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The structure of nucleosomal core particles within transcribed and repressed gene regions

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ABSTRACT

The arrangement of histones along DNA in nucleosomal core particles within transcribed heat shock gene (hsp 70) region and repressed insertion within ribosomal genes of Drosophila was analysed by using protein-DNA crosslinking methods combined with hybridization tests. In addition, two-dimensional gel electrophoresis was employed to compare the overall nucleosomal shape and the nucleosomal DNA size. The arrangement of histones along DNA and general compactness of nucleosomes were shown to be rather similar in transcriptionally active and inactive genomic regions. On the other hand, nucleosomes within transcriptionally active chromatin are characterized by a larger size of nucleosomal DNA produced by micrococcal nuclease digestion and some peculiarity in electrophoretic mobility.

INTRODUCTION

It was established that the bulk of the eukaryotic genome is organized in nucleosomes (1,2,3). At the second level of chromatin organization, the nucleosomal chain is folded into 30-nm fibers (4). Nucleosomal core particle consists of about 145 base pairs (bp) of DNA and a histone octamer containing two molecules each of histones H2A, H2B, H3 and H4.

The data on the transcribed chromatin structure are very controversial (reviewed in 5,6). It is generally accepted that increasing transcriptional activity causes progressive unfolding of chromatin structure (7). Thus, the removal of histone H1 and then the core histones from moderately to actively transcribed ribosomal (8) and heat-shock Drosophila genes (9), which histones can be displaced by RNA polymerase (10, but see Ref. 58) seems to induce unfolding of the 30 nm fiber to the 10 nm fiber and then to linearized DNA. At the same time,

nucleosomes are preserved on transcribed genes at a moderate transcriptional activity (9,11,12). Moderately transcribed chromatin possesses also some peculiarities in structure (such as an enhanced nuclease sensitivity and unusual distribution of active gene sequences within different chromatin fractions). It was assumed that these characteristics of active chromatin might be also determined by nucleosomal core structure modifications (12,14).

A question how and to what extent the nucleosome core structure is perturturbated in active chromatin regions has yet to be solved. The cores isolated from transcribed chromatin may have some peculiarities in structure since they are preferentially digested by DNAse I (15,16), show an increased affinity for HMG 14 and 17 proteins (17,18) and RNA polymerase II (19) and can be enriched in ubiquitin conjugated to histone H2A (20, but see 21). Different authors reported that the structure of nucleosomal cores is invariable (11,12) or slightly changed (12-14,22) in active chromatin. "Active" nucleosome cores have exposed H3 cystein residues (23-26), and in one case they were shown to look in electron micrographs like partly unfolded bipartite particles (23).

In this paper, to compare the primary organization of nucleosomes (the arrangement of histones along DNA) within specific active and inactive chromatin regions we used the method of locating protein contacts on DNA (27) and the "protein image" hybridization technique (9). Our results show that the primary organization of core particles is rather similar in both active in transcription and repressed chromatin regions.

MATERIALS AND METHODS

Isolation of nuclei and fractionation of micrococcal nuclease-treated nuclei

6-18 hr-old embryos of Drosophila melanogaster, Oregon R, were collected according to the method (29). Embryos were quickly frozen in liquid nitrogen and dechorionated by the procedure (30) immediately before nuclei isolation or were dechorionated before freezing which partly activated the transcription of hsp 70 genes (36). Isolation of nuclei was performed

as described in (31), in the presence of 0.5 mM diisopropyl fluorophosphate. Fractionation was conducted as described in (32).

Isolation of nucleosomal cores from crosslinked Drosophila nuclei

DNA-histone crosslinking was conducted within nuclei as described in (9). Crosslinked nuclei were then lysed in 0.5 mM t-DCTA (trans-1, 2-diaminocyclohexane-N,N,N',N'-tetraacetic acid monohydrate). The chromatin was washed twice with 0.15 M NaCl and lysed in water at a concentration of 40 A_{260} units/ml. After such a preparation of chromatin, the yield was quantitative. H1 protein was removed from crosslinked chromatin on Dowex AG-50x2 in a buffer containing 0.5 M NaCl, as described in (27). Chromatin (10 A_{260} units) without H1 was then incubated with micrococcal nuclease (1-1.5 mkg/10 A_{260} units) at a concentration of 20 A_{260} units/ml in a buffer supplemented with 0.4 mM $CaCl_2$ for different periods of time at 37°C. Digestion was terminated by adding EDTA to 2 mM and hydrolysate was then fractionated by centrifugation for 5 min at 4000 g. The DNA from the pellet and the supernatant was extracted and subjected to polyacrylamid gel electrophoresis under DNA denaturing conditions.

The hydrolisate was then concentrated by ultrafiltration through PM 30 membrane ("Amicon") and 1 ml of hydrolisate was layered onto linear sucrose density gradient (10-30%) at a concentration of 50-90 A_{260} units/ml in 10 mM Tris-Cl (pH 7.4), 0.2 mM EDTA. Gradient was centrifuged for 24 hr at 15000 g and mononucleosome fraction was then isolated and dialyzed versus 10 mM Tris-Cl (pH 7.4), 0.5 mM EDTA.

Analysis of crosslinked DNA-histone complexes

Uncrosslinked DNA was removed from crosslinked cores by phenolic extraction as described in (8). Two-dimensional electrophoresis was performed as described earlier (27).

DNA electrophoresis under denaturing conditions was performed as described in (33), DNP and DNP-DNA two-dimensional electrophoresis were conducted as described in (34).

DNA transfer from the gel to a nylon membrane and sequential hybridization were performed as described in (35). The

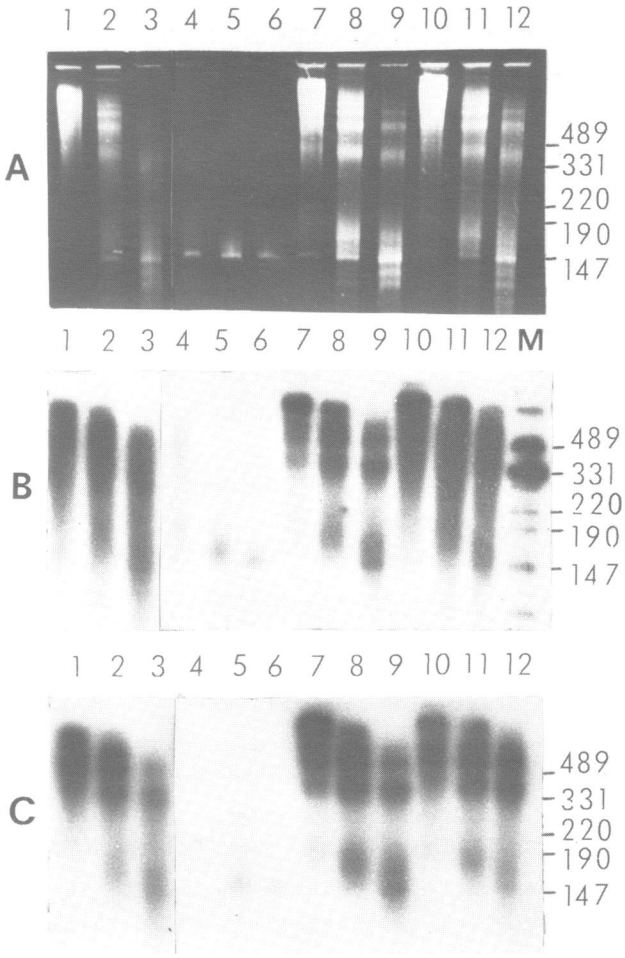

Figure 1. Fractionation of micrococcal nuclease-treated nuclei
Nuclei from dechorionated Drosophila melanogaster embryos were incubated with micrococcal nuclease at 37°C over increasing periods of time (10,20 and 40 min, correspondingly, from left to right for each set). DNA was then directly extracted from the nuclei (lanes 1-3) or chromatin and fractionated by Garrard's procedure (32) into S1 (4-6), S2 (7-9) and P (10-12) fractions. DNA was then extracted and subjected to 7% polyacrylamide gel electrophoresis under DNA denaturing conditions. An ethidium bromide fluorescence pattern is shown in A. The DNA was transferred to a nylon membrane and hybridized with ^{32}P-labeled hsp 70 gene coding region probe (B). Then the membrane was rehybridized with nontranscribed ribosomal type II insertion probe (C). Numbers at the right denote the length of marker DNA fragments (lane M).

hybridization probes were characterized in (9). These were a BamHi-Sal fragment (0.9 kb) from the hsp 70 gene coding region and EcoRI-EcoRI fragment (0.8 kb) from nontranscribed ribosomal type II insertion of Drosophila which has been cloned into M 13 phage.

RESULTS

Hsp 70 gene chromatin structure as revealed by fractionation of digested nuclei

As a model of moderately transcribed gene we investigated a partly activated hsp 70 gene (five copies in genome) in dechorionated Drosophila embryos. There was a significant increase in the rate of hsp 70 genes transcription during dechorionation of embryos although a fully activated gene transcribed still 5-6 times more intensively (9,36).

More detailed information on the chromatin structure of hsp 70 gene was obtained by chromatin fractionation into fractions S1, S2 and P (see Materials and Methods) after micrococcal nuclease digestion of nuclei and gel elestrophoretic analysis of the fractions (Fig. 1). Another aim of this study was to compare fractionation characteristics of hsp 70 gene chromatin with those of other well-known moderately transcribed genes (14,22,37). The fractionation procedure liberated about 2-4% of the total material in the S1-fraction and 80-85% in the S2-fraction. The P-fraction contained 10-15% of the total nuclear 260-nm-absorbing material (Figure 1A). The nontranscribed ribosomal type II insertion sequences were represented at the same level as the bulk of DNA in all fractions (Figure 1C).

The P-fraction was enriched in hsp 70 gene sequences (5-6 fold over total) containing about 60-80% of the total hsp 70 sequences and the S1-fraction was also enriched nearly 2-3 fold (Figure 1B). The enrichment was quantitatively measured by dot hybridization (not shown). Micrococcal nuclease-generated periodic electrophoretic pattern was greatly smeared in the total hydrolyzate and the P-fraction of hsp 70 genes (Figure 1B). Those features of hsp 70 gene chromatin structure closely

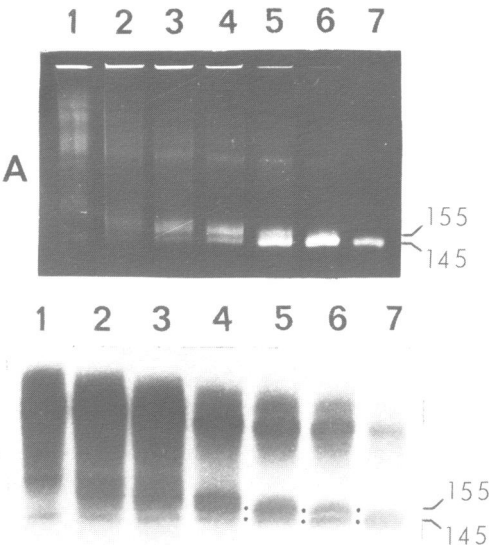

Figure 2. Detailed time-course of H1-depleted chromatin digestion by micrococcal nuclease

Chromatin without H1 from dechorionated Drosophila embryos was incubated with micrococcal nuclease over increasing periods of time (0, 2.5, 5, 10, 20,40 and 80 min, correspondingly, from left to right). DNA was then extracted and subjected to 7% polyacrylamide gel electrophoresis under DNA denaturing conditions. An ethidium bromide fluorescence pattern is shown in A. The DNA was transferred to a nylon membrane and probed with hsp 70 gene coding region (B). Numbers at the right denote the length of DNA fragments.

resemble those of previously investigated moderately transcribed genes (14,22,37).

Interestingly, the rate of digestion of the hsp 70 gene by micrococcal nuclease was very similar to that for inactive chromatin although after a full transcriptional activation the hsp 70 gene was hydrolysed by micrococcal nuclease much more rapidly (38,39). So, the hsp 70 gene is very similar to other moderately transcribed genes as regards the chromatin structure.

Some peculiarities in nucleosome structure of the moderately transcribed hsp 70 gene

Our early attempts to investigate a fine nucleosome structure in active chromatin were unsuccessful because DNA in hsp

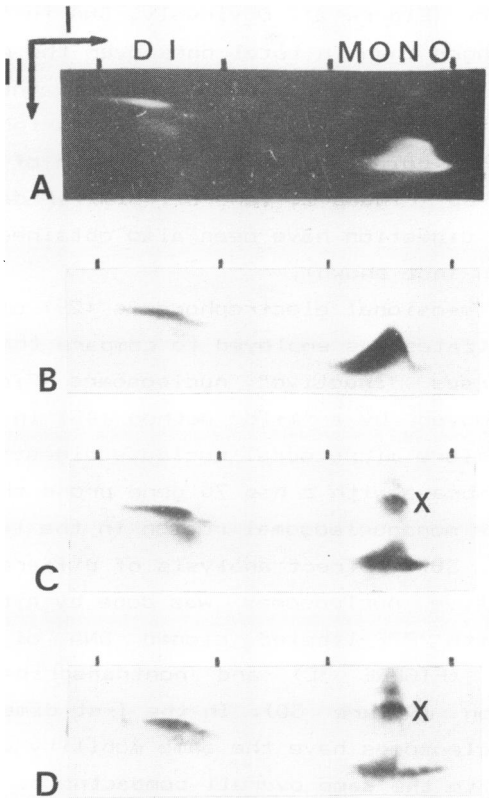

Figure 3. Two-dimensional DNP-DNA electrophoretic analysis of nucleosome core particles

Chromatin from dechorionated Drosophila embryos was mildly depleted of histone H1 in the presence of 150 mM NaCl (43) and digested with micrococcal nuclease for 40 min. The mixture was then resolved on a I-st dimension DNP electrophoresis gel. DI-dinucleosomes, MONO-mononucleosomes. After the removal of proteins, DNA was resolved in the II-nd dimension under DNA denaturing conditions. An ethidium bromide fluorescence pattern is shown in A. The DNA was then transferred to a nylon membrane and hybridized with ^{32}P-labeled probe for hsp 70 gene coding region (B). Then the membrane was rehybridized with overall ribosomal repeat probe (C) and nontranscribed ribosomal insertion probe (D). "X" marks the spots of unknown origin, which can be seen only with ribosomal probe.

70 gene nucleosomes was somewhat longer than in total nucleosomes. We investigated this fact with the aid of a more accurate electrophoretic analysis of time-courses of micrococcal

nuclease digestion (Figure 2). Obviously, DNA in "active" nucleosomes is longer than in total ones over the whole time of digestion. More specifically, nucleosomes of the hsp 70 gene have equal quantities of 145 bp- and 155 bp-long DNA at the standard digestion point where the DNA length of total nucleosomes was 145 bp (Figure 2, lane 6). Similar data on micrococcal nuclease digestion have been also obtained for H1-containing chromatin (not shown).

DNP-DNA two-dimensional electrophoresis (29) of H1-depleted chromatin hydrolizates was employed to compare the conformation of "active" versus "inactive" nucleosomes (Figure 3). Histone H1 was removed by a "mild" method (43) in the presence of 150 mM NaCl before micrococcal nuclease digestion. Hybridization of the membrane with a hsp 70 gene probe shows a specific "hump" at the mononucleosomal region in the two-dimensional picture (Figure 3B). Direct analysis of differences between total and inactive nucleosomes was done by hybridizing the same membrane with ^{32}P-labeled cloned DNA of the overall RIBOSOMAL REPEAT (FIGURE 3C) and nontranscribed ribosomal type II insertion (Figure 3D). In the I-st dimensional gel, hsp 70 gene nucleosomes have the same mobility as total ones which testifies to the same overall compactness. On the other hand, hsp 70 gene nucleosomes contain significantly longer DNA in the "hump"-region although their mobility is preserved. So there must exist certain conformational modifications in "active" nucleosomes.

The most striking observation was that mononucleosomal regions in "active" and "inactive" nucleosomes in the gel are not overlapping significantly. One can conclude therefore that there are no "inactive" nucleosomes in the hsp 70 gene chromatin.These peculiarities of "active" nucleosomes were preserved after standard 0.5 M NaCl-removal of histone H1 and after nucleosome crosslinking in nuclei (not shown).

Experimental approach for investigating the primary organization of nucleosomes within different chromatin regions

To study the nucleosome structure as it exists in nuclei, the method of locating the protein contacts on DNA (27) was combined with the "protein image" hybridization technique

(9). This method consists in crosslinking the protein to DNA partially depurinated under mild conditions (40). The crosslinking causes DNA to split in such a manner that only the 5'-terminal DNA fragment becomes attached to protein molecules. Thus, the length of a crosslinked DNA fragment shows precisely the distance of the protein crosslinking site from the DNA 5' end. This length can be assessed by two-dimensional diagonal gel electrophoresis. The method was used to study the primary organization of different nucleosomes (27,41,48) and the crosslinking data have been recently supported by crystallographic data (3,42).

DNA transfer from a two-dimensional gel to a nylon membrane and sequential hybridization with various cloned ^{32}P-labeled DNA probes enables one to determine the length of nucleosomal DNA fragments crosslinked to each histone fraction from different chromatin regions. Thus, our method of investigation of nucleosome structure in specific chromatin regions consists of four following steps:

1. Crosslinking histones to DNA in nuclei.
2. Removing histone H1 from crosslinked chromatin and isolation of nucleosomes. The removal of histone H1 significantly increases the yield and length homogeneity of nucleosomal DNA.
3. Conducting two-dimensional electrophoresis of crosslinked complexes which makes it possible to determine the length of crosslinked DNA on the diagonals of different histones.
4. Blotting of DNA fragments from the gel to a nylon membrane and sequential hybridization with cloned probes from different chromatin regions.

Let us consider some possible artefacts of this approach. There is no redistribution and proteolysis of proteins during DNA-protein crosslinking in nuclei (9). Nucleosomes crosslinked in nuclei were very similar to uncrosslinked ones as regards their DNP electrophoretic mobility and DNA length (data not shown). There are no additional breaks in the crosslinked core DNA. Therefore DNA-protein crosslinking in nuclei and subsequent nucleosome isolation do not induce any rearrangements in core nucleosome structure.

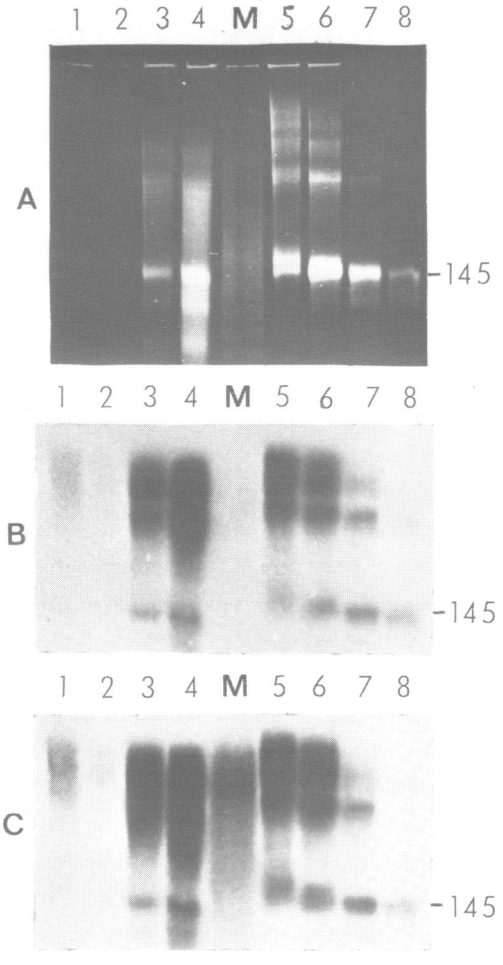

Figure 4. Fractionation of micrococcal nuclease-treated chromatin depleted of histone H1

Chromatin without H1 from dechorionated Drosophila embryos was incubated with micrococcal nuclease at 37°C over increasing periods of time (10,20,40 and 80 min, correspondingly, from left to right for each set). Chromatin was then fractionated into supernatant (lanes 1-4) and pellet (5-8) fractions. DNA was extracted and subjected to 7% polyacrylamid gel electrophoresis under DNA denaturing conditions. An ethidium bromide fluorescence pattern is shown in A. The DNA was transferred to a nylon membrane and hybridized to the same probes as in Figure 1. Numbers at the right denote the positions of nucleosomal core DNA, calculated from the length of DNA fragments obtained from DNase 1 digests of Drosophila embryos nuclei (M) (57).

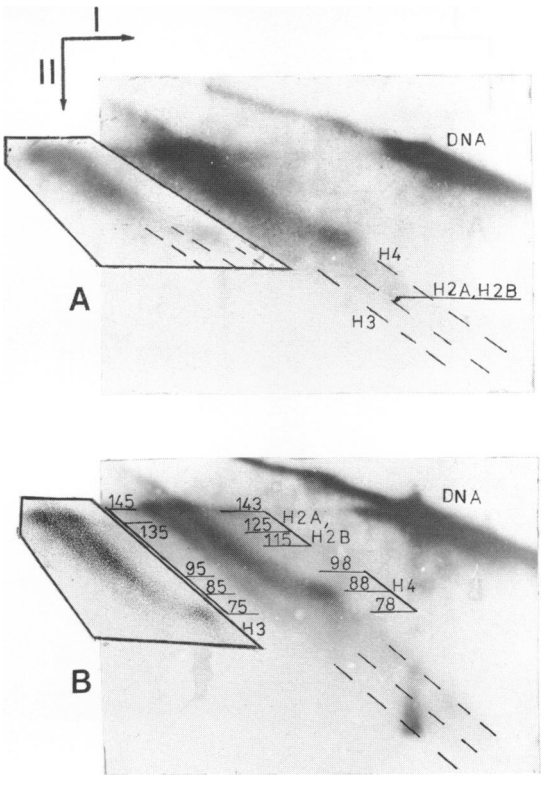

Figure 5. Two-dimensional electrophoretic analysis of histone-DNA complexes crosslinked in isolated nucleosomes

Nucleosome core particles isolated from dechorionated Drosophila embryos were crosslinked by dimethylsulphoxide. Crosslinked DNA-histone complexes were isolated and electrophoresed on a I-st dimension gel. After the digestion of proteins with Pronase, DNA was resolved on a II-nd dimension gel under DNA denaturing conditions and transferred to a nylon membrane. The membrane was analysed by hybridization with the hsp 70 gene coding region probe (A) and then with nontranscribed ribosomal type II insertion (B). The thin lines show positions in the gel and figures give the length of DNA fragments crosslinked to different histones and arranged on separate diagonals. At the left are the autoradiograms of the top part after a shorter exposure.

The yield of chromatin before micrococcal nuclease digestion was nearly quntitative (see METHODS). However based on the chromatin fractionation data obtained by us (Figure 1) and other authors (13,14,22,37), we had expected that micrococcal

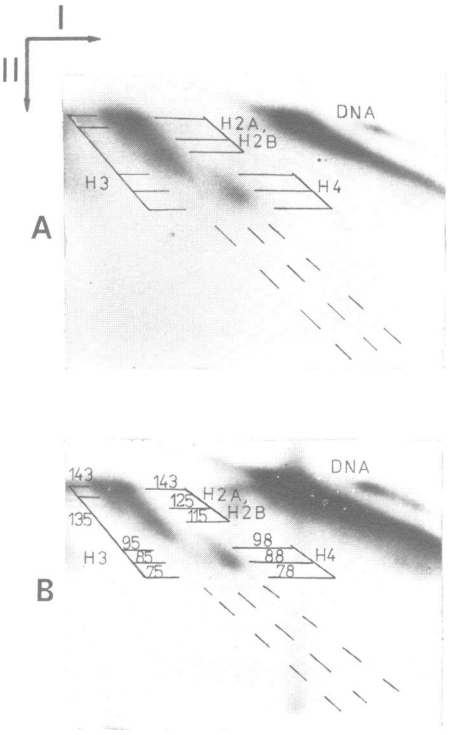

Figure 6. Two-dimensional electrophoretic analysis of histone-DNA complexes in nucleosomes crosslinked in nuclei

Nucleosome core particles were isolated from dechorionated Drosophila nuclei crosslinked by dimethylsulphoxide. Cross-linked DNA-histone complexes were then isolated and analysed by two-dimensional electrophoresis. The designations are the same as in Figure 5.

nuclease digestion should lead to some enrichment of the pellet in active gene nucleosomes so that subsequently we would be able to analyse the soluble chromatin fraction depleted of "active" nucleosomes. We have analysed this possibility by comparing the electrophoretic patterns of the active hsp 70 gene in the supernatant and pellet fractions of the H1-depleted chromatin hydrolizate (Figure 4).

From Figure 4 it is apparent that total chromatin and chromatin from active and inactive genomic regions are similarly distributed between the supernatant and pellet DNA fractions, even though the relative intensity of longer DNA fragments

can increase during hybridization (compare 4A with 4B, 4C). The pellet fractions were loaded on electrophoresis three times as much as supernatant fractions. So during digestion of H1-depleted chromatin by micrococcal nuclease the pellet was not enriched in active hsp 70 genes. The H1-containing chromatin was similarly distributed between different chromatin fractions after micrococcal nuclease hydrolysis (not shown). Obviously, the chromatin characteristics were greatly perturbed during lysis in nuclei at a low ionic strength, because no smearing of the electrophoretic pattern of the P-fraction of hsp 70 genes (Figure 1) was observed after micrococcal nuclease hydrolysis of lysed nuclei (not shown).

Arrangement of histones on DNA in "active" nucleosomes

A good resolution of two-dimensional gels can be attained only with nucleosomal DNA of homogeneous length. So in the subsequent experiments micrococcal nuclease digestion was conducted during longer periods of time. Two-dimensional electrophoretic analyses of crosslinked DNA-histone complexes are shown in Figures 5 and 6 after subsequent hybridization with probes from active and inactive chromatin regions.

After hybridization with nontranscribed ribosomal insertion, the picture was very similar to the structure of total nucleosomes obtained by direct end-labeling analysis (27,41). There are three diagonals of Drosophila histones (H3,H4 and H2A-H2B) because the mobility of histones H2A and H2B were very similar and the DNA fragments crosslinked to them could not be separated completely.

The locations of crosslinking sites for four core histones along core DNA of nontranscribed ribosomal insertion can be read directly from the positions of radioactive spots on these diagonals. The spots on the crosslinked histone H3 diagonal were smeared from top to bottom. Perhaps, this smearing was due to some slight modifications in the original method of nucleosome isolation which we incorporated in order to increase the core yield. However the overall two-dimensional pattern is very similar to the pictures obtained by the DNA end-labeling method (see for example Figure 5 in Ref. 27). One can see that the overall primary organization of nucleosomes in

the range of DNA length from 60 to 150 bp is very similar for nucleosomes crosslinked in nuclei (Figure 6) and after their isolation (Figure 5), although some fine details of the primary organization may be absent on these maps.

Hybridization of the same membrane with an hsp 70 gene probe (Figures 5A and 6A) gives a picture which is similar to the primary organization of "inactive" nucleosomes. Therefore the arrangement of histones along nucleosomal DNA is not changed in active chromatin as compared with total or inactive chromatin. Subsequently the same membranes were tested with other cloned gene probes such as overall ribosomal repeat and one-copy housekeeping gene of tubulin. We did not find any differences in the primary organization of cores on these genes (not shown).

DISCUSSION

The assumption that there are modifications in the nucleosome conformation of active chromatin was supported by nuclease digestion studies (44,45) that showed that DNase I degraded active chromatin at a higher rate. On the other hand, the sensitivity of active chromatin to micrococcal nuclease was unchanged. Therefore the authors suggested that only DNase 1 can "recognize" structural modifications in "active" nucleosomes.The preferential DNase 1 sensitivity was assigned to HMG 14,17 (17) and histone acetylation (46).

However HMG 14,17 binding to nucleosomes changes neither the overall nucleosome conformation (47) nor the primary organization of nucleosomes (48). Histone acetylation also changes the nucleosome only slightly (49). At present it is assumed that the preferential sensitivity to DNAse 1 is caused by some relaxation in supranucleosomal structure of active chromatin (50-52) presumably in consequence of histone H1 depletion in active chromatin (9,51).

On the other hand, isolated nucleosomes have been proven to have a great affinity for HMG 14,17 proteins from active chromatin (17,18,53). Our results (Figures 2,3) suggest that nucleosomes from active chromatin have longer DNA as compared with total nucleosomes after hydrolysis by micrococcal nucle-

ase both H1-containing and H1-depleted chromatin. Therefore the preferential binding of HMG 14,17 proteins to "active" nucleosomes can occur during micrococcal nuclease digestion simply due to a greater affinity of HMG 14,17 for nucleosomes with longer DNA length (54).

The longer DNA length in "active" nucleosomes can be caused by a preferential protection of the ends of nucleosomal DNA by histones H2A and H3 (27). The existence of nucleosomal conformers with different arrangement of H2A histone was shown by DNA-protein crosslinking (55) and X-ray diffraction (3,42). Obviously, nucleosomes in active chromatin may have some subtle conformational modifications which are not discerned by DNA-protein crosslinking methods. These peculiarities can be responsible for the additional protection of the nucleosomal DNA ends (Figure 2) and specific electrophoretical characteristics (Figure 3) of "active" nucleosomes. Perhaps, these structural changes are also the cause of a greater accessibility of histone H3 SH-groups to modifications in isolated "active" nucleosomes (24-26).

The above data showing that both the electrophoretic mobility and primary organization are similar in active as well as in inactive nucleosomes witnesses against the alleged existence in active genes of nucleosomes with a highly distorted structure, such as the unfolded nucleosomes found in ribosomal genes of P. polycephalum (23). A model for such unfolded nucleosomes should unavoidably incorporate considerable changes in histone contacts with DNA, in particular a simultaneous contact of histone H3 with the central and terminal regions of nucleosomal DNA would become impossible. Yet we observed no such changes in the primary organization of active nucleosomes. Preservation of electrophoretical and some other characteristics in active nucleosomes have been also demonstrated in other papers (11,12,20,21). However several authors have reported recently significant smearing in the nucleosomal repeat of some active genes (13, 22,32,37), including partly activated hsp 70 gene (Figure 1). These observations could have been interpreted on the basis of considerable distortions in the nucleosomal structure. Our data on the similar-

ity of general conformation and primary organization of nucleosomes from active and inactive genome regions have led us, however, to a different suggestion, namely that the peculiarities in chromatin structure of active genes could be better accounted for by some changes in the supranucleosomal organization that can disturbe the regular arrangement of nucleosomes during transcription.

Changes in the nucleosomal repeat could be also brought about by an association of the transcribed genes with nuclear matrix, since it is in the P-fraction of the nuclear matrix that the changes in nucleosomal repeat are most pronounced (Figure 1). The latter suggestion is supported by the observation that the periodical pattern of micrococcal nuclease digestion of the hsp 70 gene is restored after the nuclei have been lysed at a low ionic strength (Figure 4) and thereby the association with the matrix has been broken (54).

These and other characteristics of active chromatin, for example its sensitivity towards nucleases seem to arise from the depletion of histone H1 (9,51). It should be stressed here that the primary organization of core nucleosomes is not dependent on the presence of histone H1 (27,55) although cores and H1-containing nucleosomes demonstrate significantly different properties.

Our approach involves some serious experimental difficulties. First, some hsp 70 gene copies may be present in an inactive chromatin conformation and thus the characteristic features of active chromatin might be "masked". However, histone H1 is actually absent from the hsp 70 gene chromatin during moderate transcription (9), and all of hsp 70 nucleosomes have abnormal electrophoretic mobility (Figure 3). Therefore it can be assumed that there are no "inactive" nucleosomes in hsp 70 genes.

Second, variations in nuclease susceptibility and preferential aggregation of active chromatin may cause enrichment of the analysed (soluble) fraction in "inactive" nucleosomes. In fact, however, the digestion rate of moderately transcribed hsp 70 genes is the same as for inactive chromatin (Figure 1) and preferential sedimentation of "active" nucleosomes in the in-

soluble fraction was not observed after the lysis of nuclei at a low ionic strength (Figure 4). It should be stressed that in some experiments (Figure 6) crosslinking was conducted in nuclei before isolation of nucleosomes.

It should be further noted that nucleosomes must undergo considerable changes in the sites of transcription directly occupied by RNA polymerase molecules in the process of reversible removal of histones (9). The removal appears to be induced by RNA polymerase molecules (10, but see Ref. 58). The depletion of histones proceeds in parallel with the intensity of transcription increasing in the series H1>H2A,H2B>H3,H4 and can go as far as the stage of partial or complete unfolding of nucleosomes (8). However, the DNA in active chromatin regions that are free from RNA polymerase molecules, reassociate with histones and thus a compact structure of nucleosomes is restored.

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