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General Method for Isolation of DNA Sequences That Interact with Specific Nuclear Proteins in Chromosomes: Binding of the High Mobility Group Protein HMG-T to a Subset of the Protamine Gene Family†

J. Blanco,‡ J. C. States,‡,§ and G. H. Dixon*

Department of Medical Biochemistry, Faculty of Medicine, Health Sciences Centre, University of Calgary, Calgary, Alberta, Canada T2N 4N1

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ABSTRACT: A general method is described for the isolation of the DNA with which specific nuclear proteins interact in chromosomes. This method is based on the covalent photo-cross-linking of nuclear proteins to the DNA sequences, to which they normally bind, by means of irradiation with UV light and the selective retrieval of specific subsets of protein–DNA adducts by using specific antibodies. The application of this procedure to isolate the DNA sequences with which the trout high mobility group protein (HMG-T) interacts has shown that in trout liver this protein associates specifically with DNA sequences in proximity to a subset of the family of protamine genes but not with the histone or vitellogenin genes. From these observations, it appears that the HMG-T protein may be associated with inactive gene sequences.

It is currently believed that the regulation of gene expression and the conformation of chromatin are mediated by the specific interactions of nuclear proteins with DNA. However, even in the better known cases we have only an imperfect idea of when during development and where in the DNA sequence such associations take place. Some examples of such interactions, recently the focus of active investigation, are the transcriptional complex of *Xenopus* 5S ribosomal RNA genes (Brown, 1984), the binding sites of an HMG-like protein in the satellite DNA of CV-1 monkey cells (Straus & Varshavsky, 1984), the nuclease hypersensitive sites 5′ of heat-shock genes in Drosophila (Elgin, 1981; Wu, 1984), the chicken β-globin gene (Emerson & Felsenfeld, 1984), and the c-myc gene in Burkitt's lymphoma (Siebenlist et al., 1984).

It seems clear that a general procedure for the detection and mapping of the associations between nuclear proteins and specific DNA sequences would be of value in understanding the mechanisms of regulation of gene expression in chromosomes.

It has been possible for some time now to detect the sites of interaction of proteins and DNA in nucleoprotein complexes assembled artificially in vitro (Galas & Schmitz, 1978; Siebenlist et al., 1980; Ross et al., 1978; Ogata & Gilbert, 1977). However, these methods are of limited use in the more complex case of native chromosomes. Very recently, several techniques have been developed that allow the accurate localization of protein binding sites in specific DNA sequences of native chromatin. On the basis of the protective effect that proteins bound to DNA have when chromatin is digested with nucleases or irradiated with UV light, such methods have allowed the localization of nuclear protein binding sites in the nuclease hypersensitive regions 5′ of the heat-shock genes of *Drosophila* (Wu, 1984) and the *lac* repressor binding site in the *lac* operator of *Escherichia coli* DNA (Becker & Wang, 1984), the

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‡ Postdoctoral fellows of the Alberta Heritage Foundation for Medical Research.

§ Present address: IDR-720, Children's Hospital Research Foundation, Cincinnati, OH 45229.

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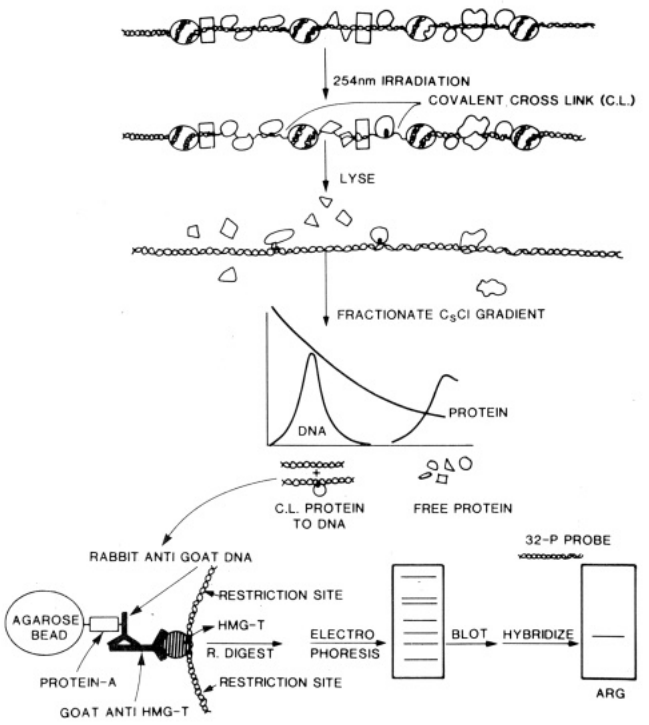

FIGURE 1: Overall experimental protocol.

nucleotide sequence recognized by the glucocorticoid receptor in the rabbit uteroglobin gene (Cato et al., 1984), and the DNA binding sites of *Drosophila* RNA polymerase II transcription factors for the hsp 70 gene (Parker & Topol, 1984a,b).

We have taken a different but complementary route to study this type of question. In our approach, illustrated in Figure 1, nuclear proteins are covalently cross-linked, by irradiation with ultraviolet light, to the DNA sequences with which they interact in intact nuclei. After unbound proteins are removed specific protein–DNA adducts are selectively retrieved with the aid of antibodies bound to a solid support and the enriched DNA segments recovered for analysis.

By use of the trout protamine gene family and the nonhistone high mobility group HMG-T protein of trout (Dixon, 1982), as a model system, the feasibility of this general approach will be demonstrated. A procedure similar to ours for the detection of the binding site of RNA polymerase in bacterial cells has recently been published (Gilmour & Lis, 1984).

Materials and Methods

Preparation of Nuclei from Trout Liver. The whole procedure was performed at 0 °C on 30-g batches of fresh trout liver in buffer A of Hewish & Burgoyne (1973) from which β-mercaptoethanol was omitted. The livers were minced with a razor blade, suspended in 20 volumes of buffer A, and homogenized by five passes of a Teflon pestle in a glass homogenizer. The resulting slurry was filtered through two layers of cheesecloth to remove connective tissue and the filtrate gently centrifuged for 2–3 min at 500 rpm in a Beckman TH41-87 rotor to remove large cell aggregates. The nuclei were then pelleted by centrifugation at 2000 rpm for 5 min and washed, first by centrifuging twice through 50 volumes of buffer A for 5 min at 2000 rpm and then through 15–20 mL of a 28% sucrose cushion made in the same buffer for 10 min at 2000 rpm.

Irradiation and Lysis. Nuclei, at a concentration of 10^7 nuclei/mL in buffer A, were irradiated in uncovered Petri dishes over ice–water with continuous agitation, for 30 min at a distance of 10 cm under two lamps (Rayonet RPR-2537A) delivering 480 $\mu W/cm^2$. After irradiation the nuclei were harvested by centrifugation at 2000 rpm for 10 min and then lysed in 6 M guanidine hydrochloride (Gdn·HCl),[1] 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.014% β-mercaptoethanol, and 0.1 mM PMSF at a concentration of 10 A_{260} units/mL. The lysis process was allowed to continue overnight at 4 °C before the lysates were used.

Phenol Extraction. For phenol extraction, nuclei were lysed in 1% SDS, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 2% β-mercaptoethanol and extracted with equal volumes of phenol equilibrated in the same buffer.

CsCl Gradients and Isolation of Cross-Linked DNA. Aliquots of the nuclear lysates containing 10 A_{260} units were layered on top of step gradients consisting of 2-mL layers of CsCl at densities of 1.76, 1.57, 1.54, and 1.32 g/mL in 6 M Gdn·HCl as modified from Shaw et al. (1973) and centrifuged in the Spinco SW41 rotor for 48 h at 35 000 rpm and 20 °C. One-milliliter fractions were collected from the bottom of the gradient tubes for analysis; otherwise, the bottom half of the gradient containing the DNA peak was collected for preparative purposes. The DNA-containing fractions from the CsCl gradients were pooled and dialyzed twice against 1 L of 0.5 M NaCl, 60 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.1 mM PMSF (wash buffer). After dialysis, the DNA sample was centrifuged for 5 min at 2000 rpm to remove aggregates and incubated with protein A–Sepharose beads (Pharmacia) that had been preloaded with rabbit anti-goat antibody followed by a specific polyclonal goat antiserum raised against trout HMG-T (Peters et al., 1979). Typically 100 mg of a protein A beads was added to 5 mg of DNA and incubated with very slow agitation for 18 h at 4 °C.

The protein A beads, now bearing DNA-bound HMG-T complexes, were then washed twice with 15 mL of wash buffer (0.5 M NaCl, 60 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.1 mM PMSF), transferred to 1.5-mL Eppendorf tubes, and washed twice more with 1 mL of the corresponding restriction nuclease digestion buffer. The DNA bound to the beads was digested with 50–100 units of restriction enzyme in 500–600 μL of buffer (from which thiol had been omitted) per 100 mg of beads, for 3–4 h at 37 °C. Controls consisting of DNA from unirradiated trout nuclei were usually digested in parallel reactions. The reaction mixtures were gently agitated every 15–20 min to keep the beads in suspension. Restriction digestions were terminated by the addition of 1 mL of cold wash buffer and the released DNA fragments removed by washing the beads 4 times with 1-mL aliquots of the same buffer. To elute the retained DNA, the beads were suspended in 500 μL of wash buffer made 1% in SDS and digested with 100 μg/mL proteinase K (Boehringer Mannheim) overnight at 37 °C. Finally, the released DNA was recovered from the beads by passage of the digestion mixture through a small glass-fiber filter made in an Eppendorf tube, the effluent phenol extracted, and the DNA concentrated by precipitation with ethanol.

Antibody Beads. Protein A–Sepharose beads (Pharmacia), 200 mg, were washed 4 times with 1 mL of 0.5 M NaCl, 60 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.1 mM PMSF (wash buffer) and then incubated with a saturating concentration of rabbit anti-goat antibody (Miles Yeda) in wash buffer for 5 h at 4 °C. Beads loaded with the first antibody were washed 4 times with 1 mL of wash buffer and then

[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

incubated for 18 h at 4 °C with either wash buffer or goat anti-HMG-T antibody (Peters et al., 1979); 5 mg/100 mg of beads), dissolved in wash buffer. Finally, the beads were washed 4 times with 1-mL aliquots of wash buffer, prior to their addition to the DNA samples. Protein A beads had the capacity to bind 30 μg of rabbit anti-goat antibody/5 mg of beads.

Affinity Purification of Antibody. Acid-extracted liver nuclear proteins (Goodwin & Johns, 1973) were separated by electrophoresis in 15% polyacrylamide–SDS slab gels and transferred (Towbin, 1979) to DBM paper (Schleicher & Schuell). Parallel strips from the DBM blot were cut and incubated with the ^{125}I-labeled goat anti-HMG-T antibody to detect the location of the HMG-T. A narrow strip containing the protein band was then cut from the filter, incubated with the anti-HMG-T antisera, and washed to remove nonspecifically bound proteins, and the retained anti-HMG-T antibody was eluted with 1 mL of 6 M Gdn·HCl for 5 min at room temperature. The extraction was repeated once more, and the combined extracts were dialyzed against 0.15 M NaCl and 60 mM Tris-HCl, pH 7.5, to reconstitute the antibody.

Agarose Gels and Hybridizations. DNA was electrophoresed in 1.2% agarose gels buffered with 0.04 M Tris–acetate, pH 8, and 2 mM EDTA. For transfer of DNA to GeneScreen filters (New England Nuclear), gels were incubated for 2 h in three changes of 500 mL in 50 mM boric acid, 5 mM sodium borate, and 10 mM sodium sulfate, pH 7.3, and then for two additional hours in the same buffer containing 10 mM methyl mercuric hydroxide. The transfer was done by electroblotting at 200 mA for 18 h, in phosphate buffer, pH 6.5.

DNA blots were air-dried, baked for 2 h at 80 °C, and prehybridized at 70 °C for at least 3 h in 0.2% PVP-10, 0.2% BSA, 0.2% Ficoll 400, 0.05 M Tris, pH 7.5, 1 M NaCl, 2 mM EDTA, 0.1% SDS, 10% dextran sulfate (M_r 500 000, and 0.5 mg/mL yeast RNA. The filters were hybridized in the same solution at 70 °C for 18 h to cloned DNA probes labeled with ^{32}P by nick translation to a specific activity of 10^8 cpm/μg of DNA. Filters were washed at 70 °C for 20 min, twice each in (1×, 0.4×, 0.2×, and 0.1×) SET (0.15 M NaCl, 1 mM EDTA, and 30 mM Tris, pH 8.0) and 0.1% in SDS, and then exposed to Kodak XAR-5 film between two intensifying screens at −80 °C.

Protein Gels and Immunodetection of HMG-T in Filters. Fifteen percent polyacrylamide–SDS slab gels, 0.075 × 14 × 16 cm, were prepared according to the system of Laemmli (Laemmli, 1970). Electrophoresed proteins were transferred to nitrocellulose membranes (BA-85) or DBM paper (Schleicher & Schuell), by electroblotting according to the method of Towbin (Towbin, 1979). Blotted filters were incubated in 5% BSA, 0.15 mM NaCl, 60 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.1 mM PMSF (quenching solution), for a minimum of 3 h at 4 °C before addition of antibodies.

HMG-T in filters was detected by incubation for 18 h at 4 °C, with fresh quenching solution, containing (1–2) × 10^6 cpm/mL of goat anti-HMG-T antisera–33% ammonium sulfate precipitate fraction, labeled with ^{125}I to a specific activity of 10^6 cpm/μg of protein and washed as indicated below, before autoradiography. For detection of HMG-T using the double-antibody technique, filters were incubated for 18 h at 4 °C in fresh quenching solution containing anti-HMG-T antisera (0.1 μg/ml) and washed twice in 0.15 M NaCl, 60 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.1 mM PMSF, then once in the same buffer plus 0.05% NP-40, and finally once in the buffer without detergent. All washes were for 20–30 min at room temperature. The bound first antibody was detected by incubation of the filter for 18 h at 4 °C in a quenching solution containing (1–2) × 10^6 cpm/mL of rabbit anti-goat antibody, labeled with ^{125}I to a specific activity of 2 × 10^7 cpm/μg of protein, washed as above, and exposed for autoradiography.

FIGURE 2: Autoradiograph of a Western blot of acid-extracted trout liver nuclear proteins fractionated by electrophoresis in a 15% polyacrylamide–SDS gel and transferred to a nitrocellulose membrane. The HMG-T(1, 2) proteins were detected with a goat anti-HMG-T antibody labeled with ^{125}I. The arrow points to protein X.

Results

Goat Anti-HMG-T Antibody. Goat antisera against trout HMG-T had been prepared previously and shown to have no detectable binding against trout histones (Peters et al., 1979). To determine further whether the antisera would react against other HMG-like proteins, acid-extracted (Goodwin & Johns, 1973) trout liver nuclear proteins were electrophresed in 15% polyacrylamide–SDS gels, blotted to nitrocellulose membranes, and challenged with the ^{125}I-labeled goat anti-HMG-T antisera. It was observed (Figure 2) that two proteins, the major faster migrating one, trout HMG-T, and a second slowly migrating unknown protein (protein X), bound appreciable amounts of antibody. To establish whether the unknown protein X was detected due to the presence of a contaminating antibody or because both proteins share common antigenic sites, the anti-HMG-T antisera was affinity-purified by using electrophoretically fractionated HMG-T bound to a DBM paper support (see Materials and Methods). The affinity-purified and crude antibodies were then used to challenge different strips from the same Western blot of acid-extracted liver nuclear proteins and the bound antibodies detected with a ^{125}I-labeled rabbit anti-goat antibody. Figure 3 shows that both purified and unpurified antibodies produced very similar patterns when bound to the blotted proteins. Furthermore, the HMG-T affinity-purified antibody appeared to bind to protein X and to HMG-T in similar relative proportions as did the unpurified antisera, indicating that binding to the higher molecular weight protein X was due to the presence of antigenic sites common also to the HMG-T. It is not clear at the moment whether protein X is a higher molecular weight HMG protein or whether it represents an

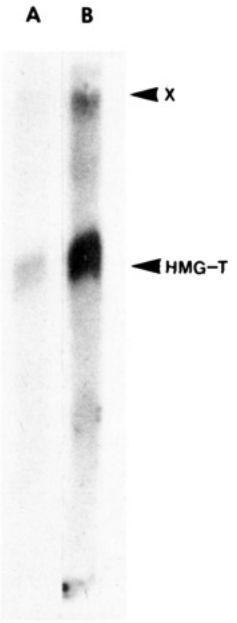

FIGURE 3: Autoradiograph of a Western blot from acid-extracted liver nuclear proteins fractionated in a 15% polyacrylamide–SDS gel and transferred to a DBM paper membrane. Two strips from the same blot were independently probed with the crude anti-HMG-T antisera (lane A) or with the affinity-purified anti-HMG-T antibody (lane B). Bound goat antibodies were detected with a ^{125}I-labeled rabbit anti-goat antibody. The arrows point to protein X and HMG-T.

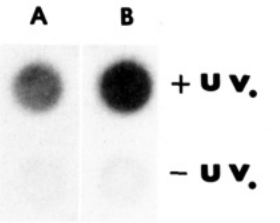

FIGURE 4: Detection of HMG-T cross-linked to DNA, in the water phase from phenol-extracted SDS lysates of nonirradiated (−UV) and irradiated (+UV) liver nuclei. Aliquots of the phenol-extracted water phases containing equivalent amounts of A_{260} units were spotted onto nitrocellulose filters and challenged with the crude goat anti-HMG-T antibody labeled with ^{125}I (panel A) or with the affinity-purified goat anti-HMG-T antibody followed by a ^{125}I-labeled rabbit anti-goat antibody (panel B).

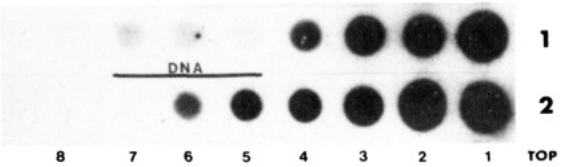

FIGURE 5: Autoradiographic detection of HMG-T in the fractions from CsCl gradients. Gdn·HCl lysates from nonirradiated (lane 1) and irradiated (lane 2) liver nuclei were centrifuged in step CsCl gradients. Aliquots from each gradient fraction containing the same volume were spotted on nitrocellulose filters after dilution of the Gdn·HCl and then challenged with the goat anti-HMG-T antibody followed by a ^{125}I-labeled rabbit antigoat antibody.

aggregate of HMG-T. In view of the presence of β-mercaptoethanol in the sample buffer, it is unlikely that protein X is a disulfide-linked dimer or higher order polymer of HMG-T.

Cross-Linking of HMG-T to DNA by Ultraviolet Light. To determine whether irradiation with ultraviolet light can cross-link the HMG-T protein to DNA in chromatin, trout liver nuclei were prepared and half of the nuclei irradiated with ultraviolet light of 254 nm as described. Irradiated and unirradiated nuclei were independently lysed in 1% SDS and repeatedly extracted with phenol until the unirradiated lysate showed no interphase. Irradiated samples consistently showed larger interphases, presumably consisting of proteins covalently cross-linked to DNA. Free nucleic acids were removed by precipitating the protein–SDS complexes in the water phase with 0.5 M KCl at 4 °C, and the SDS was removed from the pellets by repeated extractions with 95% ethanol. The resulting protein pellets were dissolved in a small volume of 6 M Gdn·HCl with sonication, diluted with Tris–saline buffer, and adjusted to the same absorbance at 260 nm, and equal amounts of each were spotted on to nitrocellulose filters. HMG-T was detected in the filters by binding either to goat anti-HMG-T antisera labeled with ^{125}I or by binding to affinity-purified anti-HMG-T antibody followed, after washing the filter, with a ^{125}I-labeled rabbit anti-goat antibody. As shown in Figure 4, irradiation of liver nuclei with ultraviolet light significantly reduces the extractability by phenol of the HMG-T from the SDS lysate, in contrast with the behavior of the unirradiated proteins which are almost entirely removed by the phenol. This result, which is in agreement with the observations of others (Smith, 1962), strongly indicates that the HMG-T was covalently attached to nucleic acids by the ultraviolet light and therefore rendered insoluble in phenol. Since it was possible that the enrichment of the HMG-T in the aqueous phase of the phenol extracts could be due to entrapment of HMG-T by heterologous protein–DNA aggregates and to show that a different approach would produce an equivalent result, irradiated and unirradiated trout liver nuclei were lysed in 6 M Gdn·HCl and centrifuged in CsCl gradients, made in 6 M Gdn·HCl. The gradients were fractionated from the bottom of the tubes, to avoid contamination by free proteins at the top of the gradient, and aliquots of the individual fractions were spotted onto nitrocellulose filters after dilution of the guanidine. HMG-T on the filters was detected with the anti-HMG-T antibody followed by the ^{125}I-labeled rabbit anti-goat antibody. Comparison of the gradient fractions from irradiated and unirradiated nuclei showed (Figure 5) that, after irradiation, larger quantities of HMG-T could be found in association with the DNA-containing fractions in the lower regions of the gradients, from which free proteins are normally excluded. This result again supports the previous conclusion that 254-nm ultraviolet light covalently cross-links the HMG-T to nucleic acids. From similar experiments it was calculated (data not shown) that approximately 1% of the total HMG-T is cross-linked to DNA by the irradiation.

Recovery of DNA Cross-Linked to HMG-T. Since irradiation of liver nuclei with 254-nm ultraviolet light covalently cross-links HMG-T to DNA, it was necessary to develop a procedure, using the antibody to HMG-T, by which those DNA fragments attached to the protein could be rescued and separated from the bulk of the DNA. Trout liver nuclei were irradiated and dispersed in 6 M Gdn·HCl and those proteins cross-linked to DNA separated from free proteins by centrifugation of the lysates through CsCl step density gradients. The regions of the gradients containing DNA were collected, dialyzed to remove excess salt, and then divided into two equal aliquots. One aliquot was incubated with protein A beads that had been saturated first with rabbit anti-goat antibody and then with goat anti-HMG-T antibody. The second aliquot was incubated with the same amount of protein A beads saturated with the rabbit anti-goat antibody only, to serve as a control for any nonspecific retention of DNA by the antibody beads. After the incubation with irradiated DNA, the beads were harvested and washed, equilibrated with the restriction nuclease buffer of choice (from which β-mercaptoethanol was omitted), and digested with restriction endonucleases to gen-

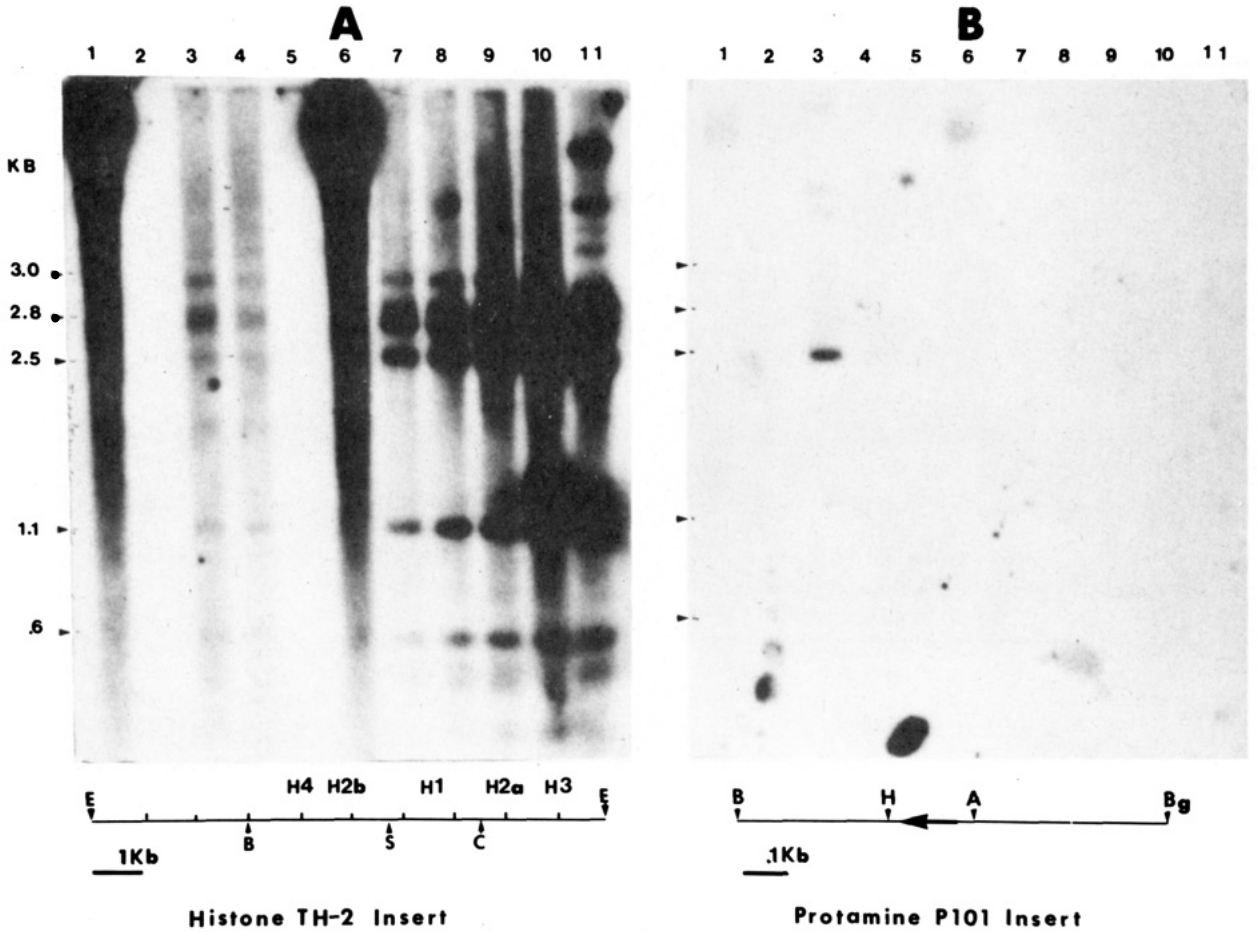

FIGURE 6: DNA obtained with the anti-HMG-T antibody beads (lane 3) and control beads (lane 4) was digested with restriction nucleases *Eco*RI, *Bam*HI, *Sac*I, and *Cla*I. After the DNA fragments released by the digestion were washed off and the DNA retained by the beads was eluted by proteinase K digestion and electrophoresed in 1.2% agarose gels, in parallel with total trout DNA (lanes 7–10), and the λ charon 4A histone clone DNA TH-2 (Connor et al., 1984) (lane 11), all DNA samples were subjected to the same digestion as the bead retained DNA. Lanes 1 and 6 are phage λ DNA *Hin*dIII digests and lanes 2 and 5 are PM-2 DNA digested with *Hae*III, as size markers. Southern blots of the gels were hybridized to the trout histone cluster DNA clone λTH-2 (Connor et al., 1984) labeled with ^{32}P and autoradiographed (panel A). After the exposure, the probe was removed and the filter hybridized to the protamine DNA clone pJP22 (Jankowski and Dixon, 1984) labeled with ^{32}P and autoradiographed (panel B).

erate DNA fragments of discrete sizes. The beads were washed again after the digestion to remove released DNA fragments not bound to HMG-T, and the retained HMG-T-bound DNA was eluted from the beads by digestion of all proteins, including HMG-T and the antibody with proteinase K in SDS. The DNA restriction fragments obtained from the beads, now free of proteins, were electrophoresed in 1.2% agarose–Tris–acetate–EDTA gels, transferred to a GeneScreen membrane and hybridized to cloned DNA probes labeled with ^{32}P, to determine whether there was any enrichment of specific DNA sequences. Figure 6A shows the results of such an experiment in which the DNA retained by the Sepharose beads was digested with the restriction enzymes *Eco*RI, *Bam*HI, *Sac*I, and *Cla*I, a digestion designed to generate a range of DNA fragments of suitable sizes from the known histone gene cluster of trout (Connor et al., 1984). The DNA blot was then probed with the ^{32}P-labeled histone cluster DNA clone λTH-2, containing the five histone genes. A comparison of lanes 3 and 4 shows that this procedure resulted in no appreciable enrichment of the histone gene containing DNA by the antibody, since the histone gene restriction fragments of 3.0, 2.8, 2.5, 1.1, and 0.6 kilobases were of equal intensity both before and after selection of the HMG-T-bound DNA by the antibody technique. However, after removal of the histone probe a second hybridization, this time to the ^{32}P-labeled DNA of the trout protamine clone pJP-22 (Jankowski & Dixon, 1984) showed (Figure 6B) that DNA fragments of 2.5, 5, and 7 kilobases containing the protamine gene had been highly enriched by the specific antibody beads, lane 3, as compared to control beads, lane 4. The filter was stripped of the protamine probe and shown by rehybridization to the histone probe to have suffered no detectable selective loss of DNA during the hybridizations (data not shown).

To determine which of the known protamine genes (Aiken et al., 1983) might have been enriched by this procedure and are, therefore, in proximity to a binding site of the HMG-T protein, DNA from irradiated nuclei was prepared by the antibody-enrichment procedure described above. The resulting DNA retained by the beads was digested with the restriction enzymes *Eco*RI and *Bam*HI, electrophoresed in agarose gels, transferred to a GeneScreen membrane, and probed with either the ^{32}P-labeled 920 base pair *Bam*HI–*Bgl*II DNA fragment, of the protamine clone pJP-22 (Jankowski & Dixon, 1984), containing several hundred bases of DNA flanking the protamine coding region or, after removal of this probe, with the 178 base pair *Ava*II–*Msp*I fragment of the same clone, which contains almost solely the protamine coding region (States et al., 1982). Figure 7 shows that only a subset of the detectable DNA sequences containing the protamine gene in total trout DNA is enriched by the antibody. A 3.5-kilobase DNA fragment that corresponds in size and restriction sites with a fragment from the protamine clone TP-15 (Aiken et al., 1983)

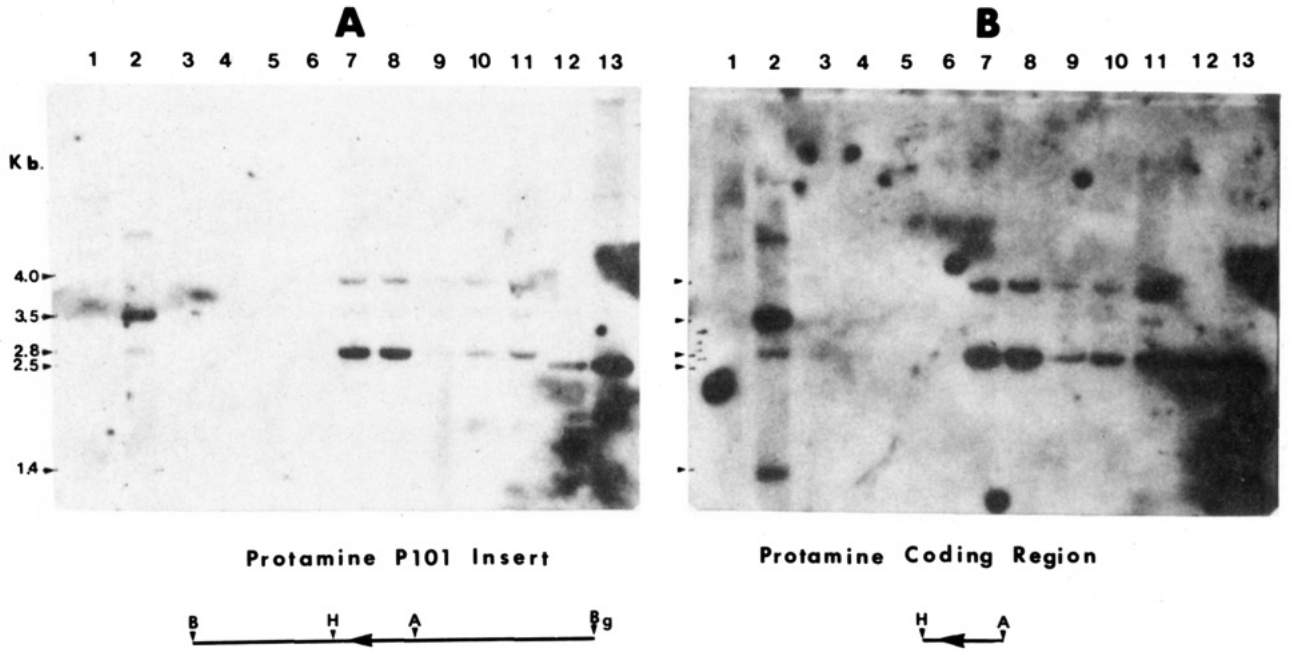

FIGURE 7: DNA obtained with the anti-HMG-T beads (lane 2) and control beads (lane 1) was digested with the restriction enzymes *Eco*RI and *Bam*HI. The DNA restriction fragments retained by the beads were eluted and electrophoresed in 1.2% agarose gels in parallel with nonirradiated total trout DNA (lanes 9–11) and 30-min-irradiated (lane 7) or 60-min-irradiated (lane 8) trout DNA subject to the same digestions as DNA retained by the beads. Lanes 12 and 13 are protamine clone TP-101 DNA digested with *Eco*RI and *Bam*HI. lanes 5 and 6 are phage λ DNA *Hin*dIII digested and PM-2 digested with *Hae*III as size markers. In panel A, the filter was hybridized to the ^{32}P-labeled *Bgl*II–*Bam*HI 920 base pair fragment of protamine clone pJP-22, and in panel B, the filter was hybridized to the ^{32}P-labeled *Ava*II–*Msp*I 178 base pair fragment of the same clone. Restriction maps of the relevant probes are given below each panel.

is the most enriched fragment of DNA containing the protamine gene. Two other DNA fragments containing this gene were also enriched by the procedure but cannot so far be assigned with certainty to any of the known fragments from the protamine clones. In Figure 7B, where the short 178 base pair *Ava*II–*Msp*I fragment, which contains essentially only the coding region of protamine, is used, enrichment is still very clear, indicating that it must be due to the protamine sequence itself being enriched rather than a flanking sequence.

Discussion

Due to the high complexity of the eukaryotic genome, the large number of proteins that constitute chromosomes, and the lability of the protein–DNA associations of functional chromatin, little progress has been made in understanding how such protein–DNA interactions modulate the expression of genes. To make further progress in this area of study, it would be valuable to have procedures to map the binding of specific nuclear proteins to the specific DNA sequences that they occupy particularly during different stages in the development of an organism.

We described here a novel and general strategy for the isolation and detection of the DNA with which nuclear proteins associate in the minimally perturbed chromosomes of intact nuclei. This method takes advantage of the formation of covalent bonds between proteins and neighboring nucleic acids when irradiated with 254-nm ultraviolet light, to stabilize protein–DNA associations, and has the ability to retrieve specific protein–DNA adducts with the aid of specific antinuclear protein antibodies bound to a solid support.

Irradiation with ultraviolet light was chosen as the means of cross-linking over other agents because of the minimal perturbation to the system that it requires (intact cells can also be used). Irradiation with ultraviolet light of mixtures of DNA and proteins results in the formation of covalent adducts of both types of macromolecules (Smith, 1962), and several amino acids have been found to participate in the cross-linking to purines and pyrimidines (Smith, 1969; Smith & Meuns, 1969). Furthermore, the photo-cross-linking process has been shown to take place at the sites of protein and DNA contact in several model systems. RNase was found to be cross-linked by ultraviolet light to pyrimidine dinucleotides at the enzyme binding site (Havron, 1977; Sperling & Havron, 1977), the *lac* repressor protein was cross-linked to the *lac* operator DNA (Lin & Riggs, 1974; Becker & Wang, 1984), and RNA polymerase could also be cross-linked to the *lac* promoter DNA (Simpson, 1979).

In the course of this work we have become aware of some potential handicaps associated with the use of ultraviolet light (unpublished results), mainly that excessive irradiation can result in the formation of interstrand cross-links that by allowing the rapid renaturation of the DNA strands reduces the efficiency of the DNA transfer between gels and hybridization membranes. This problem was minimized by carefully controlling the dose of light and by the use of methyl mercuric hydroxide as the denaturing agent before the transfer of DNA to membranes.

It should be emphasized that the polyclonal anti-HMG-T antibody used in this work is also capable of recognizing a high molecular weight protein (X) containing antigenic sites common to those present in the HMG-T. Therefore, the possibility that protein X associates with some of the protamine genes and might be responsible for the observed enrichment cannot so far be excluded. However, it has been observed (G. H. Goodwin, personal communication) that a similar higher molecular weight aggregate of calf thymus HMG-1 appears during the purification and workup of HMG-1.

The use of the anti-HMG-T antibody for the retrieval of DNA cross-linked to HMG-T (and/or protein X) has yielded DNA fragments that are highly enriched with certain protamine gene sequences, but not with the trout vitellogenin genes (data not shown) or the trout histone genes. Although hybridization with the protamine *Bgl*II–*Bam*HI fragment and the protamine coding sequence *Ava*II–*Msp*I fragments (Figure

7AB) does not constitute an absolute proof, it is indeed strong evidence for the presence of the protamine gene in the antibody-enriched DNA. These results show that by the application of this procedure it is possible to localize or "map" those DNA regions with which the HMG-T interacts. This procedure can probably be generalized to many of the nuclear proteins for which antibodies can be obtained. Estimates of the trout protamine copy number done in the past (Levy-Wilson & Dixon, 1977; Sakai et al., 1978) indicate the presence of four to eight copies per somatic cell. As judged from the restriction nuclease analysis of several trout protamine clones from this laboratory (Aiken et al., 1983), it was apparent that the protamine genes are present in the trout in at least two different flanking DNA environments that can be differentiated by their pattern of digestion with restriction nucleases. By taking advantage of this circumstance to better define which members of the protamine gene family are enriched by our procedure, and therefore presumably associated with the HMG-T protein in chromosomes, we have found the most enriched DNA fragment containing the protamine gene to correspond in size (3.5 kilobases) and restriction sites (*Bam*HI fragment) to that of protamine clone TP-15 (Aiken et al., 1983). The two other enriched DNA fragments of 2.7 and 1.5 kilobases, respectively, have not been clearly assigned to any of the known protamine clones.

It appears from our results that one of the binding sites for HMG-T in chromatin is within 3.5-, 2.7-, and 1.5-kilobase DNA fragments containing the protamine gene. Since not all the known arrangements of this gene have been found represented in the antibody-selected fraction and the relative abundance of the enriched ones is not paralleled by their relative frequency in the trout genome, it is tempting to conclude that some other members of the protamine gene family must reside in a different chromatin environment lacking the HMG-T protein. The association of HMG-T with some but not all protamine genes in liver where, as judged by our repeated failure to detect liver RNA transcripts that would hybridize with the protamine probe (data not shown), no protamine genes are expressed, and the apparent absence of the protein from the trout histone and vitellogenin genes which are expressed in trout liver all point to HMG-T as a protein that can be associated with nonexpressed regions of chromatin.

Previous results using limited micrococcal nuclease digestion of trout testis chromatin indicated that HMG-T was readily released in the early stages of digestion and appeared to be associated with the linker regions of transcribed chromatin domains (Levy-Wilson et al., 1979; Levy-Wilson & Dixon, 1979; Peters et al., 1979). However, later work indicated that HMG-T was present in two distinct subfractions of trout testis chromatin, one fraction, as shown previously, easily digested by nuclease and enriched in transcribed sequences and a second, much more resistant to nuclease, not enriched in transcribed sequences (Kuehl et al., 1980).

An accurate estimate of the degree of enrichment for the protamine gene bands from densitometer scans of the autoradiograms cannot be made at this time. However, it is at least 30-fold. Considering the observed relative intensity of the protamine gene bands in the autoradiograms of enriched and background samples and the low frequency (four to eight copies per genome) of these genes in trout, it seems reasonable to expect that with this method the study of protein–DNA interactions at the level of single copy genes should be possible.

The limits of resolution with which a given protein can be localized within a DNA sequence are determined in the experimental procedure described here by the availability of adequate restriction sites in the vicinity of the protein binding site. However, much higher resolution should be obtained by combination of our method with existing techniques for indirect end labeling and footprinting.

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Conformations of the Core Nucleosome: Effects of Ionic Strength and High Mobility Group Protein 14 and 17 Binding on the Fluorescence Emission and Polarization of Dansylated Methionine-84 of Histone H4†

Dae G. Chung and Peter N. Lewis*
Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8
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ABSTRACT: Chicken histone H4 labeled at Met-84 with the fluor *N*-[(acetylamino)ethyl]-8-naphthylamine-1-sulfonic acid has been incorporated into a nucleosome which has physical characteristics virtually identical with those of native core nucleosomes. The fluorescence emission and polarization properties of the labeled nucleosome were measured as a function of ionic strength and the binding of high mobility group (HMG) proteins 14 and 17. Also, the accessibility of the fluor to the quenching agent acrylamide was determined. It was found that the fluorescence emission changes in the range 0.1–1000 mM NaCl are rather small and indicate that no major unfolding of the octamer structure occurs around Met-84 on H4 at least. Five or perhaps six discrete states were found in that ionic strength range. Each has a different accessibility to the quenching agent. The range of accessibilities varied from 9×10^{-7} to 32×10^{-7} mol^{-1} s^{-1} for 0.1–1000 mM NaCl, respectively. Polarization measurements showed that there was little change in the rotational relaxation lifetime of the fluor at ionic strengths less than 50 mM NaCl. Above this value, the rotational relaxation lifetimes decreased from 107 to 25 ns at 600 mM NaCl, indicating a moderately increased rotational freedom for the fluor. It is suggested that the histone octamer changes its degree of compaction in the range 0.1–600 mM NaCl but that no major protein unfolding occurs. The binding of HMG 14/17 at low ionic strength (10 mM) is noncooperative and appears to increase the compaction of the histone octamer, while at physiological ionic strengths (100 mM) the binding is cooperative and the octamer becomes less compact as reflected by properties of the fluor bound to Met-84 on H4.

The functions of nucleosomes in chromatin, aside from the compaction of DNA, are still indeterminate. While the chromatin of all eukaryotes examined to date contains this ubiquitous subunit, there is much evidence for heterogeneity in nucleosome composition and structure. Nucleosomal DNA from transcriptionally competent chromatin is hypersensitive to digestion by DNase I and other nucleases (Weisbrod, 1982a,b). Histone posttranslational modifications and DNA base methylation further add to the complexity. These differences suggest that nucleosomes might be able to adopt different conformations during transcription. Whether these alternate states are characterized by unwinding of the DNA supercoil from the histone octamer, by unfolding of the histone octamer, or by some combination of both is uncertain.

Many investigators have addressed this question by examining the conformation of core nucleosomes in solutions of different ionic strength and when proteins such as H1 and HMG 14/17 are bound (McGhee & Felsenfeld, 1980; Wilhelm & Wilhelm, 1980; Burch & Martinson, 1981; Dieterich & Cantor, 1981; Daban & Cantor, 1982; Yau et al., 1983; Yager & van Holde, 1984; Ausio et al., 1984). In spite of intensive study, there is still disagreement as to whether or not the nucleosomal histone octamer experiences major unfolding at low (<1 mM) or high (>300 mM) ionic strengths. For example, Uberbacher et al. (1983) found from neutron scattering that only small conformational changes occur in the histone octamer when nucleosomes are transferred to a low ionic strength buffer. In contrast, Cantor and co-workers report that the histone core in a nucleosome becomes flexible at low ionic strength on the basis of the emission of fluors attached to Cys-110 of histone H3 (Dieterich et al., 1977, 1979). For higher ionic strengths in the range 10–600 mM, Ausio et al. (1984), on the basis of sedimentation measurements and cross-linking, found no large structural changes in the nucleosome. On the other hand, Dieterich et al. (1977, 1979) and Daban & Cantor (1982) report that major unfolding occurs by 600 mM NaCl as reflected in changes in the spectral properties of a fluor bound at the same H3 site mentioned above.

Since it has been reported that nucleosomes containing H3 modified at Cys-110 are measurably less stable than unmod-

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