Distribution of high mobility group proteins 1/2,E and 14/17 and linker histones H1 and H5 on transcribed and non-transcribed regions of chicken erythrocyte chromatin

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ABSTRACT

Quantitative analysis of distribution of chromosomal proteins on single copy DNA sequences has been further developed. Our approach consists of DNA-protein crosslinking within whole cells or isolated nuclei, specific immunoaffinity isolation of crosslinked complexes via protein and identification of crosslinked DNA by hybridisation with single-stranded DNA probes. The present study shows that transcribed chromatin of chicken embryonic erythrocyte β globin gene is characterized by about 1.5 – 2.5-fold higher density of HMG 14/17 and 2-fold lower density of H1 and H5 as compared with non-transcribed chromatin of ovalbumin and lysozyme genes, whereas HMG 1/2,E proteins were equally distributed between DNA of both transcribed and non-transcribed genes. The depletion of H1/H5 in β globin sequences was verified by the 'protein image' hybridisation technique (1). The DNase I hypersensitive site located 5' upstream from β globin gene is deficient in all the proteins assayed, what implies a drastic disruption in the nucleosomal array. Minor quantitative changes of protein pattern suggest transient local perturbation of the chromatin on transcription.

INTRODUCTION

Despite voluminous literature, an increased sensitivity of transcribed genes to cleavage by DNase I is a nearly singular persistent experimental feature, which however cannot be interpreted in terms of definite structural context. HMG 14/17 were suspected to play a key role in maintenance of DNase I sensitivity of transcriptionally competent chromatin (2). An enrichment of the nucleosomes of transcribed region with HMG 14/17 was demonstrated (3,4,5). However several studies failed to reveal any preferential binding of HMG 14/17 to transcriptionally active chromatin (6,7). It seems that the contradictions are due to differences in the experimental techniques leading to rearrangement of chromosomal proteins along DNA (8).

Linker histones seem to be involved in structural transitions of the chromatin which precede or accompany transcription. Weintraub (9) found out that after mild micrococcal nuclease (MNase) digestion of nuclei, actively transcribed sequences are enriched in particular fraction, wherein large deoxynucleoprotein particles with internucleosomal cuts lost their ability to associate with each other in low-ionic-strength buffer. Active erythrocyte β globin chromatin sediments in sucrose gradient slower than the bulk chromatin of the same size (10). The above authors (9,10) suggest that transcriptionally active chromatin is characterized by unfolding of higher order structure, mediated by changes in binding of linker histones. The altered nucleosomal ladder of active sequences resulting from mild digestion of nuclei by MNase was explained by the easier accessibility of DNA caused by selective removal of linker histones or H2A-H2B dimers during transcription (11,12). A more direct approach, namely analysis of MNase cleavage products of chromatin, was also employed. Salt fractionation has shown that aggregation-resistant fraction of chicken erythrocyte nuclei digest is highly enriched in globin sequences, depleted in H1 and H5, and contains core histones and HMG proteins in equimolar or nearly equimolar amounts (13). However, direct quantitative mapping of linker histones' density was complicated by chromatin aggregation, protein redistribution (14,15), etc.

The chemical DNA-protein crosslinking was used to circumvent these difficulties. By means of zero-length dimethyl sulphate (DMS) crosslinking it was shown that some DNA-H1 contacts are depleted on transcribed chromatin (1). Nacheva et al. (16) have specified that only the globular part of H1 is displaced from highly transcribed DNA whereas N- and/or C-terminal domains remain to be bound.

The present study combines advantages of intracellular DNA protein crosslinking, immunoprecipitation of crosslinked complexes and sensitive hybridisation for the determination of chromosomal protein density on single copy genes *in vivo*. This approach was introduced by Gilmour and Lis (17), Shick et al. (18), and Blanco et al. (19). UV-light and two chemical methods were selected for crosslinking in order to minimize possible risk

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of artefacts. Our data here demonstrate the enrichment of HMG 14/17, the depletion in both linker histones on erythrocyte active chromatin compared with inactive one and even distribution of HMG 1/2,E on both.

METHODS

Subcloning

DNA fragments were inserted into M13 mp8 and mp9 vectors as described (20): Msp I(+130)–Hind III(+1070) fragment derived from C β G13 plasmid (' β globin probe'); Pst I(–200)–Rsa I(+50) fragment from C β G13 plasmid ('promoter β globin probe'); entire Eco RI 2.4 kb fragment from pOv2.4 plasmid ('ovalbumin probe'); entire Hind III 0.8 kb fragment from pLysE plasmid ('lysozyme probe'). The C β G13 plasmid (21), pOv2.4 plasmid (22), and pLysE plasmid (23) were kindly provided by Dr. H. Martinson, Dr. P. Chambon and Dr. T. Igo-Kemenes, respectively.

Isolation of monospecific antibodies

The rabbits were immunized by initial multilocal intradermal injections (1.5–2.0 mg of each antigen emulsified with complete Freund's adjuvant) and boosted 2–4 times (0.5–1.0 mg). The titers and cross-titers were determined by ELISA (24). The panel of antigens immobilized on activated adsorbents was used for both antibody isolation and deprivation of cross-reaction. The panel consisted of HMG 1/2,E-AffiGel 10, HMG 14/17-AffiGel 10, H5-, H1- and core histone-bis-oxirane-Sepharose CL-4B, subsequently substituted by Tresyl-Sepharose CL-4B (Pharmacia Biotech. Int AB) coupled separately with all the antigens. The final antibody preparations were considered as monospecific according to data on ELISA, Western blotting, immunoprecipitation assays of ¹²⁵I-labelled DNA-protein complexes, and two-dimensional electrophoresis of ³²P-labelled adducts. The two latter methods are described below in 'Analytical immunoprecipitation' and 'Preparative immunoprecipitation' sections.

In vitro crosslinking procedure

The sampling of blood from 14-day chicken embryos in 1×SSC buffer was followed by washing the cells with 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 5 mM sodium phosphate (pH 7.3), discarding the buffy coat, filtering through a Nitex screen and suspending in the same buffer. Nuclei were prepared by lysis with 20 mM HEPES (pH 7.4), 5 mM KCl, 2 mM MgCl₂, 1% (v/v) Triton X-100 and washed with the same buffer omitting Triton X-100.

The 5mm-layer of cell and nuclei suspensions (optical density A₂₆₀ ≤ 1.0) was irradiated by UV light at 0°C for 20–30 min under gentle shaking. Ultraviolet Production illuminator Model 0–61 was used with energy flux of 350 μ W/sm² at 254 nm. After irradiation of cells, nuclei were isolated as above.

Formaldehyde/dimethyl sulphate (DMS) crosslinking was employed for whole erythrocytes according to the scheme developed in our laboratory previously (16,25,26). Briefly, the cells were incubated with 1% formaldehyde at 0°C for 18 hrs (27), washed four times with 50 mM HEPES (pH 7.4), 1 mM EDTA, 140 mM NaCl. Nuclei were prepared as described above. 8 mM DMS in 50 mM HEPES buffer was added and methylation was continued for 20 hrs at 0°C. Depurination was performed for 16 hrs at 37°C. Two agents were taken in parallel for reduction: sodium borohydride (NaBH₄) and borane-pyridine complex (C₅H₅N-BH₃), 10 mM each.

All the nuclei obtained were lysed with N-laurylsarcosinate and sonicated (26). Formaldehyde-fixed lysates were heated at 65°C for 13 hrs for complete reversal of formaldehyde-mediated crosslinks (27). Then all the lysates were subjected to ultracentrifugation in CsCl gradient (17). The lower fractions containing mainly free DNA and DNA-protein adducts were briefly dialysed against 0.1% N-laurylsarcosinate, 1 mM EDTA (pH 8.0), precipitated with ethanol, dissolved in 1% SDS and finally used for immunoaffinity procedures.

Analytical immunoprecipitation

After CsCl gradient centrifugation covalently crosslinked DNA-protein complexes were labelled at protein moieties with ¹²⁵I (28,29), purified from unincorporated radioactivity, and brought to the buffer for immunoprecipitation (IP-buffer) at final concentrations of 0.2% SDS, 1% (v/v) Triton X-100, 1% sodium deoxycholate, 50 mM tris-HCl (pH 7.5), 1 mM EDTA, 2 M NaCl, 10 mg/ml BSA, and up to 1 mg/ml DNA. 100- μ l aliquots were incubated with increasing amounts of antibodies (0.1 to 2.5 μ l) at 20°C for 2–3 hrs and then with Protein A-Sepharose CL-4B (the bed volume of 5 μ l) at 20°C for 1–2 hrs under gentle vortexing. It was followed by three washes of Sepharose beads with IP-buffer (10 min each), and three washes with 50 mM tris-HCl (pH 7.5), 1 mM EDTA, 140 mM NaCl. Bound and non-bound radioactivity was measured. Minimal antibody concentrations permitting to reach the plateau of bound radioactivity were regarded as working concentrations. We believe that adjusting antibody concentrations we kept immunoprecipitation free from putative co-isolation of cross-reactive complex, with good yield of specific product. Bound radioactivity, obtained when using working concentrations of antibodies, was visualized by DNA hydrolysis and subsequent SDS-polyacrylamide gel electrophoresis of remaining proteins to verify monospecific isolation of crosslinked adducts (26). Furthermore, specific inhibition of each antibody was tested by adding excess of cold exogenous antigen to another aliquot in the same conditions of immunoprecipitation. Non-specific adsorption of DNA-protein adducts was imitated by mock-precipitation with 2.5 μ g of non-immune rabbit IgG.

Preparative immunoprecipitation

Cross-linked DNA-protein adducts (5–15 mg of DNA) in IP-buffer, working concentrations of antibodies, and Protein A-Sepharose CL-4B (400 μ l per 1 mg antibodies) were used. The first cycle of incubation with antibodies was continued at 20°C for 6 hrs and with adsorbent for 4 hrs, and the second cycle lasted for 4 hrs and 3 hrs, respectively. The elution was carried out with 2–3 volumes of 1% SDS at 45–50°C for 20 min. After the first cycle of immunoprecipitation the eluates, containing highly enriched DNA-protein complexes, were denatured at 100°C (UV-crosslinked samples for 2 min and formaldehyde/DMS-crosslinked samples for 10 min) and subjected to the second cycle to reduce non-specific binding of free DNA. Aliquots of Protein A-Sepharose bound material were analyzed by 5' end labelling and two-dimensional electrophoresis and autoradiography as described (18).

Nylon filter blotting, hybridisation and scanning densitometry

The immunoprecipitated samples were RNase-treated, deproteinized, extracted with phenol and water-saturated ether, lyophilized and dissolved in 10 mM tris-HCl (pH 8.0), 0.1 mM EDTA. Aliquots of non-immunoprecipitated DNA crosslinked by different methods were processed the same way to obtain

internal standard for hybridisation (designated as Control DNA in immunoprecipitation data and Control protein free DNA—PFC diagonal—in two-dimensional electrophoresis). Dot-blotting of control DNAs was accomplished, using a series of seven or eight two-fold dilutions, starting with 2–3 μ g of DNA (about 1 amole of single-copy sequences). Application of DNA onto the Zeta Probe blotting membrane (Bio Rad) was done by BioDot apparatus according to supplier's recommendations. Immunoprecipitated DNA and control DNA samples were bound to one filter. Finally the filter was treated with 0.4 M NaOH at 40°C for 1 hr. Probe preparation, hybridisation, filter washing, probe elution and reprobing were carried out as described previously (30). The autoradiography was carried out using an Orwo HS-11 film and an intensifying screen at –70°C. The autoradiographs were scanned in a one-dimensional scanning mode by a computer-assisted Ultrosan XL (LKB). The hybridisation signal of a dot was estimated as an area under the

optical density peak. The densities of individual proteins on assayed sequences were compared as follows: after hybridisation of filter with ovalbumin probe autoradiograph was scanned, and 'control DNA' signals were used to plot the calibration curves (for different methods of crosslinking) with molar content of single copy DNA as absciss, and optical density as ordinate. Using this curves, signals of 'protein-DNA' were evaluated in terms of molar content. The same procedure (scanning, calibration and evaluation of molar content) were done for autoradiographs after other hybridisations. Assuming equimolar ratio of assayed sequences in control DNA, we were able to detect depletion or enrichment of these sequences in particular immunoprecipitated DNA samples.

Two-dimensional electrophoresis and electrotransfer

The bulk of free DNA was removed from formaldehyde/DMS (NaBH_4) crosslinked material by phenol extraction (to optimize electrophoretic pattern), and two dimensional polyacrylamide gel electrophoresis and electrotransfer onto Zeta Probe filter was performed as published (1,26) with the following modification. Polyacrylamide gel strip containing control free DNA track (diagonal PFC) was polymerized into second dimension gel with some displacement relative to the basic material track (diagonals PFr, C and L), and that's why control free DNA looked like an additional diagonal, strictly parallel to 'residual' free DNA diagonal on the two-dimensional picture. Two-dimensional autoradiographs were scanned at several levels in parallel to the front of the second direction, and mean values were estimated.

Figure 1. ELISA data: titration curves and cross-reaction of antibody preparations used for immunoprecipitation. **A.** anti-HMG 14/17 antibodies, **B.** anti-HMG 1/2,E antibodies, **C.** anti-H1 antibodies, **D.** anti-H5 antibodies. The following proteins were used as antigens: 1. HMG 14/17, 2. HMG 1/2,E, 3. H1 histone, 4. H5 histone, 5. core histones.

RESULTS

The validation and reliability of the method

The main requirements for present variant of immunoaffinity chromatography are antibody monospecificity, prevention of the crosslinked material aggregation and co-isolation of protein-free (uncrosslinked) DNA (the latter was present in a large excess over crosslinked complexes).

The monospecific polyclonal antibodies were isolated and purified from concomitant cross-reactive antibodies using a panel

Figure 2. Identification of product purity in immunoprecipitation. **A.** SDS-polyacrylamide gel electrophoresis of proteins, released from immunoprecipitated adducts. The material, crosslinked with UV light, was labelled with 125-iodine at protein moieties, and twice immunoprecipitated with either anti-HMG 14/17 (line 1) or anti-HMG 1/2,E (line 2) or anti-H1 (line 3) or H5 (line 4) antibodies. DNA of the samples was hydrolysed by diphenylamine/ formate treatment, and the proteins with very short oligonucleotide tails were separated by 16% gel. **B.** Two-dimensional electrophoretic analysis of free DNA content in crosslinked immunoprecipitated HMG 1/2,E-DNA and HMG 14/17-DNA. The material, crosslinked with formaldehyde/DMS (sodium borohydride), was twice immunoprecipitated with either anti-HMG 14/17 or anti-HMG 1/2,E antibodies, 5'-end-labelled and subjected to two-dimensional electrophoresis. 1 st direction—from left to right—SDS/urea polyacrylamide gel electrophoresis of crosslinked adducts. 2 nd direction—from top to bottom—SDS/urea polyacrylamide gel electrophoresis of deproteinized *in situ* DNA (32). The diagonals represent protein-free DNA (PF) and crosslinked complexes (CL) after the first (I) and second (II) cycle of immunochromatography.

of H1, H5, HMG 1/2,E, HMG 14/17 and core histones immobilized on activated adsorbents. Titers of the antisera exceeded 10^6 according to ELISA data. The titration curves of the final antibody preparations, depleted by several passings through cross-reacting antigens, coupled to adsorbents, are shown in Fig. 1. One can see that the titers of all the antibodies exceed the cross-titers by 1.5–3 orders of magnitude. The Western blotting technique for proteins of erythrocyte nuclei failed to detect cross-reactions of antibodies (not shown). Immunoprecipitated complexes could not be analyzed by Western blotting due to final products being present in small amount (less than microgram) and to the presence of IgG co-eluted with antigen from Protein A-Sepharose. To make sure the antibodies did function specifically at immunoprecipitation of crosslinked complexes, DNA- 125 I-protein adducts were immunoprecipitated

Figure 3. Physical map of chicken β globin, ovalbumin and lysozyme genes and location of hybridisation probes. The designations are as follows: GI— β globin probe, pGI—promoter β globin probe, Ov—ovalbumin probe, Lys—lysozyme probe, B—*Bam* HI sites.

on analytical scale, and proteins were analyzed by SDS-polyacrylamide gel electrophoresis following DNA degradation. The autoradiography revealed only specifically immunoprecipitated proteins (Fig. 2a). Some broadening of the bands is caused by dispersion of length of residual pyrimidine blocks at the site of crosslinking (26,31). Radioactivity was not retained on Protein A-Sepharose neither in mock-precipitation assay using non-immune rabbit IgG, nor in specific inhibition assays using cold exogenous antigens.

Two-dimensional gel electrophoresis of [32 P]-labelled crosslinked and uncrosslinked DNA characterized the Sepharose-bound radioactivity. Fig. 2b shows an examples of separation of free DNA and both HMG 1/2,E-DNA and HMG 14/17-DNA crosslinked with formaldehyde/DMS. Note that crosslinked adducts (CL) are represented by single diagonals, and that was an strong additional proof for protein specificity of immunoprecipitated products. The same autoradiographs demonstrate the ratio between crosslinked and uncrosslinked DNA in immunoprecipitates. Scanning the autoradiographs, we found that the free DNA content was reduced from initial 95–99% to about 60–80% in the material bound to Protein A-Sepharose after the first cycle of immunoprecipitation and to less than 10% after the second one. These values did not depend significantly either on the antibody or the crosslinking method used. Similar analysis of UV crosslinked adducts revealed a more marked contamination by free DNA (not shown). This contamination could be ignored taking into account some reversibility of UV-mediated crosslinking on heating before the second cycle of immunoprecipitation and electrophoresis.

Figure 4. Dot-hybridisation data of H1-DNA, H5-DNA, HMG 14/17-DNA and HMG 1/2,E-DNA. Samples, crosslinked by UV within cells (UV, a) or nuclei (UV, b), or by formaldehyde/DMS method (F/DMS, c—reduction with borane-pyridine complex; F/DMS, d—reduction with sodium borohydride), were immunoprecipitated with antibodies against H1, H5, HMG 14/17 and HMG 1/2,E, deproteinized, dot-blotted on one filter and successively rehybridised to globin (GI), ovalbumin (Ov), lysozyme (Lys) and promoter β globin (pGI) gene probes. Control DNA is non-fractionated DNA after UV (left) or formaldehyde/DMS (right) crosslinking, CsCl gradient centrifugation and deproteinization. Fig. shows the dot-blot autoradiographs after hybridisation to indicated probes, where signals of 'Control DNA' spots are of approximately equal intensity. It permits to compare visually 'protein-DNA' spots after hybridisations between each other. The spots positioned on one vertical represent the same DNA successively hybridised with various probes on the same filter.

Data on hybridisation of immunoprecipitated samples

Prior to dot-blot hybridisation, four probes (Fig. 3) were tested by Southern technique, and shown to possess high selectivity. Samples of isolated DNA were dot-blotted on one filter and successively hybridized with probes for the DNase I sensitive coding and promoter regions of transcriptionally active β globin gene and with probes for the DNase I resistant coding regions of two inactive ovalbumin and lysozyme genes.

Two upper gridirons in Fig. 4 show the hybridisation signals of DNA, obtained by immunoprecipitation of adducts using anti-H1 or anti-H5 antibodies. An important feature of this figure is the lower signals from both H1-DNA and H5-DNA after hybridisation with β globin and promoter β globin probes versus lysozyme and ovalbumin probes. Ratios of signals were the same for UV crosslinking using cell or nuclei suspension as well as for formaldehyde/DMS method. The similarity of UV data for both cells and nuclei suggests the absence of any marked migration of H1 or H5 between active and inactive chromatin regions during low-salt isolation of erythrocyte nuclei.

Fig. 5 summarizes quantitative data obtained by calculation of scanning estimates (see Methods) of the autoradiographs shown in Fig. 4. All crosslinking methods revealed that H1-DNA and H5-DNA were depleted in globin sequences as compared with ovalbumin ones to 0.5–0.65 for H1 and 0.65–0.7 for H5, respectively. The data on lysozyme gene resembled those obtained

for ovalbumin gene (hybridisation signal ratios varied from 0.85 to 1.1). H1-DNA and H5-DNA were dot-blotted onto a nylon filter in duplicate, and the ratios calculated from signals of second spots differed by no more than 0.15 in each case.

Promoter β globin/ovalbumin ratios were from 0.45 to 0.5 for H1 and from 0.4 to 0.5 for H5 in immunoprecipitation tests, but true density of linker histones on the promoter sequences must have been lower than the detected one for the following reasons. The length of the promoter region (200 bp) was shorter than the length of the probe (250 bp), and the length of the randomly sheared crosslinked DNA varied within 100–600 bp range. Therefore immunoprecipitated complex can contain promoter sequence and a protein, crosslinked to surrounding DNA. This effect (false-positive hybridisation signal) had to be negligible for longer probes.

A moderate enrichment of HMG 14/17-DNA in β globin but not promoter β globin gene sequences relative to ovalbumin and lysozyme gene sequences is illustrated at lower quarters of Fig. 4 and 5. The promoter β globin probe produced roughly equal signal as inactive gene probes. The 1.5–2.4-fold enrichment of HMG 14/17-DNA with globin versus ovalbumin and lysozyme coding sequences was in a striking contrast with the marked (up to 100-fold) enrichment of transcribed over non-transcribed sequences, reported by Dorbic and Wittig (4,5), but generally agreed with their finding that upstream boundary of HMG 14/17 location coincides with the transcriptional start (compare the

Figure 5. Relative density of H1, H5, HMG 14/17 and HMG 1/2,E on specific genes in chicken erythrocytes. Autoradiographs shown in Fig. 4 were scanned and quantitated, as described in 'Methods'. The protein density on ovalbumin sequences is taken to be a unit. The method of crosslinking is indicated below each chart (UV within cells (a) or nuclei (b), or by formaldehyde/DMS method (c—reduction with borane-pyridine complex; d—reduction with sodium borohydride)). Each subset of graph bars is the ratios between hybridisation signals of one sample on one filter.

Figure 6. 'Protein image' hybridisation of chicken erythrocyte chromatin. Chicken erythrocyte DNA was crosslinked to chromosomal proteins by formaldehyde/DMS (sodium borohydride) method. Free protein was removed by CsCl gradient centrifugation, and bulk of free DNA by phenol extraction (26). Subsequent two-dimensional gel electrophoresis demonstrates diagonals, where an angle of the slope depends on the retardative effect (molecular mass) of crosslinked protein. Designations are: **PFc**—protein-free control DNA, **PFr**—protein-free DNA remaining after phenol extraction, **C**—core histones-DNA, **L**—linker (H1 + H5) histones-DNA. Separated DNA diagonals were transferred onto Zeta Probe nylon membrane and successively hybridised to globin (**Gl**), ovalbumin (**Ov**) and promoter β globin (**pGl**) gene probes.

HMG 14/17-DNA data for β globin and promoter β globin gene on Fig. 4). The promoter β globin region had the same density of HMG 14/17 as inactive regions but the actual density could be even less due to 'false-positive hybridisation signal due to neighboring site of crosslinking', mentioned above. Moreover, promoter β globin probe overlaps the adjacent coding region, which is enriched in HMG 14/17. Neither variant of crosslinking affected appreciably signal ratios. However UV crosslinking within nuclei showed a higher presence of HMG 14/17 on globin gene region as compared with that probed by crosslinking within cells. This suggests some migration of HMG 14/17 from inactive to active chromatin during nuclei preparation.

The signals for HMG 1/2,E-DNA and densities of HMG 1/2,E on assayed genes can be also seen in Fig. 4 and 5. The similar patterns for β globin, ovalbumin and lysozyme genes suggest an equal distribution of HMG 1/2,E between transcribed and non-transcribed DNA sequences, and only promoter β globin probe has hybridized weaker to HMG 1/2,E-DNA. Again this was correct for any crosslinking procedure. The densitometric quantitation of HMG 1/2,E-DNA signals supported the fact of even distribution of these proteins between active and inactive sequences, with exception of β globin gene promoter.

'Protein image' technique

The above results on H1 and H5 densities, based on immunoprecipitation data, have been checked by the 'protein image' hybridisation method, which allows to visualize specific sequences, crosslinked with linker and core histones by two-dimensional electrophoresis and blot-hybridisation (1,26). Apparent depletion of linker histones on transcribed globin sequences is demonstrated in Fig. 6. Identification of the /L/-diagonal as H1/H5-DNA band was carried out earlier (31,32). Protein-free control DNA diagonal /PFc/ serves as internal standard (see Methods). In this variant of electrophoresis, H1-DNA and H5-DNA adducts were superimposed on the same diagonal whereas immunoprecipitation can discriminate between these adducts and thus provides an additional fact that both linker

histones crosslink weaker to active sequences. The experiment was performed on formaldehyde-fixed DMS-treated erythrocyte nuclei (the same material as for immunoprecipitation, Fig. 4, line d). The ' β globin/ovalbumin' ratios for (H1 + H5)-DNA, crosslinked by formaldehyde/DMS were close for both electrophoresis and immunoprecipitation: 0.55 and 0.6, respectively. H1 + H5 density on promoter β globin versus ovalbumin sequences was about 0.2 in 'protein images' (diagonals /L/ in Fig. 6).

So, both 'protein image' and immunoprecipitation methods are suitable for separation of DNA-protein adducts. Incidentally, one can also notice a slightly decreased (by about 30%) signal of the core histone-DNA diagonal /C/ after hybridisation to active globin probe as compared with inactive ovalbumin one (Fig. 6), and this phenomenon was observed earlier (1).

DISCUSSION

Methodological remarks

The crucial condition for correct evaluation of the chromosomal protein density on a specific sequence is to prevent rearrangement of these proteins between chromatin fragments. It is well known that HMG 14/17 exchange very easy (8), whereas the bulk of HMG 1/2,E may leak out during nuclei preparation and digestion by MNase. The DNA-protein zero-length crosslinking within whole cells prevents rearrangement of nuclear proteins, fixes intrinsic DNA-protein interactions and allows to use detergents to minimize aggregation of DNA-protein complexes. We believe that crosslinking by UV light is a reliable and feasible method. However the rate of crosslinking is rather low—about 0.05% proteins per hour in our conditions, and a higher dose would cause DNA degradation. In view of the shortcomings of UV crosslinking and in order to substantiate our results we applied basically different crosslinking method. It produces highly specific DNA-protein crosslinking due to partial DNA depurination of bases, which were methylated by DMS, and fixation of aldimine bonds between depurinated DNA residues and polypeptide imidazole group or amino group by reduction with sodium borohydride or borane-pyridine complex, respectively (16,26). Reversible and complete formaldehyde fixation of chromatin of whole cells (27,33) prevents protein rearrangement during nuclei preparation, methylation, depurination and reduction steps of DMS crosslinking, and largely during fixation itself (34,35). Fixed chromosomal proteins preserve their ability to be crosslinked to DNA by DMS treatment, since much more molar excess of formaldehyde is required for full conversion of amino groups into methyl derivatives (36). Moreover, lysines of DNA-binding domain seem to be less accessible to methylation by formaldehyde as shown for H5 (37). Agents like NaBH_4 or $\text{C}_5\text{H}_5\text{N-BH}_3$ or NaCN-BH_3 can reduce aldehydes or Schiff bases, but not methylene-bridged links. Conceivably, they have no effect on reversal of the formaldehyde crosslinks, although stabilize protein modification (methylation), introduced by formaldehyde.

And finally control experiments have shown that immunoprecipitation of crosslinked complexes meet the requirements for product purity. The basic advantage of this immunoaffinity version is stability of antibody-DNA-protein complex. Good reproducibility of the data for H1, H5 and HMG proteins associated with different chromatin regions demonstrates the usefulness of all these methods of crosslinking and fractionation.

Linker histones and HMG proteins in chicken erythrocyte chromatin

Several attempts were made to quantitate H1/H5 (or H1°) distribution on expressed and repressed genes. Linker histones were displaced from active mononucleosomes more readily in MNase-treated chromatin as described in a number of earlier reports. The oligonucleosomes depleted in H1 were preferentially soluble in low salt buffers (13,38), and the degree of solubility correlated with the DNase I sensitivity and transcriptional activity (39). Using anti-H1° antibodies and immunoaffinity chromatography of mildly hydrolysed rat liver chromatin, Mendelson et al. (40) found about 2-fold decrease of active sequences in the bound fraction. And finally, nuclear pellet-bound sequences following nuclease treatment were demonstrated to be hybridised better to active sequences and to contain roughly a half of linker histones of bulk chromatin (11,41). All these data taken together with direct electron microscopy observation (42) show that linker histones are apparently present on transcribing sequence, although some depletion is manifested too. We have found that erythrocyte H1 and H5 histones crosslink 1.5–2 times less effectively to DNA sequences of actively transcribed globin gene than to sequences of inactive lysozyme and ovalbumin genes (unfortunately we were unable to discriminate between displacement of histone and depletion of its contacts with DNA). Since the erythrocyte nucleosomal repeat is 205–210 bp long, the β globin gene length is about 1600 bp, and assuming that there is one H1 or H5 molecule per nucleosome, we suppose that our data indicate to a transient local character of the alteration of protein pattern in 3–4 nucleosomes within the region. Probably the situation here is much the same as that described by Bjorkroth et al. (42) for chromatin template of transcriptionally active Balbiani ring genes which looked like extremely dynamic structure of 5, 10 and 30 nm filaments arranged between growing ribonucleoprotein particles.

Nacheva et al. (16) found no difference in H1 densities between active and inactive chromatin using crosslinking preferentially through C- and N-terminal lysines of *Drosophila* H1 (cyanoborohydride— NaBH_3CN —variant), together with a marked withdrawal of H1 from transcribed DNA according to the data on crosslinking using histidine-specific NaBH_4 variant. It does not correlate with 1.5–2-fold depletion in linker histones using both chemical variants (crosslinking specificity of NaBH_3CN and $\text{C}_5\text{H}_5\text{N-BH}_3$ is identical) and UV light for chicken erythrocyte chromatin. Several important experimental details could be responsible for that. Firstly, amino acid sequence of chicken H1, and especially H5 is considerably differed from that of *Drosophila* H1. So we could not exclude the possibility of crosslinking of globular domain of chicken linker histones to DNA using $\text{DMS/C}_5\text{H}_5\text{N-BH}_3$ method. If this is the case, dissociation of the globular domain from DNA upon transcription should become detectable. Secondly, removal of central domain of chicken linker histones from DNA on transcription could do change the *crosslinkability* of C- or/and N-terminal lysines of chicken H1/H5, disturbing their fine spatial proximity to DNA. Note that we have detected H1/H5 depletion in globin sequences using UV light irradiation method, which usually results in DNA-protein crosslinking preferentially via lysines. And thirdly, particular properties of heat shock gene or globin gene sequences could affect the mode of linker histone binding in transcriptionally active chromatin of *Drosophila* and chicken. For example, the contradictions on nucleosome displacement by polymerase seem to be caused by such a sequence specificity (43,44).

It can be also concluded from our results that H1 and H5 show no clustering on specific gene chromatin and this could have been anticipated judging from H1 and H5 interspersion in oligonucleosomes (45). Thus H5 distribution has no preference for inactive chromatin regions. Higher H1/H1° ratio in rat liver mononucleosomes of expressed albumin gene chromatin (46) could be a result of preferential release of H1° over H1 upon nuclease digestion as it was shown for H5 (47).

Many previous publications on involvement of HMG 14/17 in transcription are controversial. The dependence of DNase I sensitivity of transcriptionally active chromatin on HMG 14/17 was advocated by Weisbrod et al. (2), but subsequently doubted (7). The preferential binding of HMG 14/17 to active nucleosomes was demonstrated by Sandeen et al. (48) and Brotherton & Ginder (49), but was refuted in other papers (6). Two following studies are of a particular importance for understanding the situation. Using low salt MNase digestion and immunoprecipitation of rat liver chromatin with anti-HMG 14/17 antibodies, Druckmann et al. (50) found no difference between poorly transcribed and non-transcribed genes, but HMG 14/17-bearing chromatin fragments of nuclei, treated with inducing agents, were enriched in both actively transcribed sequences and 5' upstream sequences of non-actively transcribed genes over repetitive sequences. An abundant enrichment of active chromatin by HMG 14/17 was detected by Dorbic and Wittig (5) using an elegant version of oviduct H1-stripped chromatin immunoprecipitation and restriction *in situ*. By interchanging the set of restriction endonucleases, they recognized clustering of HMG 14/17-bearing nucleosomes immediately downstream the transcriptional start. It is still unclear whether it should be regarded as *in vivo* distribution of HMG 14/17 or as an artefactual protein rearrangement onto binding sites of higher affinity. Both works analyzed only a portion of chromatin.

On average only every tenth nucleosome in total chicken erythrocyte chromatin has two molecules of HMG 14/17 (48). We found two-fold increase of HMG 14/17 in active chromatin. It means that every fifth nucleosome becomes associated with these proteins. It is too low a figure to expect that each nucleosome should be simultaneously activated in transcribed chromatin by HMG 14/17 binding. Assuming that HMG 14/17 and H1 share certain homology in their central regions, taken together with our data, showing that H1/H5 depletion and HMG 14/17 enrichment are of similar quantitative value, it is tempting to speculate that HMG 14/17 binding and H1 removal (partial displacement) are coupling events, occurring probably in the vicinity of moving RNA polymerase. Previously we have shown that HMG 14/17 binding does not induce gross changes of DNA-histone contacts within nucleosome and has no preference for H1-containing or H1-depleted nucleosomes (18). Hence it seems likely that HMG 14/17 facilitate histone displacement from DNA on transcription rather than modify total conformation of nucleosome. Higher enrichment of HMG 14/17 on globin gene sequences using crosslinking within nuclei as compared with crosslinking within cells supports the fact of their easy rearrangement onto accessible unfolded chromatin regions.

HMG 1/2,E is a group of DNA-binding proteins containing so-called A[−] region with the runs of approximately 40 negatively charged amino acids (51), implying tendency to protein-protein interactions. Recently a number of publications have described the properties of high-molecular-weight HMG proteins which suppose them to be implicated in transcription although so far no definite mechanism has been proposed. The proteins can facilitate displacement of histones from DNA on

transcription, overcome histone-induced or Z-DNA induced transcriptional block *in vitro* (52,53). It should be noted that HMG 1/2 are suspected of playing the role of an allosteric activator in MLTF factor dependent transcription and they can function without binding to DNA (54). On the other hand, HMG 1 binds to cruciform DNA with a high affinity (55), and effectively unwinds negatively supercoiled DNA (56). Hence, maintenance of unaltered secondary DNA structures could be postulated as a mechanism by which high molecular weight HMG proteins stimulate transcription. However, *in vitro* transcriptional and conformational effects of HMG 1/2 are usually observed at their high molar excess over DNA (57).

In a view of extremely easy exchange of HMG 1/2 within chromatin we find it difficult to comment the papers dealing with association between HMG 1/2 and transcriptionally active chromatin. The only work, wherein HMG 1/2 were fixed to DNA by UV crosslinking, reported an enrichment of HMG-T on inactive sequences of the protamine gene family as compared with active sequences of histone and vitellogenin genes in trout liver nuclei (19). These data differ from ours, since we find no enrichment or depletion of HMG 1/2,E on transcribed as compared with non-transcribed chromatin regions by any crosslinking method. Well-known dissimilarities between HMG-T and HMG-E, and moreover HMG 1/2,E (58) may be responsible for that, for example regarding sequence specificity. We suggest that either HMG 1/2,E proteins are not involved in chromatin activation or they may do it by associating with other proteins rather than with DNA. We have also found no involvement of HMG 1/2,E in the formation of transcription complex on β globin promoter in erythrocytes, possibly due to the absence of interaction between HMG 1/2,E and DNA or to HMG 1/2-independence of chicken β globin gene transcription initiation.

The promoter region of the globin gene

The depletion of all the proteins tested (including HMG proteins) in the promoter region was estimated by immunoprecipitation assays as 40–70% by their crosslinking to DNA and this is probably underestimation. The nucleosome-free state of the globin promoter has been demonstrated by nuclease digestion (59). The crosslinking experiments with the promoter of hsp 70 genes revealed nearly complete absence of all histones (1). Proteins of transcriptional machinery associating with the promoter shield DNA against interaction with other proteins.

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Note added in proof

Our data on H1/H5 are in a full agreement with results in paper 'Chromatin structure of transcriptionally competent and repressed genes' by Kamakaka and Thomas (*EMBO J.*, 9(12), 1990, in press), where analysis of UV-crosslinked and immunoprecipitated H1/H5 histones of chicken erythrocyte chromatin has been done.

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