Purification of PO-B, a Protein That Has Increased Affinity for the Pro-opiomelanocortin Gene Promoter after Dephosphorylation*

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The region -15 to -3 of the pro-opiomelanocortin (POMC) gene promoter specifically binds a transcription factor previously designated PO-B. This region of the POMC gene is involved in the control of constitutive POMC gene expression since mutation of the PO-B DNA-binding site severely reduces transcription from the POMC promoter both in vivo and in vitro (Riegel, A. T., Remenick, J., Wolford, R., Berard, D., and Hager, G. (1990) Nucleic Acids Res. 18, 4513–4521). We have now purified PO-B from HeLa cells approximately 25,000-fold to greater than 90% homogeneity by a combination of ion exchange and reversed phase chromatography. In addition we have studied post-translational modifications that alter the affinity of purified PO-B for its cognate DNA binding site. In Southwestern analysis of column fractions, two bands of apparent molecular masses of 54 and 56 kDa bound specifically to the PO-B recognition sequence. The two copurified components have indistinguishable amino acid composition, are highly hydrophobic, and are heat and acid stable. DNA-binding specificity studies suggest that PO-B does not represent any previously described transcription factor. In addition, dephosphorylation of both species with acid phosphatase induced an about 30-fold increase in DNA binding but failed to produce any significant change in electrophoretic mobility. We conclude that the purified PO-B species represent products of the same gene and suggest that the in vivo function of PO-B may be regulated by its phosphorylation status.

The pro-opiomelanocortin (POMC) gene is predominantly expressed in the anterior pituitary. The primary gene product is a large precursor protein which is processed in the pituitary into smaller polypeptide species such as ACTH and β-endorphin (1). In pituitary tissue and in pituitary tumor cell lines (AtT-20), POMC mRNA is easily detectable (2), reflecting a high level of constitutive, tissue specific transcription. Hormonal repression or induction of POMC transcription can be produced by administration of glucocorticoids or corticotropin-releasing factor, respectively (3). Approximately 780 base pairs of the POMC promoter upstream of the transcription initiation site appear to be required for high basal transcription in vivo (4) and in vitro. Furthermore this area also appears to encompass all the sequence information required for corticotrophin-releasing factor induction (5) and glucocorticoid repression of POMC transcription (6). Despite detailed knowledge of the endocrine control of POMC gene expression, the transcription factors involved in high basal, tissue specific and hormonally regulated control of POMC transcription have not been clearly defined.

We have previously described at least three areas within the first 480 base pairs of the POMC promoter which contribute to high basal transcription of the POMC gene. These are situated between -480 to -320, -70 to -50 and -15 to -3 relative to the POMC cap site (7). The more distal sites upstream of -50 appear to be involved in glucocorticoid repression of POMC gene expression. In contrast, mutation of the -15 to -3 region does not abolish the glucocorticoid repressive effect (7), but reduces constitutive transcription severely in vivo and in vitro. In nuclear extracts from a number of cell lines a specific binding factor for the -15 to -3 recognition sequence can be detected and has been designated PO-B (7). This factor is particularly intriguing since it appears to exert its effect at a site positioned between the POMC TATA box and cap site. However, the PO-B recognition sequence does not appear to have any of the characteristics of previously described initiator elements (8) since it is unable to direct transcription initiation in the absence of a functional TATA box (7). Furthermore, PO-B is not analogous to "general" transcription factors involved in initiation complex formation since these proteins do not exhibit sequence-specific DNA binding (9, 10). Indeed this region of eukaryotic promoters is infrequently associated with sequence-specific DNA binding of transcriptional activators. Whether PO-B is an ubiquitous cellular factor or is involved in one of the many hormonal (11) and immune (12, 13) signal transduction pathways that regulate POMC gene expression in the pituitary is not known. However, PO-B does represent the first characterized transcriptional activator of POMC whose function has been verified by mutational analysis.

As a first step in elucidating the mechanism of action of PO-B and its cellular control mechanisms, we have prepared a highly purified form of PO-B by a combination of ion-exchange and reversed phase chromatography. In this study...
we demonstrate that two protein species of approximately 54 and 56 kDa bind to the PO-B cognate binding site. These two proteins are highly related by a number of criteria such as amino acid composition, DNA binding specificity, hydrophobicity, acid and heat stability. Intriguingly, binding of PO-B in vitro is regulated by the phosphorylation status of the protein: dephosphorylation leads to a >30-fold increase in specific binding, suggesting that the signal transduction pathway that regulates PO-B may act through phosphatase/kinase interactions.

MATERIALS AND METHODS

Preparation of Nuclear Extracts—Nuclear extracts were prepared from the AtT-20 mouse pituitary tumor, C127 mouse mammary adenocarcinoma, and HeLa cell lines, maintained in Dulbecco's modified Eagle's medium +10% fetal calf serum, by the method of Dignam et al. (14). Large scale preparation of nuclear extracts was from HeLa cells in suspension culture maintained in minimum Eagle's medium supplemented with 10% horse serum. Briefly, the cells were cultured and lysed in Hepes (10 mM pH 7.9) at 4 °C, containing 1.5 mM MgCl₂, 10 mM KCl, 0.3 mM DTT, and nuclease. Prepared nuclear extracts were extracted in high salt buffer (20 mM Hepes, pH 7.9, 25% glycerol, 0.4 M NaCl, 1 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT). The final dialysis of the high salt nuclear extract was performed in buffer D (20 mM Hepes, pH 7.9, 20% v/v glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT). The dialysate was cleared of precipitate by centrifugation at 25,000 X g for 20 min and the supernatant was frozen at −80 °C. The protein concentration of the extract, assayed by the Bradford procedure (15), was in the range of 1–20 mg/ml.

Electrophoretic Mobility Shift Assay—Protein-DNA complexes were resolved on non-denaturing 4% low ionic strength polyacrylamide gels as described previously (16). In brief, the extracts (1–20 μg crude or pure nuclear proteins were resolved using SDS-PAGE as described previously (18). Crude or pure nuclear proteins were resolved using SDS-PAGE by either linear (10%) or gradient (10–20%) gels (10 cm x 10 cm x 1 mm) using standard procedures (19). Prior to electrophoresis acetonitrile 200-fold excess of crude nuclear proteins were transferred to nitrocellulose for 1 h at 150 mA in 6.25 mM Tris, 1.3% glycine, and 20% methanol. After transfer, the nonspecific sites on the membrane were blocked by three washes of 45 min in 10 mM Tris, pH 7.5, 0.5% nonfat dry milk skim milk, 10% glycerol, 2.5% Nonidet P-40, 0.1 mM DTT, 150 mM NaCl at 25 °C. The membrane was then rinsed in buffer (10 mM Tris, pH 7.5, 40 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, 8% glycerol, 0.125% nonfat dry milk skim milk) and was incubated in 3 ml of binding buffer containing 5 mM MgCl₂, 500,000 cpm/ml end-labeled specific oligonucleotide probe, and 10 μg/ml poly(dC)·poly(dG)-poly(dC)·poly(dG). The presence of specific binding was determined by the addition of a 100-200-fold molar excess of unlabeled specific PO-B oligonucleotide to a separate hybridization bag. To further analyze the specificity of PO-B binding Southwestern blots of crude extract and purified PO-B were also probed with the FS oligonucleotide (17) and the PO-Bm oligonucleotide (5'-AAATTTCTCCAGTGGGACGACTGGGACGACG-3') which does not bind PO-B (7) and an oligonucleotide harboring the SP1 consensus sequence (5'-GATCGGGGGCGGGCGGGGCGGCGGCGGCGGCGG-3'). Incubations were at 25 °C for 16 h with gentle agitation, followed by 3 x 15-min washes in 10 mM Tris, pH 7.5, 50 mM NaCl. The blot was then exposed for autoradiography.

Ion Exchange Chromatography—A Mono-S ion-exchange column (0.5 x 5 cm; Pharmacia LKB Biotechnology Inc.) was equilibrated with ice-cold buffer D (final dialysis buffer of nuclear extraction procedure; see above) at a flow rate of 0.5 ml/min. Nuclear extracts in buffer D were cleared from precipitated protein by a brief centrifugation, and about 5 μg of total protein were loaded in 2 ml onto the Mono-S column. The column was washed with ice-cold buffer D until the 280-nm absorbance reading returned to base line, and bound proteins were eluted with a 20 min linear gradient of 100 mM KCl to 1 M KCl in buffer D. Fractions of 0.5 ml were collected and stored frozen at −70 °C. 5–10-μl aliquots of each fraction were analyzed for the presence of PO-B by Southwestern blotting analysis.

Reversed Phase HPLC—Two subsequent reversed phase HPLC runs were carried out. The C-3 reversed phase HPLC columns (Ultraprep RPS, Beckman) were equilibrated with H₂O containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. In the first HPLC run, fractions from the ion-exchange chromatography containing PO-B were pooled and loaded directly onto a semi-prep column (10 x 250 mm) using multiple injections of 2 ml of trifluoroacetic acid preluted buffer D (0.5–1.0 ml; 1 mg/ml PO-B activity) at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected and analyzed for the presence of PO-B using Southwestern blot analysis. Proteins were visualized on trichloroacetic acid-preluted SDS-PAGE gels or on nitrocellulose membranes by staining with silver or silver/gold, respectively, using commercially available assays (enprotech-SSI, Hyde Park, MA and Amersham Corp.). Protein concentrations in crude and fractionated extracts were estimated using the Bradford procedure with BSA as a standard.

Renaturation of Purified PO-B—BSA (molecular biology grade 1 μg/ml) was added to pooled samples from fractions after the second reverse phase purification step containing PO-B activity. The samples (100 μl) were vacuum-dried briefly to remove acetonitrile reconstituted in buffer and dialyzed against 120 volumes of 10 mM Tris, pH 7.5, 100 mM KCl, 1 mM EDTA, 5% glycerol for 24 h at 4 °C. Redialyzed samples were stored at −80 °C prior to use.

Acidic Acid Analysis—The 54- and 56-kDa PO-B species were renatured in 2-10% SDS polyacrylamide gradient gels and transferred onto a PVDF membrane (Millipore) using a 10 mM CAPS buffer, pH 11, with 10% methanol. Protein bands were detected on the membrane with a brief Coomassie staining. The bands were excised from the membrane, acid hydrolyzed in 6 M HCl for 24 h at 110 °C, and analyzed on a Beckman 6300 amino acid analyzer (20) by Dr. K. Williams, Yale University.

Phosphatase Treatment of Purified PO-B—Purified, redialyzed PO-B protein was incubated with BSA (0.1 μg/ml) in 50 mM Pipes (pH 6.0) 0.2 M NaCl, 5 mM DTT, 1.5 mM MgCl₂, 10% glycerol, v/v in the presence of 0.5 unit of potato acid phosphatase/ml (Sigma; 10000 units/mg) for 2 h at 37 °C. The reaction was allowed to proceed for 16 h at 25 °C. An aliquot of the incubation solution, equivalent to 0.1 μl of the column fraction, was desalted, separated by 10–20% SDS-PAGE followed by Southwestern blot analysis (see above). Control samples contained either potato acid phosphatase alone or preboiled potato acid phosphatase.

RESULTS

Purification of PO-B

Assay of PO-B Activity—We have previously demonstrated that the region between −15 to −3 of the POMC promoter specifically binds a transcription factor designated PO-B (7). Mutation of the PO-B-binding site reduced POMC basal transcription by approximately 70% using transient transfection assays of the AtT-20 pituitary tumor cell line. This effect
was also observed in *in vitro* transcription assays with whole cell extracts from HeLa and AtT-20 cells (7). Now we describe the purification and characterization of the PO-B protein. As described previously, specific PO-B binding to its cognate site can be detected by electrophoretic mobility shift assay (7) (Fig. 1A). The PO-B binding is competed effectively by the PO-B oligonucleotide but not by a GA-rich oligonucleotide (FS) that has some sequence homology to PO-B (Fig. 1A). Although the electrophoretic mobility shift assay is an effective method of detecting PO-B, one drawback is that DNA binding is highly sensitive to minor pH and salt changes. Thus, fractionation procedures that involved changes in these parameters would require buffer adjustment of each fraction prior to the DNA-binding assay. To circumvent this problem we decided to develop the Southwestern detection assay (18) for PO-B. Using this technique two protein species of an apparent molecular mass of 56 and 54 kDa bound to a radiolabeled PO-B probe in AtT-20, C127, and HeLa nuclear extracts (Fig. 1B). The small differences between the mobility of the bands for each cell type (e.g. lanes 2 versus 3) were due to salt differences in the extracts and were not observed consistently. Competition by a 200-fold molar excess of nonlabeled PO-B probe significantly reduced the signal observed in the Southwestern analysis (Fig. 1B, lane 4). To further validate Southwestern analysis as a specific detection method, an oligonucleotide harboring a PO-B mutation (PO-Bm) which does not bind PO-B *in vitro* and suppresses POMC gene expression both *in vivo* and *in vitro* (7) was used. This mutated PO-B probe did not detect any proteins similar to the wild-type probe (Fig. 1C, lane 1). Furthermore an SP1-binding site oligonucleotide detected only a predominant species at approximately 100 kDa (Fig. 1C, lane 2) which is the expected molecular mass range of SP1-binding species (21).

We have repeated this analysis with a number of oligonucleotide-binding sites for characterized transcription factors (AP1 (22), AP2 (23), IRE (24), and ISRE (17, 25)). None of these oligonucleotides was able to compete for PO-B binding (analyzed by electrophoretic mobility shift or Southwestern analysis) nor bind to the 54- or 56-kDa species (data not shown). Although we have not defined the limits of sensitivity of the Southwestern analysis, it is possible to detect PO-B in 1 μl of crude HeLa nuclear extract (1-20 μg of protein) and <10 fmol of purified protein are sufficient to give a signal in this assay. Thus it appears that Southwestern analysis of PO-B is sufficiently sensitive and specific to be used as a detection method for PO-B.

**Ion Exchange Chromatography**—As an initial step we decided to use a cation exchange column because PO-B had previously been fractionated effectively on an analytical gravity flow phosphocellulose column (7). We decided to use HeLa cells in suspension culture as a source of PO-B because they grow rapidly and are less demanding in terms of serum and media requirements than AtT-20 cells. The 54- and 56-kDa PO-B species are detected in both these cell lines (Fig. 1B). Furthermore mutation of the PO-B element in the POMC promoter has similar phenotypic effects in HeLa and AtT-20 whole cell extracts in *in vitro* transcription assays (7).

Crude nuclear extract in the final dialysis buffer of the

![Figure 1](image-url)

**Fig. 1.** Electrophoretic mobility shift and Southwestern blot analysis of PO-B using crude nuclear extracts. A, HeLa nuclear extracts (5 μg) were incubated with a radiolabeled oligonucleotide representing the PO-B cognate DNA-binding site in the absence or presence of an increasing amount of unlabeled competitor (details described under "Materials and Methods"). The specific PO-B-DNA complex is indicated as are bands due to a nonspecific (NS) complex and free fragment. B, specific PO-B DNA binding was also analyzed with Southwestern blot analysis. AtT-20 (lane 1), C127 (lane 2), and HeLa (5 μg) (lane 3) extracts, were resolved on 10% denaturing SDS-polyacrylamide gels and electroblotted onto nitrocellulose. The blot was incubated with radiolabeled PO-B oligonucleotide (lanes 1, 2, and 3) or radiolabeled PO-B oligonucleotide in the presence of a 200-fold excess of cold competitor (lane 4). Details of the procedure are described under "Materials and Methods." Molecular mass standard markers (in kilodaltons) are indicated. C, Southwestern analysis of crude nuclear extract from HeLa (5 μg) on a 10–20% SDS-PAGE gel probed with either oligonucleotide PO-Bm (lane 1) or and oligonucleotide harboring the SP1 consensus binding site (lane 2).
Dignam and Roeder procedure (14) was applied directly to a Mono-S cation exchange column and proteins were eluted with a linear gradient of 0.1–1.0 M KCl. The active fractions eluted at approximately 0.25–0.3 M KCl as indicated in Fig. 2A. We determined that PO-B present in these fractions remained active at room temperature for at least 24 h. Therefore all subsequent purification steps were performed at 22 °C.

Reversed Phase Chromatography—The PO-B-containing fractions retrieved from the Mono-S column were pooled and were loaded directly onto a semipreparative C-3 column. Proteins bound to the column were eluted with a linear 0–60% acetonitrile gradient (Fig. 2B). Protein concentration was monitored by absorbance at 280 nm (Fig. 2B). The 54- and 56-kDa PO-B protein species were eluted at approximately 50% acetonitrile in four fractions as assayed by Southwestern analysis (Fig. 2B, lower panel). We subjected the pooled fractions that contained PO-B to a second analytical scale reverse phase chromatography. The samples were loaded after residual acetonitrile had been removed by vacuum extraction. In this second reversed phase run proteins were eluted by a shallow linear gradient of 35–65% acetonitrile (Fig. 2C). Using this method the bulk of the protein eluted at the beginning of the run, whereas PO-B protein once again eluted in four fractions at approximately 50–55% acetonitrile (Fig. 2C, lower panel). The protein concentration in these fractions was below the detection limit of the 280 nm absorbance (Fig. 2C).

Estimate of the -Fold Purification—To assess the purity of PO-B after the second reverse phase we compared the protein profile of aliquots of peak fractions from each stage of purification. The amounts of protein loaded on the SDS-PAGE gel were determined from pilot experiments to give approximately equal PO-B signals after Southwestern analysis. The protein in the gel was assayed using silver stain analysis (Fig. 3). The most purified fraction after the second reversed phase step contains predominantly two silver-stained proteins with apparent molecular masses of 56 and 54 kDa. Approximately 2 ng of protein was loaded into this lane. Therefore if we assume that the lower detection limit of protein in these gels is 50 pg/band (26) we can conclude that this preparation of PO-B is greater than 90% homogeneous.

To estimate the -fold purification obtained, we titrated with a crude and a purified preparation, how much of either was required to give a signal of equal strength in the Southwestern analysis. In these experiments, 20 μg of total protein from crude extract (Fig. 4, lane 1) and 600 pg of purified protein of either PO-B species (Fig. 4, lane 2) gave a signal of equal strength. These protein concentrations were assessed by the Bradford assay of crude extract and by total amino acid analysis of pure protein (see “Materials and Methods”). We thus estimate that we achieved about a 25,000-fold purification. In a typical purification run the start-up material would contain 100 mg of total nuclear protein which we estimate contains about 3 μg of PO-B (from Southwestern analysis above). Our final preparation of PO-B from this run contained 0.1 μg of purified protein as assessed by total amino acid

![Fig. 2. Purification of PO-B. A. Mono-S ion exchange chromatography: nuclear extracts (approximately 5 mg of total protein) were loaded onto the pre-equilibrated Mono-S columns (see “Materials and Methods”). After washing, bound proteins were eluted with a linear gradient from 100 to 1000 mM KCl. Fractions (300 μl) were collected and an aliquot (10 μl) of each fraction assayed for PO-B-binding activity using Southwestern analysis. UV absorbance and the salt gradient are shown in the upper panel and the Southwestern analysis of fractions containing PO-B binding activity (boxed area of protein profile) in the lower panel. B, C-3 reversed phase HPLC: active fractions from the Mono-S column were pooled and applied directly onto a semipreparative column (250 x 100 mm), and bound proteins were eluted from the column with a linear gradient of 0–60% acetonitrile as described under “Materials and Methods.” Eluted proteins were monitored by absorbance at 280 nm, and 10 μl of the 1-ml fractions were assayed for PO-B activity by Southwestern analysis (see Fig. 1B). The boxed area in the upper panel represents the peak fractions shown in the Southwestern analysis in the lower panel. C, second reversed phase HPLC: active fractions from the first reversed phase were pooled, vacuum-dried, and applied to a second reversed phase analytical column (details under “Materials and Methods”). Bound proteins were eluted with a shallow linear gradient of 35–65% acetonitrile as described under “Material and Methods.” Eluted proteins were detected at 280 nm but were below sensitivity in most of the fractions. Each fraction (1 ml) was analyzed for PO-B as described above. Southwestern analysis of fractions 48–58 (boxed area, upper panel) is indicated in lower panel (lanes 1–10).
Purification of Transcriptional Activator of POMC Gmc.

In four separate purification runs we recovered between 1 and 5% of PO-H present in the initial preparation after the final Southwestern blot of purified material. The major loss of PO-H occurs during the ion exchange chromatography step.

A rough calculation of the abundance of PO-B can be made based on the amount of protein recovered and the number of cells used initially to prepare the nuclear extract. From approximately $10^9$ cells we recovered 5 µg of purified PO-B. Assuming an average molecular mass of 55 kDa we estimate a cellular abundance of approximately 6000 molecules of PO-B per cell.

Specificity of Purified PO-B DNA Binding

To determine if the specificity of purified PO-B was similar to that of the crude extracts we assayed the ability of a series of transcription factor binding sites (SP1, AP1, AP2) to bind to the purified protein. In a typical experiment the FS consensus sequence was unable to compete for PO-B binding (Fig. 1A) but binds to a number of species in the 40–50-kDa range in crude extracts (Fig. 4, lane 3). However no FS binding species was detected in the PO-B purified fractions (Fig. 4, lane 4). In fact only the PO-B oligonucleotide was able to detect the 54- and 56-kDa species in crude and purified fractions (Fig. 4, lane 1 versus lane 2).

Finally we tested if the two protein species observed on the silver-stained gel corresponded to the two bands observed after binding in the Southwestern procedure. Three lanes of the purified sample were run on a 10–20% SDS-polyacrylamide gradient gel and blotted onto nitrocellulose. The blot was cut in half dividing the middle lane longitudinally and protein was detected on one half by silver-gold staining (Fig. 5, lanes 1 and 2) and on the other by the Southwestern procedure (Fig. 5, lanes 2 and 3). The bands detected by each procedure were coincident indicating that the protein species visualized by silver staining are most likely to be the ones binding to the PO-B recognition sequence. Also consistent with this conclusion is the observation that a single DNA binding species is detected after each band has been cut out of a Coomassie-stained gel, and protein electroeluted and analyzed by Southwestern blotting (data not shown).

Experiments using electrophoretic mobility shift assays with purified PO-B indicated that DNA-binding activity of PO-B was undetectable after the protein had been in contact with acetonitrile. However, this process was not irreversible since Southwestern analysis of the reversed phase fractions detected specific PO-B DNA binding. Therefore we attempted to renature purified PO-B by overnight dialysis. After this renaturation step the electrophoretic mobility of the purified PO-B/DNA complex was indistinguishable from that obtained with crude extracts (Fig. 6). These data taken together

![Fig. 3. SDS-polyacrylamide gel analysis of protein fractions.](image)

![Fig. 4. Southwestern analysis of crude extract and purified PO-B.](image)

![Fig. 5. Coincidence of bands detected by protein staining and Southwestern analysis.](image)
with the results presented in Fig. 5 strongly suggest that the 56- and 54-kDa proteins that we have purified are responsible for specific binding to the PO-B recognition sequence.

**Role of Phosphorylation in PO-B DNA Binding**

We were particularly interested to determine if the two PO-B forms observed were due to post-translational modifications. It has previously been demonstrated that glycosylation (27) and phosphorylation (28-33) of transcription factors can be important modifications for the activity of these proteins. Furthermore, these post-translational modifications can produce considerable differences in the electrophoretic mobility of proteins (29). Initial experiments using digestion of purified PO-B with N- and O-endoglycosidases did not alter the mobility of either species (data not shown). To test for differences in phosphorylation status, purified protein was digested with potato acid phosphatase and probed by Western analysis. No significant change in the electrophoretic mobility of the proteins was observed after dephosphorylation. However, phosphatase treatment increased the PO-B DNA-binding activity by over 30-fold as analyzed by laser scanning densitometry of the two species (Fig. 7). The increase in DNA binding was observed for both the 54- and 56-kDa species (Fig. 7). The effect was not due to a protein specie fortuitously present in the potato acid phosphatase preparation since this enzyme gives no signal on a Southern blot when run in the absence of purified PO-B. Boiled enzyme did not produce an increase in DNA binding (data not shown). We have also obtained an increase in DNA binding affinity using acid phosphatases from different sources (data not shown).

**Relatedness of the 54- and 56-kDa Species**

The 54- and 56-kDa PO-B species copurify throughout all biochemical fractionation procedures and the binding specificity of both forms is identical. Furthermore, dephosphorylation of both species increases DNA binding. These data taken together indicate that these species probably represent modified forms of the same protein. To investigate this further we performed total amino acid analysis on the 54- and 56-kDa species (Table I). For each amino acid or group of amino acids analyzed, the percent composition for each species is almost identical within the precision of the HPLC analysis (20). Although these data do not prove that these proteins are the same it does not preclude this conclusion and is consistent with the other physicochemical data that indicates that these proteins are highly related.

**DISCUSSION**

In the present study we have used Western blot analysis to follow the phosphorylation of PO-B, a transcription factor that binds to the −15 to −3 region of the POMC promoter. We have previously demonstrated that mutation of this site causes a 70% decrease in POMC basal transcription both in vivo and in vitro transcription extracts from a number of cell lines (7). PO-B activity was particularly pronounced in HeLa cell extracts (7) and hence our decision to purify and
characterize PO-B further from this cell line. We had previously demonstrated that the retarded complex formed with the cognate PO-B DNA binding site was similar with nuclear extracts from the AtT-20 pituitary tumor, C127 mouse mammary adenocarcinoma and HeLa cell lines (7). This is consistent with the observation in this study of similar 54- and 56-kDa species detected by Southwestern analysis in these different cell types. Mutation of the PO-B binding site reduces POMC transcription by about 3-fold in HeLa, AtT-20, and C127 whole cell extracts (7). Furthermore, purified PO-B is capable of producing the same electrophoretic mobility shift complex as that observed with crude nuclear extracts. Taken together these data imply that the 56- and 54-kDa proteins are components of the specific retarded DNA-protein complex obtained with the PO-B DNA binding site. A separate issue is whether the 54- and 56-kDa PO-B-binding species represent modified forms of the same protein or different gene products. The current data strongly suggest that the former view is correct. First, the 56- and 54-kDa species copurify in all fractionation procedures that we have used. Second, the amino acid analysis of the two species is highly similar. Third, the binding specificity of both species is identical. Southwestern analysis of the purified fractions leads to a concomitant increase in DNA binding of the 54- and 56-kDa species but no change in the electrophoretic mobility of either species. Our preliminary data also imply that the differences are not due to glycosylation. We are currently determining if other post-translational modifications are involved in producing the two PO-B species. Conversely they may be products of alternate splicing as has been shown to occur for the CREB and ΔCREB transcription factors (33). Interestingly under non-denaturing conditions only a single species of approximately 110 kDa is observed with Southwestern analysis. This observation might suggest that the two forms of PO-B copurify as a dimer and are separated during denaturing electrophoresis. Homo- and heterodimer formation has been described for a number of cellular transcription factors (34, 35).

The rare abundance of PO-B is consistent with that of other described cellular transcription factors (36, 37). Furthermore, PO-B fractionates in high (50-60%) acetonitrile implying that this is a highly hydrophobic protein, also a characteristic of a number of other transcription factors (37). In fact, a number of these proteins have molecular masses in the range of 45-60 kDa (37) leading us to question whether PO-B was actually one of these previously characterized proteins. In addition, homology searches of other DNA consensus sequences had revealed some similarities to the PO-B DNA binding site. However, in extensive cross competition and Southwestern analysis with the AP1 (22), AP2 (23), Sp1 (37) and a variety of interferon and viral response elements (17, 24, 25), no competition for PO-B DNA binding site in either crude or purified fractions was observed. This was true for both the 56- and 54-kDa species. This suggests that PO-B is a novel protein that has not been previously characterized. In fact we have now obtained an N-terminal amino acid sequence from the 54-kDa species. Homology searches with available sequences in GenBank indicate no significant similarity with any protein in this database.

The role of dephosphorylation in PO-B mechanism of action is intriguing especially since phosphorylation of other transcription factors is required for high DNA affinity and transcriptional activation (28-32). Some of these phosphorylation events are accompanied by significant changes in mobility on SDS-PAGE (29). Phosphorylation of other factors, as is the case with PO-B, causes no appreciable change (38). Some recent studies demonstrate that progressive phosphorylation in vitro is accompanied by progressively larger differences in SDS-PAGE mobility (29). This might imply that PO-B is undergoing dephosphorylation at relatively few amino acids. Clear conclusions on this point await further in vitro studies. However, our data do imply that there must be significant overlap in the purification profiles of the phosphorylated and dephosphorylated forms of PO-B. In fact the phosphorylated and dephosphorylated forms of the 54- and 56-kDa species must have the same mobility on SDS-polyacrylamide gels since no other major protein species is observed on the silver stains of the purified product. Whether the variable levels of the phospho and dephospho versions of PO-B alter its transcriptional activating ability is currently under investigation.

Currently we do not know the signal transduction pathway that regulates PO-B in vivo. Therefore we can only speculate that PO-B may be activated by a specific cellular phosphatase or conversely deactivated by a specific cellular kinase. The number of cellular phosphatases characterized is small (39) especially compared with the extensive knowledge on cellular kinases. Recently a number of specific tyrosine phosphatases have been characterized and cloned (40, 41). However, we failed to detect PO-B using anti-phosphotyrosine antibodies in Western blot analysis, indicating that PO-B may be a substrate for serine/threonine kinase activity. The availability of more specific enzymes should make experiments on the role of dephosphorylation in PO-B mechanism of action more feasible. It has been postulated that for each specific cellular kinase there exists an opposing specific phosphatase (42); thus one might predict that a specific cellular kinase may reduce PO-B DNA binding. We are currently determining if any of the known cellular kinases are able to alter PO-B DNA-binding affinity in vivo and in vitro.

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