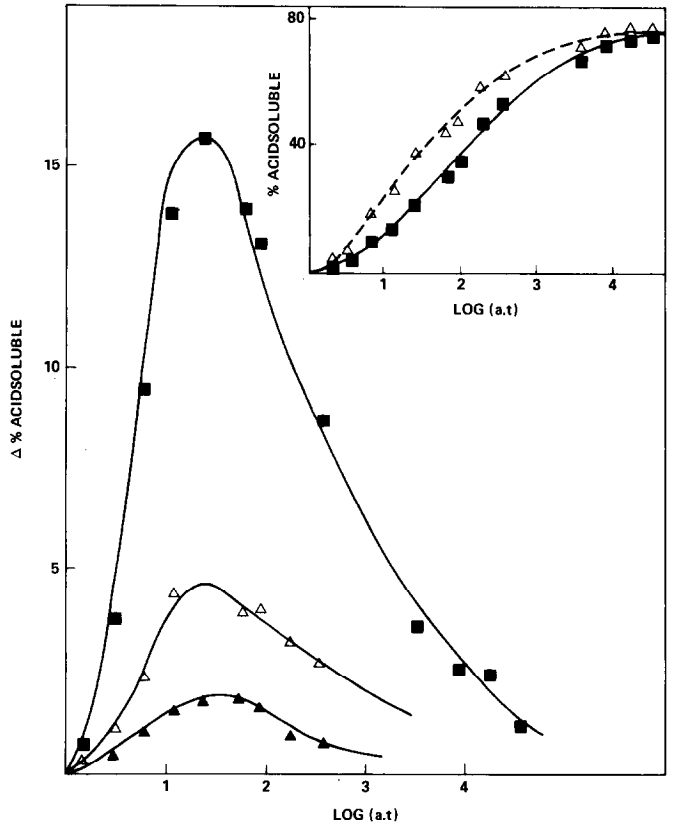

Fig. 1 - Kinetics of maturation of newly replicated chromatin - Kinetics of micrococcal nuclease digestion at 37°C. Nuclei were isolated from CHO cells doubly labelled with [^{14}C]-thymidine (0.02 µCi/ml, 55 mCi/mMole) for 24 h and [^{3}H]-thymidine pulse (10 µCi/ml, 50 Ci/mMole) for 20 s. The production of acid-soluble material is shown as a function of the extent of digestion, expressed as the logarithm activity of enzyme per sample (units x digestion time (min) Δ···Δ [^{3}H] label ■—■ [^{14}C] label (insert). The difference between acid-soluble material of both types of material is calculated and plotted as a function of chase time : 0 min ■—■ 1 min Δ—Δ 5 min ▲—▲.

lower amplitude, is observed when the pulse duration is varied, i.e. the longer the pulse labelling time the lower the maximum amplitude of the difference curve (data not shown).

2. Isolation and analysis of a nuclear fraction enriched in newly replicative chromatin : Differences in the accessibility of micrococcal nuclease to replicating chromatin as compared to bulk chromatin may be related to differences in their structural organization. For this reason, we have isolated a nuclear fraction enriched in newly replicated chromatin to compare its structure with a fraction of mature chromatin.

As described by Worcel et al. (4) the oligonucleosomes leaking from nuclei during the digestion process and which are recovered in the supernatant S1 after a low speed centrifugation are enriched in replicating material. The mature chromatin is recovered in the supernatant S2 after the lysis of the nuclei pellet. Figure 2 shows the variation of the amount of replicative material and parental chromatin as a function of the digestion time in both S1 and S2 fractions. It is clear that the S1 supernatant is enriched in replicative material as evidenced by the higher [^{3}H]/[^{14}C] ratio. The reverse is observed in S2 which mainly contain parental chromatin.

A - Size of the repetitive unit of newly replicated and mature chromatin :

We have measured the repetitive unit in S1 and S2 supernatants by DNA electrophoresis. The repetitive unit of new DNA fragments (157 bp) is smaller

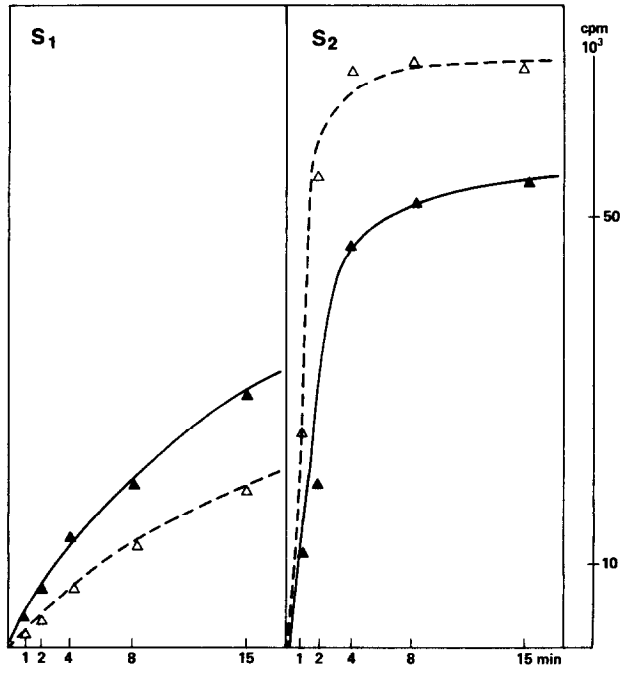

Fig. 2 - [^{3}H] cpm (——) and [^{14}C] cpm (---) plotted as a function of digestion time. 10^{8}nuclei are digested by 60 units of micrococcal nuclease at 37°C. S1 and S2 were obtained as described in Methods.

TABLE I - Number of DNA base pairs in the oligomers contained in fractions S_1 and S_2 obtained from unfixed and formaldehyde (2%) fixed cells.

Oligomers	monomers	dimers	trimers	tetramers	repetitive
Fraction	number of bases pairs				unit
S_1 from unfixed cells	145	290	455	670	157
S_2 from unfixed cells	160	320	570	800	195
S_1 from fixed cells	180	390	600	780	195
S_2 from fixed cells	180	380	580	770	190

than that of bulk chromatin (195 bp). However it is known that DNA digestion by micrococcal nuclease may induce sliding of nucleosomes along the chromatin fiber (14). Since replicating chromatin is more rapidly digested than bulk chromatin, this phenomenon could be responsible for the difference observed in the repetitive unit of both types of material.

To prevent the nucleosomes sliding, the chromatin was fixed with formaldehyde prior to digestion. These nuclei were then digested with micrococcal nuclease to obtain 10 % of acid-soluble bulk DNA and the repetitive unit measured on supernatants S1 and S2 as before (Table I).

The repetitive unit was found to be 195 bp and 190 bp, in S1 and S2 respectively, values very similar to those obtained for unfixed bulk chromatin.

B - Fractionation of S1 on sucrose gradient : the fraction enriched in replicative chromatin (S1) was sedimented on an isokinetic sucrose gradient as described in Methods. The absorbance profile of this gradient (figure 3, curve A) shows that a peak of material of lower molecular weight than mononucleosome (prenucleosomal peak) appears in addition to the familiar oligonucleosome pattern. [^{3}H] and [^{14}C] cpm were measured along the gradient (curve B and C respectively in figure 3). These profiles show that the prenucleosomal material is highly enriched in replicative material, a situation which is no longer observed in the mononucleosome peak for the digestion conditions used in these experiments. When supernatant S1 as obtained from cells incubated in the same conditions as this sample but without the micrococcal nuclease, no prenucleosomal peak was observed. That the prenucleosomal material was made of newly replicated material was confirmed by two sets of experiments : firstly the prenucleosomal peak was

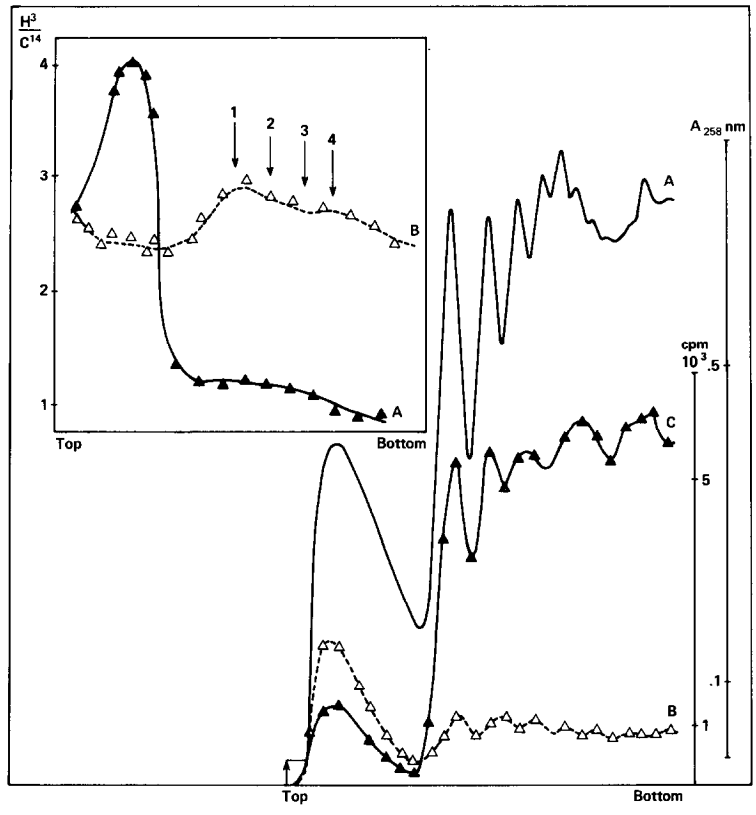

Fig. 3 - Sedimentation profile on an isokinetic 5-60 % sucrose gradient of the fraction S1.
This fraction was obtained after the digestion of 10^8 nuclei by 30 units of micrococcal nuclease for 2 min. at 37°C.
A absorbance at 258 nm.
B [^{3}H] cpm.
C [^{14}C] cpm.
insert : Relative enrichment of newly replicated DNA in the fraction S1.
Variation of [^{3}H] cpm/[^{14}C] cpm ratio of two digests.
A - supernatant S1 obtained from nuclei pulse labelled for 30 s with [^{3}H]-thymidine.
B - Supernatant S1 obtained from nuclei labelled for 30 s [^{3}H]-thymidine followed by a 10 min. chase.

absent from supernatant S1 obtained from cells arrested in Go at saturation density under controlled conditions of growth (22); secondly the evolution of the [^{3}H]/[^{14}C] ratio along the gradient with increasing chase times was determined. Figure 3 (insert) shows the results obtained after a 10 min. chase : the ratio [^{3}H]/[^{14}C] was about 4 in the prenucleosomal peak in the absence of maturation and only 1 for the other fractions. After 10 min of maturation in the absence of [^{3}H]-thymidine it decreased to about 2.5, whereas this value increased to 3 in the mononucleosome peak and decreased slightly for the other fractions.

DISCUSSION : In agreement with results published by others (3,6,7,20,21) on different systems we observed that the replicative chromatin from CHO cells

was more sensitive to micrococcal nuclease than bulk chromatin. We have used this property to try to estimate the maturation time of replicating chromatin into bulk chromatin. Using short pulse labelling and chase experiments we were able to show that the hyper-sensitivity of replicating chromatin to micrococcal nuclease was abolished within a few minutes. This strongly supports the suggestion that the loss of hyper-sensitivity to micrococcal nuclease with increasing chase times is due to maturation of chromatin. The greater sensitivity of replicating chromatin to micrococcal nuclease may have several origins. One of them could be a difference in the basic structure of nucleosomes. The repetitive unit of replicating chromatin has been studied by several authors and found either identical to (5,13) or shorter (3,7,9-12) than that of bulk chromatin. Under conditions used by Levy et al. (7) or Murphy et al. (9), we found a much shorter repetitive unit in replicating chromatin than in mature chromatin. However when the nuclei were fixed with formaldehyde to prevent sliding of nucleosomes, this difference was abolished. Hence our results imply that the repetitive unit of replicating cellular chromatin is not different from that of bulk chromatin, and the increased sensitivity to micrococcal nuclease must originate from other causes.

Worcel et al. (4) have shown that histone H1 is deposited on the replicating material much later that the other histones, Schlaeger (23) has found that histone H1 is more weakly bound to chromatin during replication. Both results could explain the hyper-sensitivity to micrococcal nuclease. Another reason could be that nascent DNA fragments in the vicinity of the replication fork are not yet protected as in the nucleosomal structure from the action of nuclease. The fractionation of a nuclear digest enriched in newly replicated material led us to isolate a [^{3}H] labelled DNA fragment the molecular weight of which was lower than that of mononucleosomes. Such fragments were absent in DNA from cells arrested in Go, and their enrichment in newly synthetized labelled DNA desappeared after relatively short chase experiments. The labelled DNA was then found predominantly in mononucleosomes and to a less extend in higher order oligomers. This result is in good agreement with the rate at which hyper-sensitivity to micrococcal nuclease described in the first part of the paper is lost.

Preliminary characterization (unpublished results), of this prenucleosomal replicative material shows that it is composed of both DNA and proteins. High resolution electrophoresis of DNA after [^{32}P] labelling showed a series of discreet bands in the range of 30 base pairs. Among proteins, several histones fractions, namely H3 and H2B, were found, a result different from what was observed in SV40 replication (13). These results strongly suggest that the prenucleosomal material at least partially constituted of structural

intermediates between parental and newly synthetized nucleosomes, but clearly more experimental data is needed to further characterize this material.

ACKNOWLEDGEMENTS :

This work was supported in part by grants from CNRS (CP 033 416) and DGRST (81 L 0726). We are grateful to Dr. H. Beverley OSBORNE for critical reading of the manuscript.

REFERENCES :

1. Hewish, D.R. and Burgoyne, L.A. (1973) Biochem. Biophys. Res. Com. 52, 504-510.
2. Seale, R.L. (1976) Cell, 9, 423-429.
3. Seale, R.L. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2717-2721.
4. Worcel, A., Han, S. and Wong, M.L. (1978) Cell, 15, 969-977.
5. Cremisi, C. (1979) Microbiol. Rev. 43, 297-319.
6. Jalouzot, R., Briane, D., Ohlenbusch, H.H., Wilhelm, M.L. and Wilhelm, F.X. (1980) Eur. J. Biochem. 104, 423-431.
7. Levy, A. and Jakob, K.M. (1978) Cell, 14, 259-267.
8. Munroe, S.H. and Latt, S.A. (1977) Exp. Cell. Res. 110, 299-313.
9. Murphy, R.F., Wallace, R.B. and Bonner, R.B. (1980) Proc. Natl. Acad. Sci. U.S.A., 77, 3336-3340.
10. Yakura, K. and Tanifuji,S. (1980) Biochem. Biophys. Acta, 609, 448-455.
11. Galili, G., Levy, A. and Jakob, K.M. (1981) Nucleic Acids Res. 9, 3991-4005.
12. Jackson, V., Marshall, S. and Chalkley, R. (1981) Nucleic Acids Res. 9, 4561-4581.
13. Cusick, M.E., Herman, T.M., De Pamphilis, M.L. and Wassarman, P.M. (1981) Biochemistry, 20, 6648-6658.
14. Spadafora, C., Oudet, P. and Chambon, P. (1979) Eur. J. Biol. 100, 225-235.
15. Annunziato, A.T., Schindler, R.K., Thomas, C.A., Seale, R.L. (1981) J. Biol. Chem. 256, 11880-11886.
16. Annunziato, A.T., Schindler, R.K., Riggs, M.G., Seale, R.L. (1982) J. Biol. Chem. 257, 8507-8515.
17. Compton, J.L., BELLARD, M. and CHAMBON, P. (1976) Proc. Natl. Acad. Sci. USA, 73, 4382-4386.
18. Mac Carty, Jr.K.S., Vollmer,R.T., Mac Carty, K.S. (1974) Anal. Biochem. 61, 165-183.
19. Gorka, C. and Lawrence, J.J. (1979) Nucleic Acids Res. 7, 347-359.
20. Hildebrand, C.E. and Walters, R.A. (1976) Biochem. Biophys. Res. Com. 73, 157-163.
21. Klempnauer, R.H., Fanning, E., Otto, B. and Knippers, R. (1980) J. Mol. Biol. 136, 359-374.
22. Chabanas, A., Gilly, C., Vaury, C., Lawrence, J.J. (1980), 2d International Congress on Cell Biology, Berlin.
23. Schaeger, E.J. (1982) Biochemistry 21, 3167-3174.