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Subnucleosome particles containing high mobility group proteins HMG-E and HMG-G originate from transcriptionally active chromatin

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ABSTRACT

Subnucleosome particles SN2 and SN3 containing short DNA fragments and non-histone proteins of the high mobility group, HMG-G and HMG-E respectively, were purified from the chromatin preparations of mouse L cells partially digested with staphylococcal nuclease. Labeled DNAs prepared from these particles were hybridized to an excess of nuclear RNA. The binding of subnucleosomal DNA was about 3-fold higher comparing to total cellular DNA fragmented to the same size. Special control experiments showed that DNA·protein complexes present in subnucleosomes SN2 and SN3 preexisted in nontreated nuclei. The conclusion has been drawn that non-histone proteins HMG-G and HMG-E are associated with the DNA of transcriptionally active chromatin and are released by nuclease as subnucleosomes.

INTRODUCTION

Besides mononucleosomes and oligonucleosomes, chromatin preparations solubilized by partial digestion with staphylococcal nuclease contain smaller nucleoprotein particles referred to as subnucleosomes /1-5/. Eight components were described originally but now, as the resolving power of gel electrophoretic analysis has been increased, it becomes clear that the number of different subnucleosomes is much higher /5/. Some of them (SN7 and 8) contain histones and may originate from nucleosomes /4/. However, most of the subnucleosome particles contain no octamer histones but certain non-histone proteins /5/, in particular those belonging to the high mobility group (HMG proteins) first described by Johns and coworkers /6,7/. We detected eight HMG proteins in mouse cells and designated them with Latin letters in the order of increasing mobility in acetic acid-urea gel electrophoresis. Two of them, HMG-G

and HMG-E, apparently corresponding to HMG-17 and HMG-14 from calf thymus /7/ were found to be the only protein components of two subnucleosomes, SN2 and SN3 respectively /5/. These particles are liberated early in the course of chromatin digestion. This may be due to the localization of the corresponding HMG proteins in the internucleosomal regions of transcriptionally active chromatin which was found to be more accessible to nuclease digestion /8-11/. To check this possibility, we purified SN2 and SN3 particles, isolated DNA from them, and tested this DNA for hybridization with nuclear RNA. The DNA prepared from SN2 or SN3 particles was bound much more as compared to total nuclear DNA. Thus, DNA fragments in SN2 and SN3 are enriched in transcriptionally active DNA, and HMG-G and HMG-E proteins seem to be predominantly located in transcriptionally active chromatin. The results of experiments indicating that these HMG proteins are not rearranged in the course of nuclease digestion are also presented supporting the above conclusion.

MATERIALS AND METHODS

Cultivation of cells and their labeling. L cells were grown to confluence in the Eagle medium containing 0.25% lactalbumin hydrolysate and 5% calf serum.

The cells were labeled with [Me-^{3}H] thymidine (15 Ci/mmole; Isotope, USSR) or carrier-free[^{32}P] orthophosphate (Amersham, England) to a final concentration of 20 and 40 μCi/ml, respectively, for 20-24 hrs. In the latter case, the cells were washed before labeling with the phosphate-free Eagle medium and labeled in the same medium.

Preparation of nuclei and chromatin. The cells were washed three times with a 0.14 M NaCl solution, then with a versene solution and scraped from the dished with a rubber policeman. Thereafter, the cells were washed again with 0.14 M NaCl and treated with a lysis buffer containing buffer A (0.04 M NaCl, 2 mM $MgCl_2$, 10 mM triethanolamine (TEA)·HCl, pH 7.6), 0.5% NP40, and 1 mM phenylmethylsulfonyl fluoride. Nuclei were collected by centrifugation, washed four times with buffer A to

remove NP40, and used either for preparation of chromatin or for immediate nuclease digestion. In the latter case, they were additionally washed with 0.15 M NaCl, 2 mM $MgCl_2$, 5 mM TEA·HCl, pH 7.6 and purified by centrifugation throuth 1.7 M sucrose containing buffer A and treated with staphylococcal nuclease in buffer A containing 1 mM $CaCl_2$. The nuclease digestion was terminated by cooling to 0^oC and the first supernatant (SI) was obtained by low speed centrifugation and 50 mM Na-EDTA was added to a final concentration of 2 mM. Then the pellet of digested nuclei was washed with buffer A and resuspended in 1 mM Na-EDTA, pH 7.6, to solubilize the digested chromatin. The second supernatant (SII) was collected by centrifugation after dialysis against 1 mM EDTA, pH 7.6, and the both supernatants were analysed electrophoretically.

Chromatin was prepared as described previously /1,4/. The procedure involved extensive extraction of nuclei with 0.15 M NaCl, 2 mM $MgCl_2$, 5 mM TEA·HCl, pH 7.6, and washing in 5 mM TEA·HCl, pH 7.6. Thereafter, chromatin was digested with different nucleases. In some cases, it was prefixed with 1% HCHO /12/. The latter was removed by dialysis against 2 mM TEA·HCl, pH 7.6.

DNase digestion of chromatin. Unfixed or prefixed chromatin (1.0–1.5 mg DNA per ml) was treated with either staphylococcal nuclease (5 µg/ml, 15020 u/mg, Worthington) or DNase I (2 µg/ml, 2414 u/mg, Worthington) or DNase II (10 µg/ml, 36460 u/mg, Worthington) in 0.1 mM $CaCl_2$, 1 mM TEA·HCl, pH 7.6, for different time intervals. The digestion was stopped by chilling the sample on ice and simultaneously adding 50 mM Na-EDTA, pH 7.6, to a final concentration of 1 mM. The extent of digestion was measured by assaying the acid-soluble DNA fraction with 5% TCA. In some cases, highly ^{32}P-labeled naked DNA (0.5 µg or $1.5x10^5$ cpm/ml) was added to an unlabeled chromatin preparation (1.5–2.0 mg DNA/ml) before digestion. The nuclease degradation of this naked labeled DNA was analysed by autoradiography of the electrophoretically separated DNA-containing material.

Polyacrylamide gel electrophoresis. We used low ionic strength electrophoresis in polyacrylamide gel to fractionate soluble DNP /3,4/. The fractionated material was visualized by staining DNA in an ethidium bromide solution or by autoradiography of ^{32}P-labeled DNA.

Preparation of subnucleosomal DNA. Preparative gel electrophoresis of DNP particles was used to obtain significant quantities of DNA fragments from subnucleosomes SN2 and SN3 /4/. ^{32}P- or ^{3}H-labeled DNA was extracted from polyacrylamide gel strips with a solution containing 0.1 M NaCl, 0.1% SDS, 10 mM tris-HCl, pH 7.6, 5 mM Na-EDTA (NETS), deproteinized by phenol treatment (twice), precipitated with ethanol and collected by centrifugation.

Preparation of pre-mRNA. Nuclear RNA was isolated and purified from L cell nuclei by the hot phenol method /13/. The purification process included treating the RNA with pancreatic DNase to digest contaminating DNA, sucrose gradient ultracentrifugation, and passing the RNA through a Sephadex G-150 column to remove degraded RNA and DNA. The purified RNA was collected by ethanol precipitation and dissolved in water. The concentration was ca. 20 mg/ml.

Hybridization of subnucleosomal DNA to excess of pre-mRNA. ^{3}H- or ^{32}P-labeled DNA was dissolved in water and denatured at 100°C. Each reaction mixture contained 10-15 ng of tested DNA (2000-5000 cpm) and different amounts of RNA in 0.30 M NaCl, 0.030 M Na citrate, pH 7.0, 0.01% SDS, 2 mM Na-EDTA. The final volume was 30 µl. The time of DNA-RNA hybridization did not exceed 6 hrs in order to prevent RNA degradation. Incubation was carried on at 65°C.

Assay of hybrids with S1 nuclease. Hybrid formation was assayed with S1 nuclease essentially by the method of Bellard et al. /10/. Hybridization samples were diluted 50-fold in the nuclease buffer (0.3 M NaCl, 0.03 M Na acetate, pH 4.6, 1 mM $ZnSO_4$), S1 nuclease was added, and the samples were incubated for 1 hr at 45°C. The nuclease-resistant material was determined after washing of the material with cold 5% TCA.

Assay of hybrids by gel filtration. Alternative method was used for determination of hybridized complexes. In this case, hybridization reaction mixtures were diluted in 0.1 ml of NETS buffer, incubated at 65°C for 3 min, and applied to a 25-ml column of Sephadex G-150 equilibrated with NETS buffer. RNA-DNA hybrids were eluted in the excluded volume being well separated from unhybridized small DNA fragments (see below).

Purification and analysis of HMG proteins. HMG proteins were extracted from chromatin by 0.35 M NaCl /7/, precipitated with 4 volumes of ethanol and separated in preparative polyacrylamide gel electrophoresis in urea-acetic acid medium /5/.

The bands of HMG proteins E and G were excised and proteins were eluted by homogenization of gel strips in 0.1% SDS, 50 mM TEA·HCl, pH 7.6, precipitated by ethanol and washed with acetone. The purity of proteins was checked by two-dimensional gel electrophoresis /5/. The proteins were hydrolyzed in 5.7 N HCl for 24 hrs at 115°C and amino acid composition was determined in "Technicon" amino acid analyser.

It was shown previously /5/ that HMG-G and HMG-E prepared in this way corresponded to the only protein components present in SN2 and SN3 subnucleosomes respectively.

RESULTS

1. Early release of subnucleosomes SN2 and SN3 upon digestion with different nucleases

We have found previously that subnucleosomes SN2 and SN3 appear at the early stage of chromatin digestion with staphylococcal nuclease /1,4/. This work is currently underway using different nucleases and examining more carefully the kinetics of subnucleosomal release.

One can see from Fig. 1 that SN2 and SN3 occur in chromatin digests in all cases where either staphylococcal nuclease or DNase I or DNase II were used. They appear at the early stage of digestion when 5-10% of DNA becomes acid soluble.

Two-dimensional electrophoretic analysis /5/ confirms

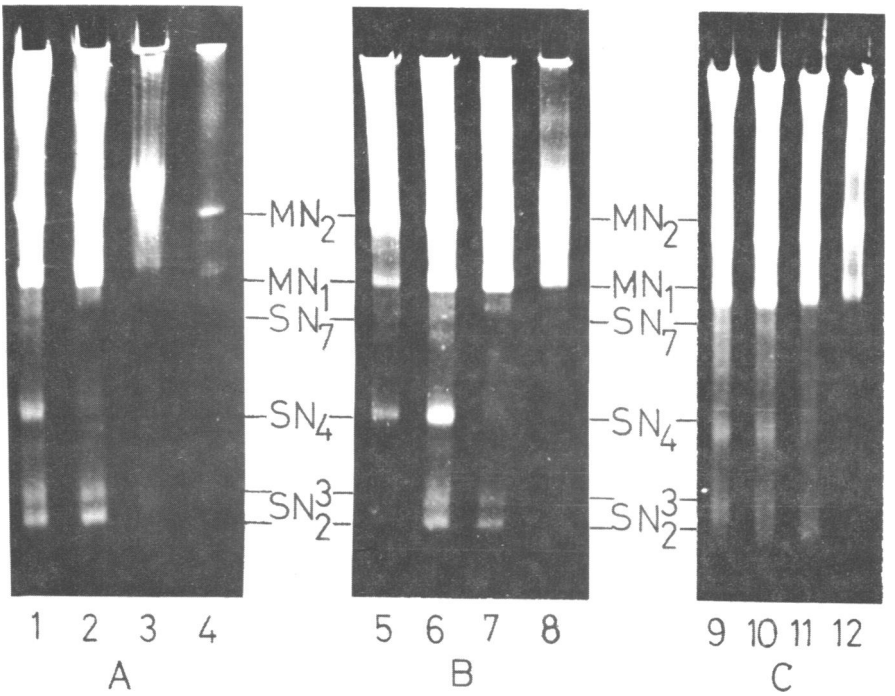

Fig. 1. Electrophoretic analysis of subnucleosomes produced by digestion with different nucleases.

Chromatin was treated with (A) DNase I, (B) Staphylococcal nuclease and (C) DNase II to 3-6% (slots 1,5,9), 9-19% (slots 2,3,6,7,10,11), and to 25-35% (slots 4,8,12) of acid soluble ^{3}H-DNA followed by direct loading of material soluble in the 1 mM Na-EDTA containing buffer onto a 6% polyacrylamide gel /3,4/.

the presence of HMG-G and -E in subnucleosomes SN2 and SN3 produced by DNase I (data not shown).

It was reported that HMG proteins appeared upon either staphylococcal nuclease or pancreatic DNase I treatment of the nuclei, in the supernatant even when the nuclei were not lyzed by Na-EDTA treatment /14,15/. One may suggest that in these conditions SN2 and SN3 were released from the nuclei. To check this possibility, the supernatant obtained after mild digestion of the nuclei by staphylococcal nuclease was collected and analysed by DNP electrophoresis (Fig. 2). Virtually

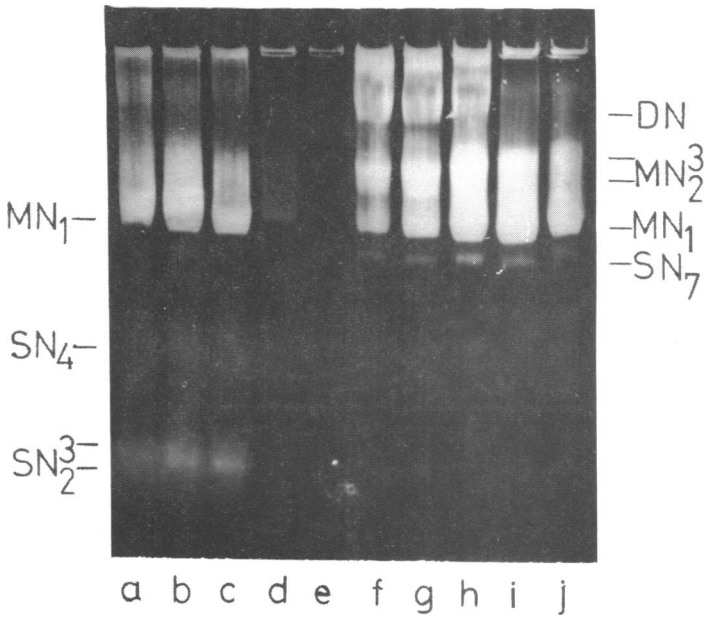

Fig. 2. Solubilization of subnucleosomes from non-lyzed nuclei by nuclease treatment.

Purified nuclei (~1.2 mg of DNA per ml) were digested with staphylococcal nuclease in 0.04 M NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM TEA-HCl, pH 7.6, for different periods of time. The supernatant fractions were collected (see Methods). The nuclear pellet was extracted with 1 mM Na EDTA, pH 7.6. The supernatants were analyzed electrophoretically (6% polyacrylamide gel).

(a-e) the first supernatants: 6%, 11%, 18%, and 35% of acid-soluble DNA, respectively;

(f-j) the second supernatants: 6%, 11%, 18%, and 35% of acid-soluble DNA, respectively.

no oligonucleosomes and mononucleosomes MN2 and MN3 containing histone H1 were found. They remained inside the cell nuclei. Some core nucleosome particles (MN1) lacking H1 were present. On the other hand, visible amounts of subnucleosomes, in particular SN2 and SN3, were revealed. Thus, early release of HMG proteins in the supernatant upon nuclear digestion may be explained by the cleavage of subnucleosome particles, at least SN2 and SN3, from chromatin and their extraction from the nuclei.

The nuclear pellet was lyzed in the Na-EDTA containing medium and analysed electrophoretically. It was found to contain usual amounts of mono- and oligonucleosomes and almost no subnucleosomes with an exception of SN7 (Fig. 2).

The highest yield of SN2 and SN3 was obtained upon the use of staphylococcal nuclease and therefore this procedure was chosen for isolating these particles and preparing DNA from them.

2. Hybridization of subnucleosomal DNA to pre-mRNA of L cells

The main question of the present work is what part of DNA in SN2 and SN3 particles is complementary to RNA transcribed in the cells from which these particles have been obtained.

This question may be answered by hybridization of DNA from subnucleosomes with nuclear RNA. As the DNA prepared from subnucleosomes is very short, ranging from 25 to 30 base pairs in length, special techniques for hybrid recovery should be elaborated.

DNA was labeled in vivo with [^{3}H]thymidine or with [^{32}P] orthophosphate. As a control, total labeled mouse DNA fragmented by staphylococcal nuclease treatment was fractionated by polyacrylamide gel electrophoresis and the fraction of 25-30 base pairs in length was used in the hybridization reaction.

After annealing, the hybrids were recovered either with the aid of a conventional technique based on S1 nuclease treatment or by a new technique involving gel filtration through Sephadex G-150. The latter technique (see Fig. 3) is convenient when very short nucleic acid fragments are hybridized to an excess of long ones. One can see that all RNA and DNA+RNA hybrids are recovered in the excluded volume while non-hybridized DNA fragments are eluted later. The background of the technique is rather low. In most cases, both techniques gave similar results. DNA fragments from subnucleosomes SN2 and SN3 were purified by phenol extraction of electrophoretically separated DNP particles. The densitometric analysis of ^{32}P-labeled digested chromatin showed that the amount of DNA complexed with HMG-G and -E to form subnucleosomes SN2 and SN3 did not exceed 1.5-2.0% of total incubated DNA (data not

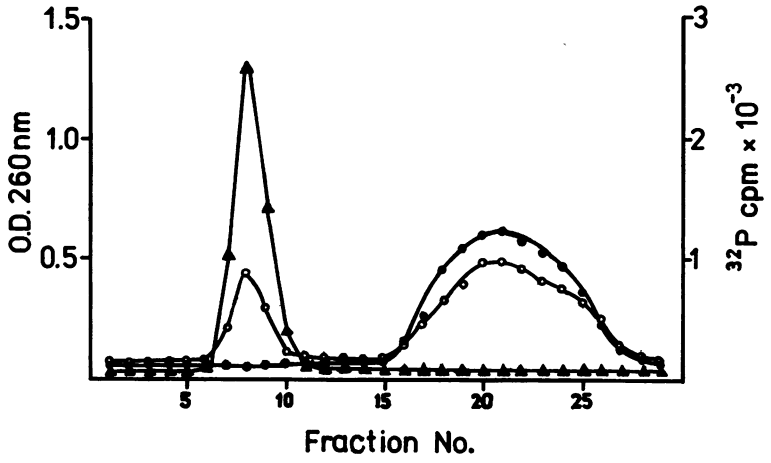

Fig. 3. Separation of subnucleosomal DNA hybridized with pre-mRNA from nonhybridized DNA by gel filtration.

The hybridization mixture was diluted to 0.1 ml with 0.1 M NaCl, 0.01 M tris-HCl, pH 7.5, 5 mM Na-EDTA, 0.1% sodium dodecylsulfate (NETS), incubated at 65°C for 3 min and loaded onto a Sephadex G-150 column equilibrated with a NETS solution. Elution was performed in the same solution.

(●),(○) - the radioactivity profiles of DNA purified from SN2 subnucleosomes before and after hybridization, respectively;
(▲) - optical density of pre-mRNA after hybridization.

presented). The purified DNA fragments were used in the hybridization reaction with pre-mRNA.

One can see (Fig. 4) that pre-mRNA binds up to 18-20% of the DNA present in SN2 or SN3 particles under the saturation conditions. At the same point, only 6-7% of total DNA is involved in the hybridization reaction. The kinetics of the reaction is such as could be expected from the known complexity of nuclear pre-mRNA. The saturation is reached at a C_0t value equal to $(1-2)\times10^3$ moles x l^{-1} x sec.

It is not excluded that the efficiency of the hybridization is lowered due to the small size of DNA fragments. To check this possibility, we repeated the same experiment but in the presence of total DNA taken in excess. Really, only 53% of

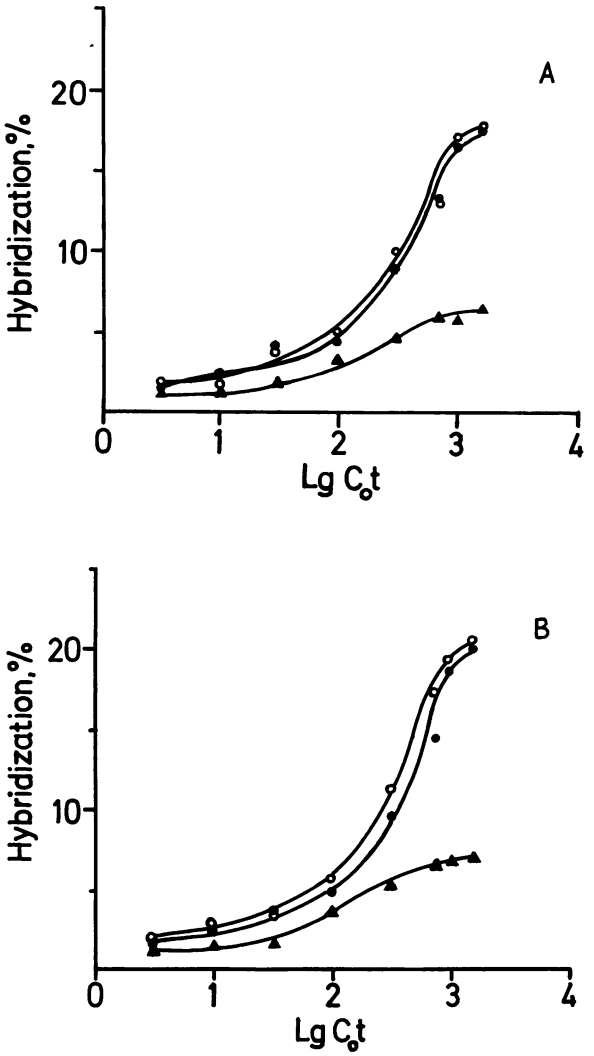

Fig. 4. Hybridization of purified SN2 and SN3 subnucleosomal DNA to pre-mRNA.

[^{3}H]- or [^{32}P] labeled subnucleosomal or total DNA was hybridized to an excess of pre-mRNA from L-cells as described in Methods and the hybrids were recovered using (A)S1 nuclease treatment (see Methods) or (B) a gel filtration technique (for details see legend to Fig. 3).

Hybridization of: (o) SN2 DNA; (•) SN3 DNA; (▲) total DNA.

either total DNA sheared to 20-30 nucleotide fragments or DNA from SN2 particles was bound to total cellular DNA where all sequences were represented. If the figure obtained is considered as a 100% limit for the reaction efficiency, the corrected hybridization of DNA to pre-mRNA would be 37% and 13% for SN2 and SN3 and for total DNA, respectively. However, it should be considered also that longer DNA fragments may replace already bound short pieces of DNA from hybrids during DNA-DNA hybridization and this may lead to a decrease in apparent hybridization. Anyhow one may conclude that the DNA from SN2 and SN3 is several times enriched in transcribed sequences as compared to total nuclear DNA.

3. Control experiments on the preexistence of DNA.protein complexes recovered in subnucleosomes

One may suggest that subnucleosomes SN2 and SN3 are formed in the course of chromatin digestion and fractionation by redistribution of HMG proteins or by their interaction with free DNA-pieces.

To check the possibility of such artifacts, two types of control experiments were performed.

First, in order to prevent protein redistribution, isolated chromatin was fixed with formaldehyde before digestion by staphylococcal nuclease. One can see (Fig. 5) that here subnucleosome particles are recovered in gel electrophoresis at exactly the same positions as subnucleosomes SN2 and SN3 obtained from unfixed samples. Their content is somewhat increased comparing to mononucleosomes. Thus, prefixation stabilizing the nucleoprotein structure of chromatin does not prevent the appearance of subnucleosomes studied upon nuclease digestion.

In the second control experiment, free DNA highly labeled with ^{32}P was added to a chromatin preparation before its digestion with staphylococcal nuclease. The gels were autoradiographed after electrophoresis. No labeled DNA bands were found in the regions where SN2 and SN3 were migrated (data not shown).

The sensitivity of the technique was enough to detect

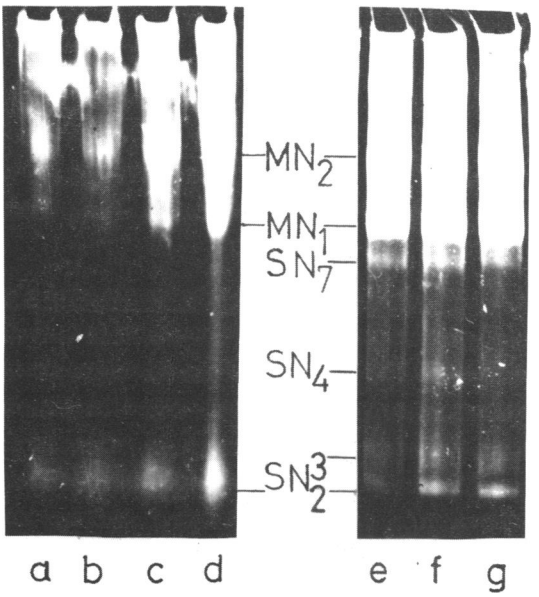

Fig. 5. Subnucleosomes from chromatin stabilized by formaldehyde prefixation.

^{3}H labeled chromatin (~ 200 µg/ml) was fixed with 1% HCHO for 70 hrs at 4°C, dialyzed against 5 mM TEA-HCl, collected by centrifugation and treated with staphylococcal nuclease (10 µg/ml) at a concentration of ca. 1.0 mg/ml of DNA.

(a) 3%, (b) 8%; (c) 10%, and (d) 15 % hydrolysis to acid soluble DNA fraction, respectively.

(e,f,g) control treatment of unfixed chromatin with nuclease to 4%, 12%, 17% acid soluble DNA.

Electrophoresis was performed in 6% gel.

0.1% of added DNA. The total amount of the latter was 4×10^{3} times less than that of DNA present in the chromatin preparation. Thus, if free DNA is involved in subnucleosome formation, the label should be detected in subnucleosomal bands. The negative result makes such possibility unlikely.

4. Comparison of HMG-E and G with calf thymus HMG proteins

It was important to determine to which of the known calf thymus HMG proteins those isolated from SN2 and SN3 did cor-

respond. According to the mobility in SDS and acetic urea gel (see refs. 5,16), they are similar to HMG-14 and HMG-17 of Goodwin et al. /7/. Now the amino acid composition of purified HMG-E and G was determined (Table 1). It was found to be close to that of HMG-14 and 17 /17/. Thus, these two HMG proteins we worked with probably correspond to HMG-14 and HMG-17. Two-dimensional electrophoretic analysis /5/ confirms possible relation between these proteins.

DISCUSSION

We have shown in this paper that subnucleosomes SN2 and SN3 originate from certain nucleoprotein complexes preexisting in nondigested chromatin. This conclusion is supported by the following results: (i) SN2 and SN3 appear after nuclease digestion of prefixed chromatin (see Fig. 5); (ii) free DNA added to chromatin is not involved in the formation of these subnucleosomes; (iii) sequences present in SN2 and SN3 are nonrandom and differ from those in total DNA. Therefore, one

Table 1 Comparison of the amino acid composition (moles %) of mouse HMG-E and HMG-G and calf thymus HMG-14 and 17 /17/

Residue	Mouse		Calf	
	HMG-E	HMG-G	HMG 14	HMG 17
Asp	5.3	9.6	8.1	12.0
Thr	5.2	2.9	4.2	1.2
Ser	8.2	5.4	7.8	2.3
Glu	16.0	10.8	17.1	10.5
Pro	9.1	11.2	8.5	12.9
Gly	11.8	10.6	6.5	11.2
Ala	9.9	14.2	14.5	18.4
Val	4.2	4.7	4.2	2.0
Cys	-	-	0.7	-
Met	-	-	-	-
Ile	Trace	Trace	0.5	-
Leu	2.8	2.1	2.0	1.0
Tyr	Trace	-	0.4	-
Phe	Trace	-	0.6	-
Lys	19.2	18.5	19.0	24.3
His	Trace	-	0.3	-
Arg	8.0	9.7	5.6	4.1

may conclude that HMG-E and HMG-G proteins are associated with the same DNA sequences either in original chromatin or in isolated SN2 and SN3 subnucleosomes.

It has been shown previously that subnucleosomes are released early upon digestion with staphylococcal nuclease /1, 4/. Now we observed the same phenomenon in experiments where DNase I and DNase II were used for chromatin digestion. Early digestion with DNase I is one of the most well documented features of transcriptionally active chromatin /8,9,18/.

It has been found by several authors that mild DNase I or staphylococcal nuclease treatment of nuclei selectively released a characteristic set of nonhistone proteins among which HMG proteins prevailed /14,15/. Similarly, we observed that subnucleosomes SN3 and SN2 were solubilized from nuclei upon brief staphylococcal nuclease treatment while no considerable solubilization of H1-containing mono- and oligonucleosomes took place. The absence of H1-containing nucleosomes in the first supernatant (SI) was shown by two-dimensional electrophoresis (not shown). Thus, some HMG proteins extracted from nuclei upon nuclease digestion of active chromatin are combined with short DNA fragments forming subnucleosome particles.

The hybridization experiments with nuclear pre-mRNA demonstrate the enrichment of SN2 and SN3 particles in DNA of transcribed chromatin. Although the presence of some non-transcribed sequences in these subnucleosomes cannot be excluded at least the bulk of SN2 and SN3 DNA is origined from the transcriptionally active chromatin. Thus, the conclusion can be drawn about the interaction of HMG-E and G (which possibly correspond to HMG-14 and 17 of Goodwin et al. /17/) with the DNA of active chromatin.

This conclusion is in a good agreement with the results obtained by other authors using different approaches.

Levy and Dixon /19/ have shown that nucleosomes appearing early in the digestion of trout sperm nuclei contain almost no H1 but H6 protein that corresponds to HMG-17. These nucleosomes were found to contain DNA efficiently hybridizing to mRNA.

In our previous experiments not all HMG-G was found in

SN2 /5/. About a half of it was present in monosomes moving slightly ahead MN-2 band and also with MN-3 band. These mononucleosomes are possibly equivalent to those described by Levy and Dixon /14/. However, in mice we were unable to purify them and perform hybridization experiments with their DNA. Nevertheless, the HMG-G containing regions of mononucleosomes seem to be related to SN2 particles /5/.

Weisbrod and Weintraub /20/ have demonstrated the loss of preferential DNase I digestion of active chromatin upon preliminary extraction with 0.35 M NaCl, the procedure known to remove HMG proteins. The addition of HMG-14 and 17 back to chromatin reconstituted this property. We found previously that 0.35 M NaCl extraction of chromatin prevented further appearance of SN2 and SN3 in nuclease digests /5/. All these data support the idea of the two HMG proteins (14 and 17 or E and G) interacting with transcriptionally active chromatin.

Our previous data gave evidence in favour of HMG binding to the linker regions of chromatin /5/. Taking into account the abundance of SN2 and SN3 DNA fragments (1-2% of total DNA) and their length ($\sim$30 base pairs) /4/, one may calculate that nucleosomes containing HMG-E and G in the linker region comprise from 6 to 12% of all nucleosomes. This figure is of the same order as the expected amount of active chromatin /21/.

It is not clear now whether HMG-E and G replace H1, or both HMG proteins and histone H1 are present in the linkers of active chromatin. The first suggestion seems to be more likely but further experiments are necessary. The possibility of isolating specific complexes of HMG proteins with short DNA fragments makes it possible to answer several questions about the organization of transcriptionally active chromatin.

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