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Detection of Two Chromatin Proteins Which Bind Specifically to the 5′-Flanking Region of the Rat Prolactin Gene

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We employed a protein gel blotting procedure to search for nuclear proteins from rat pituitary cells that bind preferentially to the 5′-flanking region of the rat prolactin gene. By incubating gel blots of chromatin proteins from GH_3 rat pituitary tumor cells with a ^{32}P-labeled prolactin genomic clone, we detected two major binding proteins with molecular weights of approximately 44,000 and 48,000, designated NP44 and NP48, respectively. Both NP44 and NP48 are minor chromatin proteins which are extracted at low salt concentrations (0.4 M NaCl) and exhibit a range of slightly acidic isoelectric variants. NP44 and NP48 were detected at similar levels in chromatin extracts of GH_3 cells, the prolactin-negative GC cell variant of the GH_3 cells, and normal rat pituitary tissue. Considerably lower levels of these two proteins were found in chromatin extracts from rat liver and rat C_6 glial cells. NP44 and NP48 exhibit DNA sequence specificity, as evidenced by their strong binding to the upstream flanking region of the prolactin gene, but only very weak binding to plasmid DNA, rat prolactin or growth hormone cDNAs, or upstream flanking regions of two other rat genes. By analyzing subclones of a rat prolactin genomic clone, we established that NP44 and NP48 bind to at least two sites, which are located between 0.4 and 2.0 kilobases (region I) and between 2.0 and 4.8 kilobases (region II) upstream of the transcription initiation site. These findings are discussed in the context of a possible functional association between the strong binding of NP44 and NP48 to the prolactin 5′-flanking region and pituitary-specific expression of the prolactin gene.

The prolactin gene is subject to tissue-specific regulation, since it is expressed almost exclusively in the pituitary gland. In addition, expression of this gene in the GH_3 rat pituitary cell line or normal pituitary cells or both is regulated by a large number of factors (1). These include peptide (7, 24, 46), steroid (19, 31, 40, 47), and thyroid (20) hormones; Ca^{2+} (12, 45, 47); cyclic AMP (18); and phorbol esters (35). Such developmental or hormonal regulation involves complex interactions among chromatin and nuclear matrix proteins and between these proteins and specific DNA sequences. Much indirect and some direct evidence exists for a broad range of functions of nuclear proteins which regulate the dynamic structural characteristics of chromatin, ultimately leading to differential gene expression. Regulatory proteins could control gene expression via any of a number of mechanisms, including increases in promoter efficiency (38), initiation and maintenance of an altered chromatin structure (for a review, see reference 5), nucleosomal structure (30, 43) or phasing (34), or chromatin-nuclear matrix interaction (3).

An understanding of specific protein-DNA interactions should help to clarify the mechanism of action of gene regulatory proteins. There are basically two possible approaches to such an analysis in eucaryotes. When a DNA-binding protein such as the glucocorticoid receptor has been purified, it can be employed as a probe to determine the specific DNA sequence(s) to which it binds (29, 33). However, in the case of a gene such as that for prolactin, in which there is no prior knowledge of the chromatin proteins which bind to it specifically, it would be useful to have available a technique for employing a genomic clone as a probe to search for and characterize such proteins. The protein blotting technique developed by Bowen et al. (4) combines the resolving power of gel electrophoresis with a DNA binding assay. Hence it should be possible by this technique to begin with no prior knowledge of specific protein-DNA interactions and obtain information about both DNA sequences which bind specific chromatin proteins and the physical properties of the proteins themselves. Following the report of Bowen et al. (4), protein blotting was employed to detect and partially characterize proteins binding to a *Drosophila* heat shock gene (14) and to the mouse β-globin gene (37).

Nuclear proteins which might be involved in regulation of prolactin gene expression have been detected previously. These include a phosphorylated GH cell chromatin protein designated BRP (24) and calmodulin-binding proteins in the nuclear matrix of GH cells (B. A. White, manuscript in preparation). However, no chromatin proteins which interact specifically with the prolactin gene have been identified previously. In the study reported here, we employed the protein blotting technique of Bowen et al. (4) to detect two GH_3 cell chromatin proteins (NP44 and NP48). Each of these two proteins, which are present at high levels in pituitary cells but not in liver or glial cells, binds specifically to two regions upstream of the rat prolactin gene. These observations suggest that NP44 and NP48 may be involved in tissue-specific regulation of prolactin gene expression.

MATERIALS AND METHODS

Preparation of chromatin fractions. GH_3 and GC cells were grown in suspension culture as described previously (50). Typically, 5×10^8 cells were pelleted, washed once in phosphate-buffered saline, and suspended in 9 ml (final volume) of 10 mM Tris hydrochloride (pH 7.4)–15 mM

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KCl–1.5 mM $MgCl_2$. The suspended cells were split into two 4.5-ml aliquots and lysed by the addition of 500 μl of 5% Nonidet P-40 (NP-40) and incubation on ice for 5 min. The cell lysates were centrifuged for 5 min at 1,000 × *g*, and the centrifugates (crude nuclear pellets) were washed twice in 5 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.5)–0.5 mM spermidine–1 mM EDTA–0.25 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid–5 mM NaCl–2 mM dithiothreitol–10% glycerol (NW buffer). Washed nuclei were suspended in 5 ml of NW buffer containing 0.1% Triton X-100, layered over 5 ml of NW buffer containing 30% sucrose, and centrifuged for 20 min at 10,000 × *g* in a Sorvall HB-4 rotor. The nuclear pellets were lysed in 1 ml of 10 mM Tris hydrochloride (pH 7.5)–0.2 mM EDTA–0.075 M NaCl–0.1% Sarkosyl by homogenization in a 2-ml glass-glass homogenizer and centrifuged for 3 min at 7,000 × *g* in a Beckman microfuge 12. The chromatin pellet was serially extracted by homogenization in 5 mM sodium phosphate (pH 7.0) containing increasing concentrations of NaCl in the range of 0.2 to 0.6 M, with each extraction followed by centrifugation as described above. The protein fraction most frequently used in this report is referred to as the 0.2 to 0.4 M NaCl chromatin extract. This fraction is a 0.4 M NaCl extract of a chromatin pellet that was previously extracted with 0.2 M NaCl. The post-0.6 M NaCl chromatin pellet was suspended in 500 μl of 50 mM Tris hydrochloride (pH 7.4)–5 mM $MgCl_2$ and digested with 5 μg of DNase I at 37°C for 15 min. Fractionation of C_6 glial cells was performed as described above. The procedure for fractionation of rat tissues (Sprague-Dawley, retired female breeders) was as described above, except that tissue lysis was done by carrying out Dounce homogenization with a B pestle in the presence of 0.5% NP-40. All reagents contained 0.2 mM paramethylsulfonyl fluoride as a protease inhibitor.

Further fractionation of the 0.2 to 0.4M NaCl chromatin extract proteins was achieved by column chromatography. The extract was digested with 10 μg each of DNase I and RNase A per ml at 37°C for 15 min and loaded onto a Sephadex G-75 column (1.8 by 23 cm), and 1-ml fractions were collected. The void volume and the expected elution volume of NP44 and NP48 were determined with blue dextran and ovalbumin (molecular weight, 45,000), respectively. Fractions of 30 μl from the 1-ml fractions were used for gel electrophoresis and gel blotting.

Preparation of prolactin genomic clones. Clone pPRL-G_1 (see below) is a subclone of λPRL-1, which was generously supplied by David Steffen (Worcester Foundation for Experimental Biology). λPRL-1 was isolated by employing rat prolactin cDNA clone pPRL-1 (22) to screen a library of complete *Eco*RI fragments of Fisher rat lymphoma DNA cloned into bacteriophage Charon 4A. Restriction enzyme analysis of the insert in λPRL-1 showed that it contains an 11-kilobase (kb) *Eco*RI-*Eco*RI fragment of prolactin genomic DNA. The hybrid plasmids employed were constructed as follows. For pPRL-G_1, a 10-kb *Bam*HI-*Eco*RI fragment of the insert in λPRL-1 was cloned into the multiple cloning region of plasmid pUC8 (39). The inserts in the following clones were all derived from the insert in plasmid pPRL-G_1 and were cloned (unless indicated otherwise) into the multiple cloning site of plasmid pUC9. For clone a, a 5.2-kb fragment was excised by complete *Bam*HI plus partial *Pst*I digestion and cloned into pUC8. Clone b contains a 1.55-kb *Bam*HI-*Pst*I fragment. Clone c contains two discontinuous fragments, a 5'-flanking 3.4-kb *Pst*I-*Pst*I fragment, and an intragenic 0.5-kb *Pst*I-*Eco*RI fragment. Clone d contains a 2.0-kb *Pst*I-*Pst*I fragment. Clone e contains a 0.86-kb *Pst*I-*Hin*cII fragment. For clone f, pPRL-G_1 was cut with *Hin*dIII, followed by religation in an increased volume. Clone g contains a 2.8-kb *Pst*I-*Pst*I fragment.

^{32}P labeling of DNA. Plasmid DNA probes were labeled with [^{32}P]dCTP by nick translation, as described previously (17), to a specific activity of 1×10^8 to 2×10^8 cpm/μg and purified from unincorporated nucleotides by Sephadex G-50 chromatography.

Protein blotting and protein-DNA incubation. Protein fractions were resolved either on one-dimensional sodium dodecyl sulfate (SDS) gels (16) containing a linear 7 to 15% polyacrylamide gradient or by two-dimensional gel electrophoresis as described previously (28) with a 3 to 10 ampholyte range and electrophoresis for 1,600 V · h in the first dimension. Proteins were then electrophoretically transferred from the gel to nitrocellulose at 80 V for 2 h with a previously described buffer system (36). Other transfer conditions were exactly as described previously (41). Protein blots were preincubated overnight at 37°C in 5 to 20 ml of 10 mM Tris hydrochloride (pH 7.4)–0.1 mM EDTA–50 mM NaCl (DPB buffer), containing 10 μg of denatured, sheared salmon sperm DNA per ml and 1% bovine serum albumin, in a Seal-a-Meal freezer bag. The preincubation buffer was removed, and an equal volume of DPB buffer containing salmon sperm DNA, but without bovine serum albumin, plus double-stranded ^{32}P-labeled DNA (2×10^6 to 5×10^6 cpm) was added, followed by incubation for 1 h at 37°C. On some occasions it was convenient to incubate blots longer (e.g., overnight), but binding was essentially complete by 30 min. The blots were then washed in DPB buffer containing 0.05% NP-40 (usually several washes in 200 ml at room temperature with shaking), blotted on Kim-Wipes, and exposed to XAR-5 (Kodak) film at −80°C with an intensifying screen.

RESULTS

Detection of two prolactin DNA-binding proteins by protein blotting. The prolactin genomic clones employed in these studies are shown in Fig. 1.

Chromatin prepared from lysed rat pituitary GH_3 cell nuclei was serially extracted with increasing NaCl concentrations, and the extracted proteins were analyzed by SDS-polyacrylamide gel electrophoresis. It is evident from a stained gel (Fig. 2A) that each fraction contained numerous proteins and that most of the H1 variants and core histones (Fig. 2A, regions marked 1 and 2, respectively) were extracted primarily at NaCl concentrations of 0.6 M or higher. After transfer of the proteins to nitrocellulose and incubation with a ^{32}P-labeled rat prolactin genomic probe (pPRL-G_1), several protein bands displayed readily detectable DNA binding (Fig. 2B). Results of many replicate experiments showed that the extent of binding by most proteins varied considerably (see below). In contrast, strong binding to prolactin genomic sequences was exhibited consistently by two proteins (NP44 and NP48) approximately 44 and 48 kilodaltons (kDa), respectively, which are eluted from chromatin between 0.2 and 0.4 M NaCl (Fig. 2B, arrows). The 0.2 to 0.4 M NaCl chromatin fraction therefore was employed for all subsequent experiments. The strong binding exhibited by NP44 and NP48 was not simply due to their presence at high levels in chromatin, as appears to be the case for the histones, which are abundant nuclear DNA-binding proteins. In fact, comparison of Fig. 2A and B (see also Fig. 8) indicates that neither NP44 nor NP48 is an abundant chromatin protein.

The ability to detect these proteins in chromatin prepared

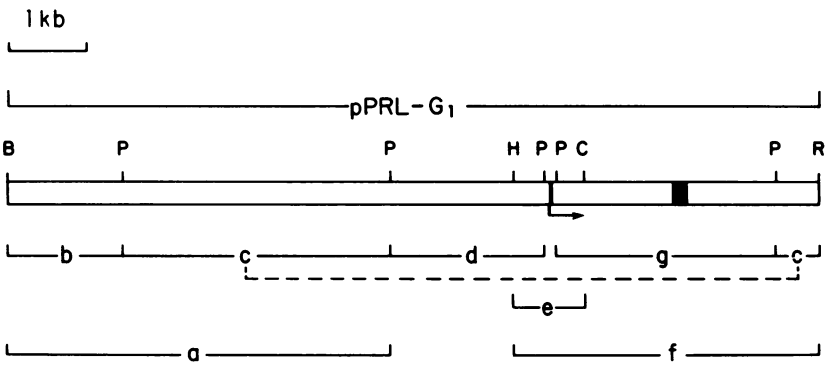

FIG. 1. Prolactin genomic clones employed for protein blotting experiments. The two shaded regions represent the first two exons of the rat prolactin gene, and the arrow shows the transcription start site. Clone c contains two discontinuous fragments (see text). The clones are shown schematically as linear DNA, but they were employed for protein blotting experiments in the form of circular hybrid plasmids. Abbreviations for the restriction map of the prolactin gene are as follows: B, *Bam*HI; P, *Pst*I; H, *Hin*dIII; C, *Hin*cII; R, *Eco*RI.

from various tissues and cell types was examined (Fig. 3). As before, the proteins were readily detected in GH_3 cell chromatin (Fig. 3, lanes 1, 4, and 8). Interestingly, they were also detected at high levels in chromatin from GC cells (Fig. 3, lane 2), a variant of the GH_3 cells containing undetectable levels of prolactin mRNA (1, 7). NP44 and NP48 are also detectable in normal pituitary chromatin (Fig. 3, lane 3) at about the same levels as in GH_3 cells. By contrast, in chromatin isolated either from rat liver (Fig. 3, lane 5) or rat glioma (C_6) cells (Fig. 3, lane 7), NP44 and NP48 were present at considerably lower levels. The observation that both proteins were readily detectable in chromatin prepared from a mixture of liver and GH_3 cells (Fig. 3, lane 6) shows that the low apparent levels of these proteins in liver was not due to a liver protease which degraded NP44 and NP48 during chromatin preparation. The absence of protease action during preparation of chromatin proteins from both liver and C_6 cells was also indicated by the presence of numerous large discrete protein bands in silver-stained gels of these proteins (data not shown). In preliminary experiments, we were unable to detect NP44 or NP48 in rat kidney chromatin preparations (data not shown), further demonstrating the tissue-specific distribution of these two proteins. However, our detection in preliminary experiments of sizable amounts of NP44 and NP48 in chromatin prepared from a rat osteosarcoma cell line (data not shown) indicates that the pituitary gland may not be the only rat tissue which contains high levels of these proteins.

NP44 and NP48 exhibit DNA sequence-specific binding. In addition to the reproducibility with which NP44 and NP48 appear as major binding proteins, they also exhibit preferential binding to prolactin genomic DNA relative to several other types of cloned DNA sequences. Figure 4 shows a comparison between the ability of a prolactin upstream genomic clone (clone d) and plasmid DNA to bind to chromatin proteins. Inspection of the bands in Fig. 4A and of

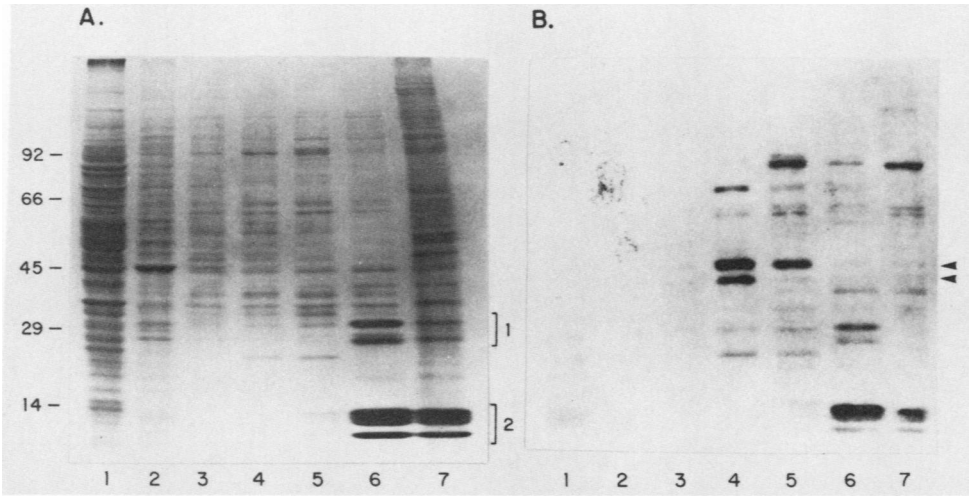

FIG. 2. Detection by protein blotting of two prolactin genomic DNA-binding proteins. (A) GH_3 cell fractions stained with Coomassie brilliant blue. Cells and chromatin were fractionated, and the proteins in each fraction were resolved on SDS gels as described in the text. The size markers (in kDa) are shown. Lane 1, NP-40 cell lysate supernatant; lane 2, Sarkosyl nuclear lysate supernatant; lanes 3 through 7, chromatin extracted at the following NaCl concentrations: 0.2 M (lane 3); 0.3 M (lane 4); 0.4 M (lane 5); 0.6 M (lane 6); post-0.6 M (lane 7). Regions 1 and 2 correspond to histone 1 variants and core histones, respectively. (B) Autoradiogram of binding of ^{32}P-labeled prolactin genomic hybrid plasmid pPRL-G_1 to a nitrocellulose blot of a duplicate of the protein gel shown in panel A. Arrows denote positions of NP44 and NP48 in lanes 4 and 5.

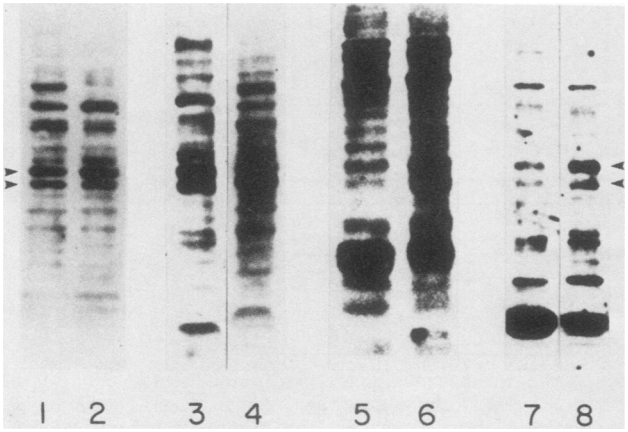

FIG. 3. Detection of NP44 and NP48 in various rat cell types. Equal amounts (30 μg of protein) of 0.2 to 0.4 M NaCl chromatin extracts from the indicated samples were transferred to nitrocellulose and incubated with ^{32}P-labeled pPRL-G_1. Results of four separate experiments are shown (lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8), each employing a separate GH_3 cell chromatin preparation. Lane 1, GH_3 cells; lane 2, GC cells; lane 3, pituitary gland cells; lane 4, GH_3 cells; lane 5, liver cells; lane 6, 1:2 mixture (by weight) of GH_3 cells and liver; lane 7, C_6 glial cells; lane 8, GH_3 cells. Arrows denote positions of NP44 and NP48.

the corresponding densitometric traces in Fig. 4B shows that plasmid DNA and clone d bound to some GH_3 cell chromatin proteins to a similar extent (e.g., proteins D and F). In contrast, NP44 and NP48 bound strongly to clone d but exhibited barely detectable binding to plasmid DNA.

The ability of these proteins to bind to several other types of DNA sequences was examined (Fig. 5). As before, both NP44 and NP48 bound strongly to prolactin gene-flanking DNA sequences. However, these proteins did not detectably bind to rat prolactin or growth hormone cDNA, plasmid DNA, or 5′-flanking sequences of the genes for rat whey acidic protein or β-casein. These results show that NP44 and NP48 bind to specific DNA sequence(s) that are present upstream of the rat prolactin gene but that are absent from other rat gene-flanking regions.

NP44 and NP48 bind to two sites upstream of the prolactin gene but to none within the first 3.3 kb of the gene body. The binding of these proteins to sequences upstream of and within the prolactin gene was investigated (Fig. 6). Both clones a and d bound to NP44 and NP48, demonstrating the existence of two upstream binding sites. The results with clones b and c localize the more upstream site to a region (region II) between 2.0 and 4.8 kb upstream of the transcription initiation site. The lack of binding of clones e and f localizes the proximal binding site to region I, between 0.4

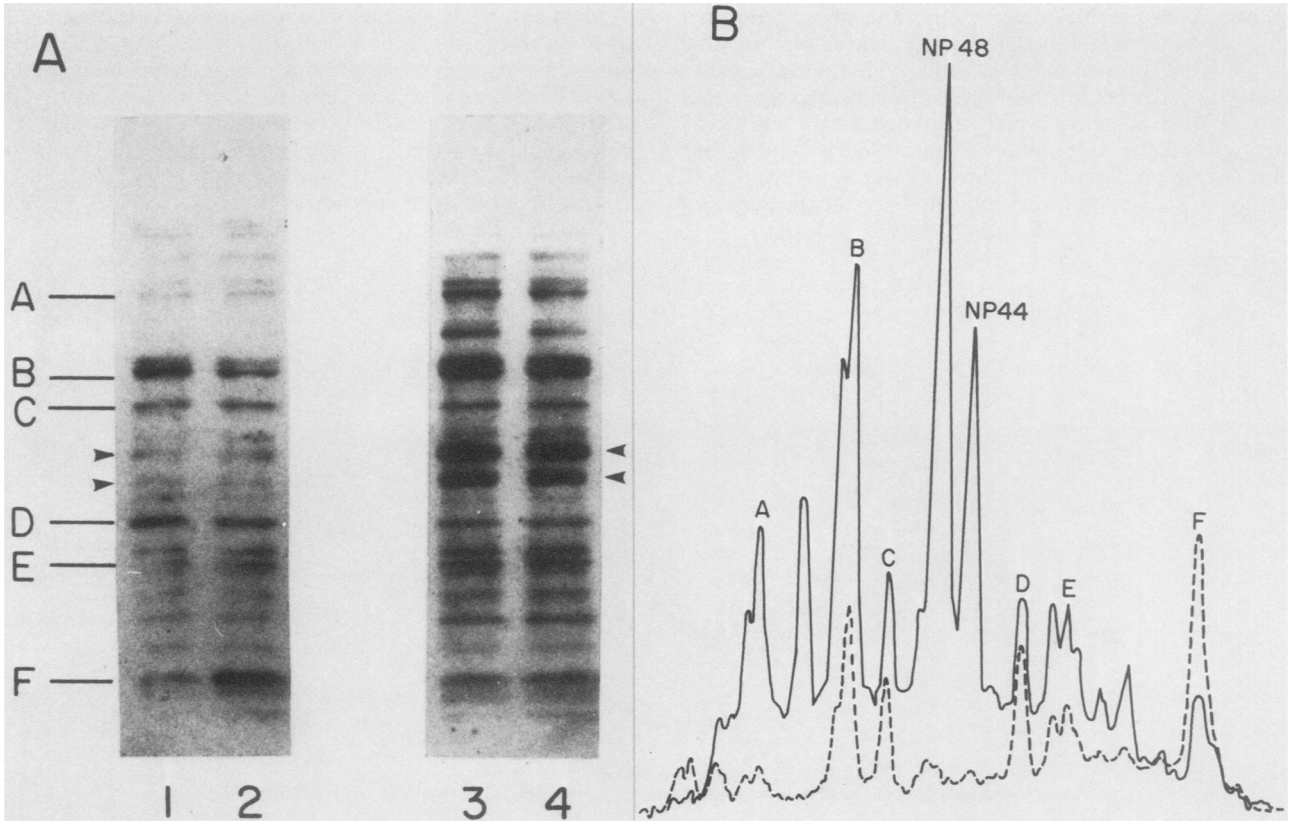

FIG. 4. DNA sequence binding specificity of NP44 and NP48. (A) Autoradiogram of binding of ^{32}P-labeled plasmid pBR322 (lanes 1 and 2) or clone d (lanes 3 and 4) to 0.2 to 0.4 M NaCl chromatin extact proteins. Four identical aliquots of this extract from GH_3 cells were resolved on the same gel and transferred to nitrocellulose. Two lanes were incubated with either 8×10^6 cpm of pBR322 or 4×10^6 cpm of clone d, labeled to essentially identical specific activities. A through F indicate reference proteins arbitrarily chosen for comparison and alignment of peaks on the densitometric scan. Arrows denote the positions of NP44 and NP48. (B) Densitometric scan of lane 2 (-----) and lane 4 (——) in panel A.

and 2.0 kb upstream of the initiation site. Finally, the lack of binding of clones e, f, and g implies that NP44 and NP48 do not bind to the first 3.3 kb of the prolactin gene body, which has a total length of about 10 kb (8, 21).

Further physical characterization of NP44 and NP48. From the results presented above (see Fig. 6), it can be seen that these polypeptides each consistently exhibited equally strong binding to prolactin DNA probes. This observation suggests the possibility that NP44 and NP48 exist in their native state as part of a multisubunit protein. However, the following results provide evidence against the existence of such a structure. NP44 and NP48 are apparently not held together by interchain disulfide bond(s), since they were still detected as separate polypeptides by protein blotting when mercaptoethanol was omitted from the gel sample buffer (data not shown). To examine further the native structure of these polypeptides, proteins in the 0.2 to 0.4 M NaCl chromatin fraction from GH_3 cells were fractionated under nondenaturing conditions by Sephadex G-75 column chromatography, and individual fractions were analyzed by protein blotting (Fig. 7). It can be seen that NP44 and NP48 were detected in the protein fractions corresponding to the 40- to 50-kDa range (Fig. 7, lanes 3 and 4), but not in the excluded protein fractions (Fig. 7, lanes 1 and 2). By contrast, a number of other DNA-binding proteins in this chromatin fraction were apparently aggregated, as evidenced by their presence in the excluded protein fractions.

The physical properties of NP44 and NP48 were further characterized by two-dimensional gel electrophoresis. Multiple variants of both proteins were reproducibly observed in the electrofocusing dimension, with isoelectric points in the range of 6.2 to 7.2 (Fig. 8B, arrows). None of these variants corresponded to a protein detectable by staining (Fig. 8A). Thus, NP44 and NP48 each appears to be composed of a series of nonabundant polypeptides that are equal in size but that possess different overall charges.

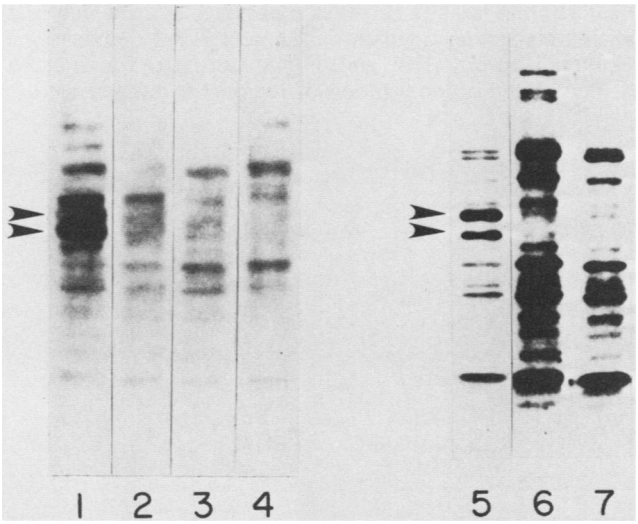

FIG. 5. Binding of NP44 and NP48 to DNA fragments of various genes. The same number of counts per minute of the indicated plasmid DNAs, labeled to essentially equivalent specific activities, was incubated with replicate strips of protein gel blots. The plasmids employed and their inserts were as follows: lane 1, pPRL-G_1, prolactin genomic DNA; lane 2, pPRL-1, rat prolactin cDNA (22); lane 3, pBR322-GH_1, rat growth hormone cDNA (9); lane 4, plasmid pUC9; lane 5, clone a, prolactin genomic DNA; lane 6, p5WAP1-2.4, rat whey acidic protein genomic DNA containing about 2 kb of immediate 5′-flanking DNA (6); lane 7, pCλβ-2.8, rat β-casein genomic DNA containing about 2 kb of immediate 5′-flanking DNA (15). Lanes 1 through 4 were from the same blot and were autoradiographically exposed for 16 h. Lanes 5 through 7 were from a second blot. Lane 5 was exposed for 16 h, and lanes 6 and 7 were exposed for 40 h. Arrows denote positions of NP44 and NP48.

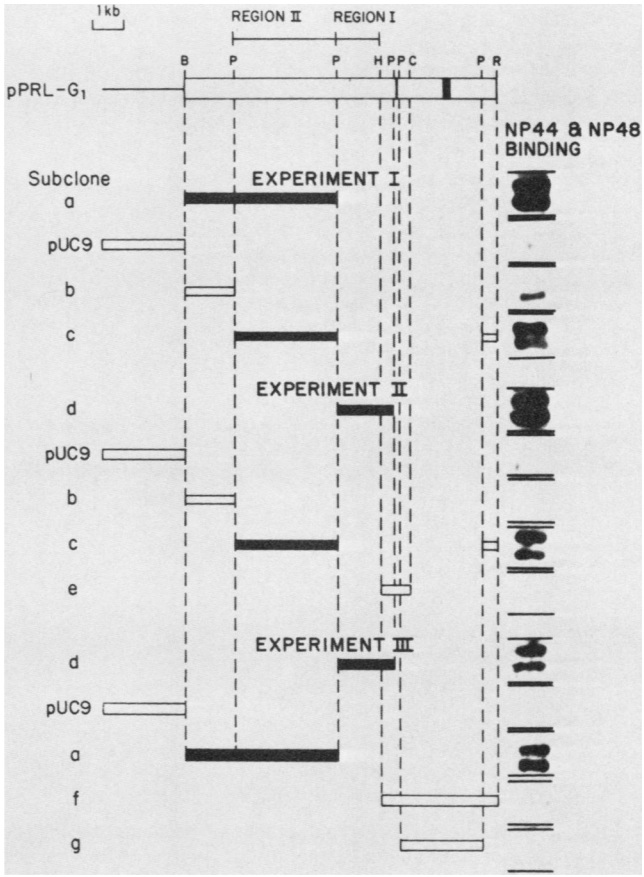

FIG. 6. Use of prolactin genomic subclones to map binding sites for NP44 and NP48. The map of clone pPRL-G_1 and its subclones is identical to that shown in Fig. 1. The indicated clones were labeled to essentially equivalent specific activities (1×10^8 to 2×10^8 cpm/μg), and the same number of counts per minute of each clone was incubated with individual lanes from the same piece of nitrocellulose of a protein gel blot of the 0.2 to 0.4 M NaCl chromatin fraction of GH_3 cells. The autoradiograms on the right show the binding of individual clones to NP44 and NP48 observed in three separate experiments. The solid bars in the clone maps indicate the DNA fragments which were observed to bind to NP44 and NP48.

DISCUSSION

We employed protein blotting to identify two chromatin proteins, NP44 and NP48, which both exhibit DNA sequence-specific binding to two upstream flanking regions of the prolactin gene. NP44 and NP48 are nonabundant chromatin proteins which, since they are removed by a low (0.4 M NaCl) salt concentration, are relatively loosely bound to chromatin. Another class of DNA-binding proteins, the high mobility group proteins, are also extracted from chromatin at this salt concentration (44). However, NP44 and NP48 appear to be unrelated to the high mobility group proteins, since they are larger in size (Fig. 2), and, unlike the high

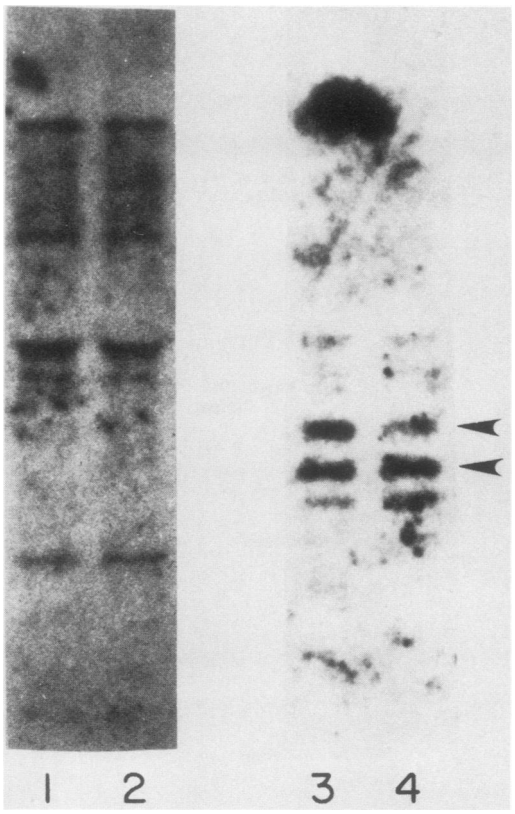

FIG. 7. Sephadex G-75 chromatography of NP44 and NP48. The 0.2 to 0.4 M NaCl chromatin extract of GH_3 cells was fractionated by Sephadex G-75 column chromatography. Aliquots were analyzed by protein gel blotting with ^{32}P-labeled pPRL-G_1 used as a probe. Lanes 1 and 2 are from succesive peak void volume fractions, and lanes 3 and 4 are from succesive fractions containing proteins eluting in the 40- to 50-kDa range.

mobility group proteins, they are precipitated by 2% trichloroacetic acid (data not shown).

We found that protein blotting is useful not only for localizing binding sites for specific DNA-binding proteins (see below) but also for physical characterization of the proteins themselves. Thus, one-dimensional SDS-polyacrylamide gel analysis yielded an estimate of the molecular weights of NP44 and NP48. Analysis on two-dimensional gels showed that each polypeptide consists of a series of mildly acidic isoelectric variants. Finally, analysis by protein blotting of chromatin proteins previously fractionated under nondenaturing conditions indicated that neither polypeptide exists in its native state as part of a multisubunit protein.

The observation that NP44 and NP48 consistently bind about equally well to specific DNA sequences is striking. It is possible that when the binding sites for these proteins are more accurately defined, proximal but distinct binding sites for each protein will be revealed. However, it seems more likely that these are related proteins, i.e., that NP44 is a proteolytic product of NP48 or that one is a covalently modified form of the other. This would be consistent both with the tight correlation of their DNA-binding characteristics and with their similar overall charges and acid solubility. The significance of the multiple isoelectric forms of each protein is not known. It may be that interconversion among these forms plays a role in gene regulation. Resolution of these questions will await isolation of the proteins and preparation of corresponding antibodies.

Clones representing various regions upstream of and within the rat prolactin gene were employed to identify two regions containing binding sites for NP44 and NP48: region I between −2.0 and −0.4 kb, and region II between −4.8 and −2.0 kb. The results of other experiments, in which end-labeled restriction fragments of clone d were bound to and recovered from NP44 and NP48 and size fractionated, yielded a further localization of region I to between −1.27

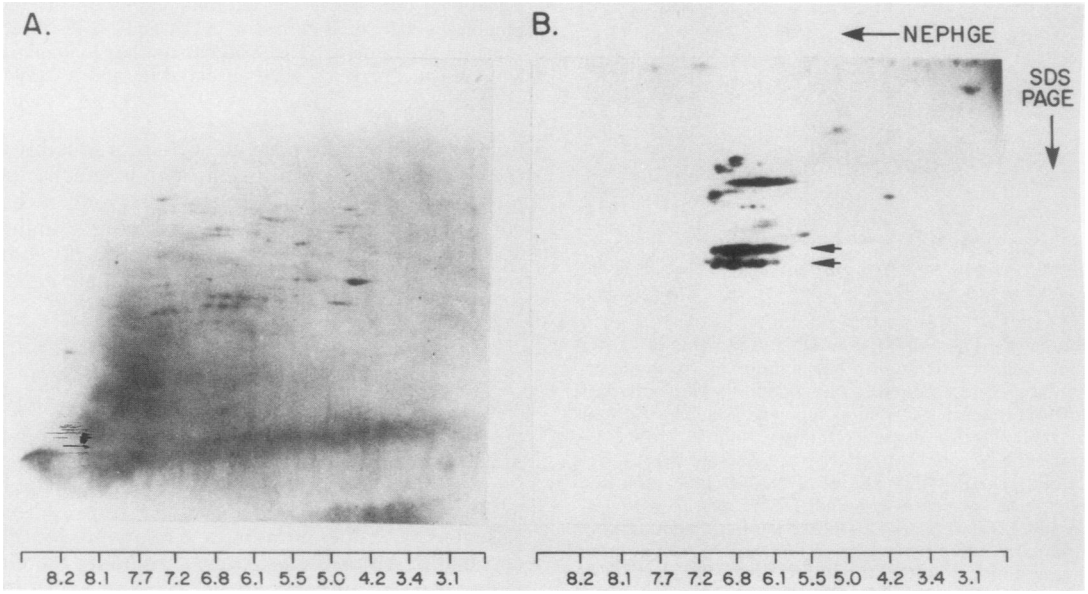

FIG. 8. Detection of NP44 and NP48 after two-dimensional gel electrophoresis. (A) Amido black stain (32) of a nitrocellulose blot of the 0.2 to 0.4 M NaCl chromatin fraction. (B) Binding of ^{32}P-labeled pPRL-G_1 to a nitrocellulose blot prepared under the same conditions as described for panel A. Arrows denote positions of NP44 and NP48. NEPHGE, nonequilibrium pH gel electrophoresis; SDS-PAGE, SDS-polyacrylamide gel electrophoresis. The pH of the electrofocusing gel (first dimension) is shown at the bottom of each slab gel.

and −0.62 kb (data not shown). We do not currently know whether the sequences of the binding sites in regions I and II are related. It should be noted that the existence of more than one DNA binding site for a protein does not exclude the possibility that the sites have different sequences (25). We also do not know whether the binding site within either of these regions contains DNA sequences repeated elsewhere in the genome. Repeated sequences have been observed within a 2.5-kb stretch of the rat prolactin gene-flanking region containing region I (42). However, since the distribution of the repeated sequences(s) within this 2.5-kb stretch has apparently not yet been examined, it is not known whether the binding site for NP44 and NP48 in region I is unique or repeated. Since region II has been observed to contain both unique and repeated sequences (42), the same uncertainty exists for the binding site in region II. Our recent observation of strong binding of these proteins to the rat growth hormone gene region (unpublished data) suggests that rat genes other than the prolactin gene may contain binding sites for NP44 and NP48. However, our observation of undetectable binding of these proteins to approximately 2 kb of upstream flanking sequences of the rat β-casein and whey acidic protein genes (Fig. 5) implies that NP44 and NP48 do not bind to the upstream flanking regions of all rat genes.

It is notable that no binding site for NP44 and NP48 was detected closer than about 600 bases upstream of the prolactin transcription initiation site. Distal upstream binding proteins have been detected previously for at least two other eucaryotic genes. Jack et al. (14) have detected three proteins which bind 800 to 1,000 base pairs upstream of the transcription initiation site of the *Drosophila* HSP 70 heat-shock protein gene, more than 700 base pairs upstream of the binding site for a heat-shock activator protein (48). Interestingly, as with NP44 and NP48, all three proteins bound to a single DNA fragment (14). Results of more recent studies (23) have shown that the binding site reported by Jack et al. (14) is flanked by DNA sequences attached to the nuclear scaffold, suggesting a possible role for this binding site in the regulation of chromatin loop domain structure. Distal protein binding sites have also been detected at 6.1 and 3.9 kb upstream of the chicken lysozyme gene initiation site (27). The finding in that study of additional protein binding sites 2.8 and 6.2 kb downstream of the poly(A) site of the lysozyme gene again raises the possibility that such binding sites are important in chromatin-nuclear matrix attachment and in the regulation of chromatin loop structure.

Regulatory sequences for the prolactin gene have not yet been identified. However, the relatively large distance of the binding sites for NP44 and NP48 from the prolactin transcription initiation site is consistent with recent results suggesting the existence of potential regulatory sequences further upstream of this gene than many of the presently known eucaryotic gene regulatory elements. DNase I-hypersensitive sites may correspond to regulatory DNA sequences which bind specific proteins (10, 11, 49). Such a site has been detected in rat pituitary tumor chromatin, lying 1.8 kb upstream of the prolactin gene transcription initiation site (10). In addition, a binding site for the estrogen receptor recently has been shown to lie between 1.2 and 2.0 kb upstream of the initiation site for the rat prolactin gene (21). Although the functional significance of this binding was not determined, it seems likely that it is involved in the known regulation by estrogen of prolactin gene transcription (19, 31). In the same study (21), two stretches of alternating purine-pyrimidine sequences were noted, at positions −1428 to −1260 and −1909 to −1848. Such sequences can form the Z-DNA conformation, which can bind specific proteins (26) and may have regulatory properties (13). Thus, although the binding sites for NP44 and NP48 are located relatively distally upstream, they are in the vicinity of several DNA sequences which are potential regulatory elements for the rat prolactin gene.

We do not know currently the functions of NP44 and NP48. The findings that induction of prolactin gene expression in Ca^{2+}-deprived GH_3 cells by incubation with Ca^{2+} (47) led to no changes in the apparent levels of these two proteins (data not shown) and that the detection of these proteins in the prolactin-negative GC cell variants imply that the proteins could be necessary but not sufficient for regulation of prolactin gene expression. Furthermore, although NP44 and NP48 exist at high levels in pituitary-derived cells, they do not exhibit absolute cell-type specificity. This is similar to previous observations that chromatin extracts of several different cell types displayed highly DNA sequence-specific binding to regions flanking the chicken lysozyme gene (27). Thus, NP44 and NP48 may belong to a class of nuclear docking proteins which function as guiding intermediates between other cell type-specific proteins and specific regions of DNA. A test of this hypothesis will require purification of these proteins and further delineation of the DNA sequences required both for binding of NP44 and NP48 and for tissue-specific regulation of prolactin gene expression.

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