RAPID ASSEMBLY OF NEWLY SYNTHESIZED DNA INTO CHROMATIN SUBUNITS PRIOR TO JOINING OF SMALL DNA REPLICATION INTERMEDIATES

C. E. Hildebrand and R. A. Walters

Cellular and Molecular Biology Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87545

Received September 10,1976

SUMMARY: Nuclei from cells having the replicating DNA pulse-labeled with [^{3}H]thymidine and the nonreplicating DNA uniformly labeled with [^{14}C]thymidine were treated with micrococcal nuclease according to procedures which have been used to study the subunit structure of chromatin. Sedimentation analyses of chromatin from nuclease-treated nuclei, together with measurements of the size of newly synthesized DNA, indicate that (1) chromatin subunits near the replication fork are more susceptible to nuclease attack than subunits in nonreplicating chromatin; (2) newly synthesized DNA is rapidly assembled into chromatin subunits prior to joining of small DNA replication intermediates; and (3) within 10 min after synthesis, DNA in newly replicated chromatin acquires a susceptibility to nuclease treatment similar to that of nonreplicating chromatin.

INTRODUCTION

Recent advances in studying the fine structure of eukaryotic chromatin have led to a generalized subunit structure of chromatin (1-16). However, the mechanisms by which this repeating subunit or nucleosome (6) structure is replicated remain to be elucidated (17,18). It is clear that chromatin replication in eukaryotic cells requires not only duplication of the DNA but also coordinate synthesis of chromatin proteins and association of these proteins with the nascent DNA duplexes (18-23). We report here studies of the incorporation of replicating DNA into the subunit structure of chromatin. The procedures employed in this report are unique in that they provide by sedimentation analysis of nuclease-treated chromatin a direct comparison of the assembly of newly replicated DNA into nucleosomes with the subunit organization of nonreplicating chromatin.

MATERIALS AND METHODS

Chinese hamster cells (line CHO) were maintained as described previously (24) in Ham's F-10 medium lacking thymidine. Cells with uniformly labeled DNA were obtained by two consecutive 24-hr treatments with 0.005 μCi/ml [^{14}C]-thymidine (Schwarz/Mann, 55.3 mCi/mmole), followed by a 2-hr chase in fresh label-free medium. Cells were pulse-labeled with 50 μCi/ml [^{3}H]thymidine (New England Nuclear Corp., 40-60 Ci/mmole). The pulses were stopped by pouring the culture over crushed, frozen medium plus 3 mM thymidine containing 30 mM KCN (adjusted to pH 7.0). In the pulse-chase experiments, the KCN was left out of the pulse-stopping medium. Nuclei were prepared using a nonionic deter-

gent (NP-40), as described previously (25), washed twice in ice-cold buffer [0.01 M Tris-Cl (pH 7.4 at 24°C), 0.01 M NaCl, 0.0015 M $MgCl_2$], and finally resuspended in buffer A, as described by Hewish and Burgoyne (1). At this stage, all ^3H and ^{14}C radioactivity in the nuclei is insoluble in cold 5% trichloroacetic acid (TCA).

Nuclei were treated with 110 units/ml of micrococcal nuclease (Worthington Biochemical Corp.) at 1.9 x 10^7 nuclei/ml in buffer A (1) with 0.1 mM $CaCl_2$. It should be noted that the $CaCl_2$ concentration was one-tenth that commonly used in chromatin digestion studies (10,16). After incubation at 37°C for the times indicated in the text, nuclease action was terminated by dilution of the reaction mixture with one volume of ice-cold 2 mM EDTA (pH 7.0), and the samples were stored in ice. Within 5 min, nuclease-treated nuclei were harvested by centrifugation at 2000 x g for 15 min. The total suspension before centrifugation and the supernatant and pellet fractions following centrifugation were analyzed both for total ^3H and ^{14}C radioactivity and for cold 5% TCA-insoluble ^3H and ^{14}C radioactivity. The pellet of nuclei obtained as described above after nuclease treatment was resuspended in 0.5 ml 0.2 mM EDTA (pH 7.0), causing lysis of nuclei and release of chromatin. Duplicate 25-µl aliquots of the suspension of lysed nuclei were taken for radioactivity determinations, and the remaining 0.45 ml of the suspension was used for sedimentation analysis of chromatin, as described by others (10,16). The size of the pulse-labeled DNA was analyzed by alkaline sucrose gradient sedimentation, as reported earlier (26).

RESULTS AND DISCUSSION

Reports from several laboratories have indicated that the "native" structure of chromatin may be altered during isolation and shearing procedures (27,28). Hence, in order to minimize perturbation of the "native" chromatin structure, the studies described herein have been performed using isolated nuclei as substrate for micrococcal nuclease digestion.

To examine the incorporation of newly replicated DNA into chromatin subunits, nuclei from cells pulse-labeled with [^3H]thymidine for 0.5 min were digested with micrococcal nuclease for 2.5 min, and the chromatin was analyzed in isokinetic sucrose gradients (see Fig. 1). Several major features should be noted regarding the data in Fig. 1. First, the peaks sedimenting at fractions 9, 12, and 14 correspond, respectively, to the monomer, dimer, and trimer of the chromatin subunit organization characterized for several cell types in other laboratories (4,7,8,10,14) and for chromatin of CHO cells by Strniste et al. of this Laboratory (16). Second, the pulse-labeled chromatin is rapidly degraded to monomer and dimer nucleosomes, compared with the uniformly labeled chromatin. In fact, Fig. 1 shows that 46% of the ^3H label in the gradient is in the mononucleosome peak, compared with 6% of the ^{14}C uniform label. In Table 1, these percentages are recorded for comparison with the percentages of ^3H pulse-labeled DNA and ^{14}C uniformly labeled DNA degraded to acid-soluble products during the 2.5-min nuclease treatment. It should be noted that, for the 2-min pulse, both the percentage of pulse-labeled DNA rendered

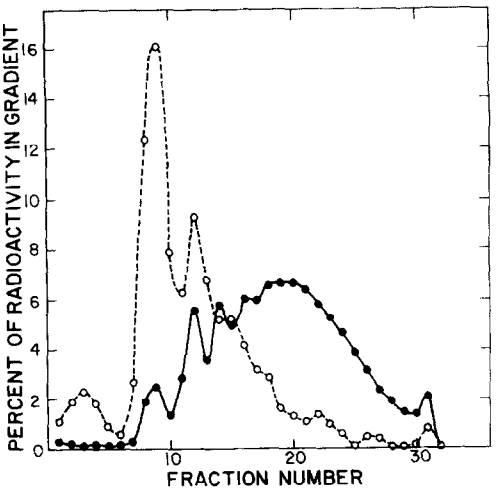

Fig. 1. Isokinetic neutral sucrose gradient analysis of chromatin following micrococcal nuclease digestion of the nuclei from pulse-labeled CHO cells. Cells were uniformly prelabeled with [^{14}C]thymidine (—●—) and pulse-labeled with [^{3}H]thymidine (--O--) for 0.5 min. Micrococcal nuclease digestion of the isolated nuclei was performed for 2.5 min. Chromatin was analyzed as described elsewhere (10,16) with a Spinco type SW27 rotor in a Model L3-50 (Spinco) preparative ultracentrifuge at 4°C. Gradients were centrifuged for 14.5 hr at 26,000 rpm.

TABLE 1. Data for Digestion of DNA to TCA-Soluble Products and for Degradation of Chromatin to Mononucleosomes during 2.5-min Nuclease Treatment[a]

Pulse Length (min)	% Labeled DNA TCA-Soluble		% DNA in Mononucleosome Peak[b] Relative to Total in Gradient	
	^{3}H	^{14}C	^{3}H	^{14}C
0.5	52	13	46	6.0
2.0	21	13	27	5.3

[a]All data shown in this table for each pulse time were obtained from the same preparation following termination of nuclease treatment.

[b]Data in this column are taken from Fig. 1 for the 0.5-min pulse and from similar sedimentation studies for the 2.0-min pulse.

acid-soluble and the percentage of ^{3}H label in the mononucleosome peak decreased, compared with the 0.5-min pulse, indicating a rapidly decreasing nuclease sensitivity of newly replicated chromatin with increasing pulse length. Finally, returning to Fig. 1, the small peak of ^{3}H radioactivity

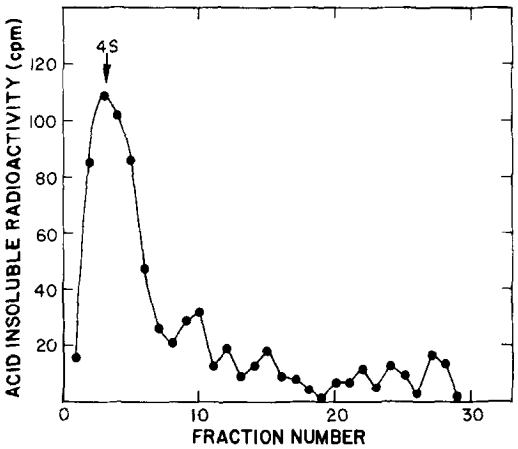

Fig. 2. Alkaline sucrose gradient analysis of DNA from CHO cells pulse-labeled for 0.5 min with [^{3}H]thymidine, as described above. Cell preparation, sedimentation analysis, and cold TCA-insoluble radioactivity determination were performed as previously described (26). Gradients were calibrated with λ-phage and T2 phage DNAs. Net cpm were corrected for background. The pellet fraction containing material sedimenting due to wall effects (26) (~25% of total) has been omitted from the figure.

appearing between fractions 1 and 6 may be nuclease-resistant submonomer assembly intermediates involved in the replication process or may be small DNA degradation products resulting from incomplete nuclease digestion, although the discreteness of the distribution argues against this possibility. Studies to examine these possibilities are being performed.

Examination of the size of the DNA synthesized during the 0.5-min pulse revealed that 52% of the DNA sedimented at ~4S, similar to the size of the eukaryotic "Okazaki fragment" (Fig. 2) [for a review, see Edenberg and Huberman (29)]. It is evident that very little pulse-labeled DNA has matured to higher molecular weight intermediates during the 0.5-min pulse. Hence, the results in Fig. 2, in combination with those of Fig. 1, suggest that newly replicated DNA is rapidly assembled into the subunit structure of chromatin at times preceding the joining of the newly synthesized DNA strands into high molecular weight DNA.

To estimate the time required for newly replicated DNA (chromatin) to display a nuclease digestion profile similar to that of nonreplicating chromatin, uniformly prelabeled cells were pulse-labeled for 1 min, harvested, washed, and chased in fresh medium for times up to 30 min. The results for nuclease digestion of nuclei after 0- and 10-min chase are shown in Fig. 3A and Fig. 3B, respectively. By inspection of these data, it is clear that 0-

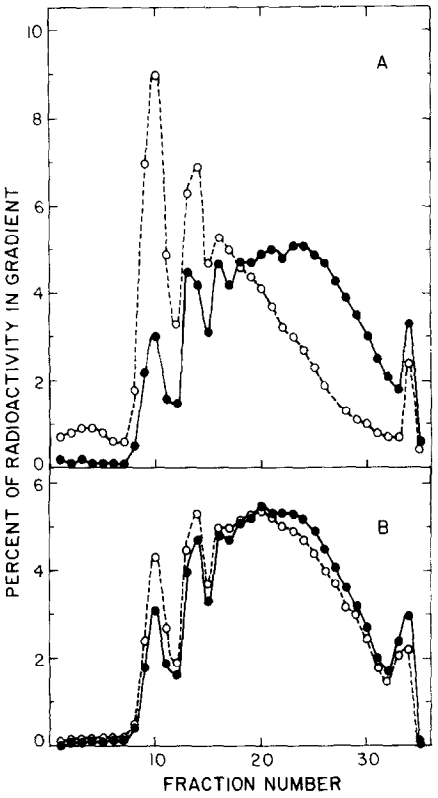

Fig. 3. Isokinetic neutral sucrose gradient analysis of chromatin following micrococcal nuclease digestion of the nuclei from cells pulse-labeled (A) and chased for 10 min (B). Nuclei from CHO cells prelabeled with [^{14}C]thymidine (—●—), pulse-labeled with [^{3}H]thymidine (--○--) for 1 min, and chased for 0 min (A) or 10 min (B) were digested with micrococcal nuclease for 2.5 min and analyzed as described in Fig. 1. Comparison of the percentage of ^{3}H and ^{14}C label (relative to total in the gradient) in the monomer chromatin subunit in (A) and (B) shows that, in the 0-min chase profile (A), these numbers are 27% for ^{3}H and 9% for ^{14}C radioactivity and that, in the 10-min chase, these numbers are 12% for ^{3}H and 9% for ^{14}C radioactivity. These percentages were obtained assuming a symmetrical distribution about the monomer subunit peak. Gradients were centrifuged as described in Fig. 1 for 15.25 hr.

min chase chromatin shows differential nuclease sensitivity of newly replicated chromatin, compared with that of nonreplicating chromatin. Further, the 10-min chase data indicate that most of the DNA synthesized in the 1-min pulse has matured to a configuration displaying nuclease sensitivity similar to non-replicating chromatin. This finding is in close agreement with conclusions reached by Weintraub (19) using chick erythroblasts and by Seale (20) and Seale and Simpson (21) using HeLa cells.

While the maturation of newly replicated chromatin to a configuration having the same nuclease sensitivity as nonreplicating chromatin requires

times of the order of minutes (Fig. 3), the incorporation of newly replicated DNA into nucleosomes requires only seconds (Fig. 1). Our results also indicate that incorporation of newly replicated DNA into nucleosomes occurs when newly synthesized DNA is predominantly the size of the lowest molecular weight replication intermediate (~ 4S by alkaline sucrose gradient analysis, see Fig. 2). Hence, the increased nuclease sensitivity of newly replicated chromatin may be attributed to (1) preferential nuclease attack at the single-stranded gaps between unjoined nascent 4S DNA molecules; (2) enhanced nuclease degradation at the unjoined 4S ends due to the presence of RNA primers (30), since micrococcal nuclease has a specificity for both RNA and DNA (31); (3) an absence of protection of replicating DNA by chromatin proteins; or (4) a combination of the above.

It is interesting to note that the size of the 4S eukaryotic "Okazaki fragment" is similar to the size of DNA in the chromatin subunit (17,29). The existence of small (4S) DNA replication intermediates has been accommodated recently by a model describing chromatin subunit replication (17). In addition to the features of that model, it is possible that the absence of rotational constraints on the newly replicated duplex DNA molecule at single-stranded gaps (at the ends of the 4S replication intermediates) might facilitate nucleosome assembly, since the folding or "kinking" of DNA suggested for its arrangement in the nucleosome (15,32) may require some twisting (or supercoiling) of the DNA duplex (33). This suggestion would help to explain the rapid assembly of small DNA replication intermediates into nucleosomes prior to joining of the newly synthesized DNA strands.

Preliminary experiments have indicated a correlation between the maturation of newly synthesized DNA strands into successively larger replication intermediates and the maturation of newly replicated chromatin into a structure similar to that of nonreplicating chromatin. Further investigations of this relationship are in progress. The possible involvement of histone modifications and nonhistone proteins in maturation of newly replicated chromatin must also be considered.

ACKNOWLEDGMENTS

The authors wish to thank L. T. Ferzoco and J. L. Hanners for skillful assistance. The authors are also indebted to their colleagues, Drs. J. A. D'Anna, S. C. Rall, A. G. Saponara, and G. F. Strniste, for valuable discussions of this work. These investigations were performed under the auspices of the U. S. Energy Research and Development Administration.

REFERENCES

1. Hewish, D. R. and Burgoyne, L. A. (1973) Biochem. Biophys. Res. Commun. 52, 504-510.

2. Olins, A. L. and Olins, D. E. (1974) Science 183, 330-332.
3. Kornberg, R. D. (1974) Science 184, 868-871.
4. Noll, M. (1974) Nature 251, 249-251.
5. Van Holde, K. E., Sahasrabuddhe, C. G., and Shaw, B. R. (1974) Nucleic Acids Res. 1, 1579-1586.
6. Oudet, P., Gross-Bellard, M., and Chambon, P. (1975) Cell 4, 281-300.
7. Lacy, E. and Axel, R. (1975) Proc. Natl. Acad. Sci. 72, 3978-3982.
8. Oosterhof, D. K., Hozier, J. C., and Rill, R. L. (1975) Proc. Natl. Acad. Sci. 72, 633-637.
9. Baldwin, J. P., Boseley, P. G., Bradbury, E. M., and Ibel, K. (1975) Nature 253, 245-249.
10. Finch, J. T., Noll, M., and Kornberg, R. D. (1975) Proc. Natl. Acad. Sci. 72, 3320-3322.
11. Lohr, D. and Van Holde, K. E. (1975) Science 188, 165-166.
12. McGhee, J. D. and Engel, J. D. (1975) Nature 254, 449-450.
13. Varshavsky, A. J. and Georgiev, G. P. (1975) Mol. Biol. Reports 2, 255-262.
14. Woodcock, C. L. F., Safer, J. P., and Stanchfield, J. E. (1976) Exp. Cell Res. 97, 101-110.
15. Finch, J. T. and Klug, A. (1976) Proc. Natl. Acad. Sci. 73, 1897-1901.
16. Strniste, G. F., Okinaka, R. T., and Rall, S. C. (1976), manuscript submitted.
17. Hewish, D. R. (1976) Nucleic Acids Res. 3, 69-77.
18. Elgin, S. C. R. and Weintraub, H. (1975) Ann. Rev. Biochem. 44, 725-774.
19. Weintraub, H. (1973) Cold Spring Harbor Symp. Quant. Biol. 38, 247-256.
20. Seale, R. L. (1975) Nature 255, 247-249.
21. Seale, R. L. and Simpson, R. T. (1975) J. Mol. Biol. 94, 479-501.
22. Jackson, V., Granner, D., and Chalkley, R. (1976) Proc. Natl. Acad. Sci. 73, 2266-2269.
23. Seale, R. L. (1976) Proc. Natl. Acad. Sci. 73, 2270-2274.
24. Walters, R. A., Gurley, L. R., Tobey, R. A., Enger, M. D., and Ratliff, R. L. (1974) Radiation Res. 60, 173-201.
25. Hildebrand, C. E. and Okinaka, R. T. (1976) Anal. Biochem. (in press).
26. Walters, R. A. and Hildebrand, C. E. (1975) Biochim. Biophys. Acta 407, 120-124.
27. Noll, M., Thomas, J. O., and Kornberg, R. D. (1975) Science 187, 1203-1206.
28. Nicolini, C., Baserga, R., and Kendall, F. (1976) Science 192, 796-798.
29. Edenberg, H. J. and Huberman, J. A. (1975) Ann. Rev. Genetics 9, 245-284.
30. Waqar, M. A. and Huberman, J. A. (1975) Cell 6, 551-557.
31. Alexander, M., Heppe, L. A., and Hurwitz, J. (1961), J. Biol. Chem. 236, 3014-3019.
32. Crick, F. H. C. and Klug, A. (1975) Nature 255, 530-533.
33. Germond, J. E., Hirt, B., Oudet, P., Gross-Bellard, M., and Chambon, P. (1975) Proc. Natl. Acad. Sci. 72, 1843-1847.