Mammalian β-Polymerase Promoter: Large-Scale Purification and Properties of ATF/CREB Palindrome Binding Protein from Bovine Testes

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ABSTRACT: The mammalian DNA repair enzyme β-polymerase is encoded by a single-copy gene that is expressed in all tissues and cell lines studied to date. A protein fraction with high binding affinity for an ATF/CREB-like binding element, GTGACGTCAC, at −49 to −40 in the core β-polymerase promoter has been purified to near-homogeneity from a nuclear extract of bovine testes. The major binding activity, as monitored by gel mobility shift assay, is recovered in 20% yield by a procedure involving oligonucleotide affinity chromatography. The purified protein yields DNase I footprinting and gel shift binding patterns indistinguishable from the activity in crude extracts. The final fraction activates transcription in an in vitro transcription reaction. The native molecular weight of the purified binding activity is about 100–120K as measured by gel filtration. SDS–PAGE of the purified fraction revealed that it contains several polypeptides in the molecular weight range of 30–52K, yet two of these peptides (M_r 49K and 52K) are predominant. Specific binding to the palindrome is salt-sensitive and is consistent with the formation of nine ion pairs (from log K_A vs log KCl plots) and has a K_A at 200 mM KCl of 5.8×10^{11} M^{-1}. Kinetic studies with synthetic oligonucleotides as binding ligands indicate that the purified protein can bind tighter to or discriminate between the β-polymerase ATF/CREB element and similar elements derived from somatostatin and chorionic gonadotropin genes.

DNA polymerase β (β-pol)[1] is a vertebrate "housekeeping" enzyme generally considered to be involved in DNA repair [for reviews, see Fry and Loeb (1986) and Wilson et al. (1988)]. It has been found in all cell and tissue types that have been examined, although the levels of mRNA may vary significantly between tissue types (Hirose et al., 1989; Nowak et al., 1989). The 5′ region of the gene for this enzyme has been cloned from human (Widen et al., 1988) and mouse (Yamaguchi et al., 1987), and the promoter from each has been identified and characterized. By use of transient expression assays, the human promoter has been shown to be positively regulated by adenovirus transactivating proteins (Widen et al., 1988) and negatively regulated by the HTLV-I *tax* protein (Jeang et al., 1990). In addition, the *ras* oncogene can increase expression from the β-pol promoter (Kedar et al., 1990) as can DNA damage caused by alkylating agents but not by other different acting DNA damaging agents (Fornace et al., 1989; Kedar et al., 1991). The promoter from the human gene is G+C rich, lacks typical TATA and CCAAT box sequences, and has several consensus sequences for the transcription factor Sp1 (Widen et al., 1988). From 40 to 49 bp upstream of the major transcription start site there is a 10 bp palindrome of sequence GTGACGTCAC. This sequence is conserved between the human and mouse promoters and has been shown to be important for the expression of the human β-pol promoter (Widen et al., 1988; Jeang et al., 1990; Kedar et al., 1990).

Many viral and cellular gene promoters contain elements similar in sequence to the palindrome found in the β-pol promoter. There is a family of transcription factors that are able to bind to some or all of these elements (Hai et al., 1988, 1989; Hoeffler et al., 1988; Gonzalez et al., 1989; Maekawa et al., 1989; Gaire et al., 1990; Kara et al., 1990). Variously called ATF, for activating transcription factor, or CREB, for cyclic AMP response element binding protein, these proteins fall into the leucine zipper class of transcription factors and bind to DNA as homo- or heterodimers (Hai et al., 1989). The amino acid sequences of several of these factors as predicted from cDNA clones show that although the proteins are related in the DNA binding and protein dimerization domains, the other regions may be completely different.

Detailed studies of ATF/CREB proteins have revealed at least two important general findings. The first is that the different proteins may function differently in regulating transcription. For instance, a protein named CREB contains a transcriptional activator domain, and is regulated by phosphorylation of specific amino acid residues (Yamamoto et al., 1988). However, another family member with similar DNA binding specificity, ATF-2, does not contain a similar activator domain and does not have the same phosphorylation sites as CREB (Liu & Green, 1990). This protein must interact with additional factors, such as the adenovirus E1A protein, to activate transcription. CREB does not appear to have such a requirement. The second point is that some of the proteins bind differently to elements that appear to be very similar. Another CREB-like protein isolated by Hoeffler et al. (1988) is able to bind to ATF/CREB elements found in the somatostatin and chorionic gonadotropin promoters but not to similar elements from bovine parathyroid hormone and glucagon genes. These elements all have the same 8 bp palindrome, TGACGTCA, but different flanking sequences, indicating that the flanking sequences are important for determining binding specificity.

Since there are members of the ATF/CREB family with slightly different binding specificities and with different transcriptional activities, it is likely that genes with similar

[1] Abbreviations: β-pol, DNA polymerase β; CG, chorionic gonadotropin; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; Glu, glucagon; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBP, palindrome binding protein; PMSF, phenylmethanesulfonyl fluoride; PTH, parathyroid hormone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SMS, somatostatin.

regulatory sequences may be regulated differently. Thus, the various members of the ATF/CREB family may be able to discriminate between different promoters. In HeLa cells, there are at least six different family members expressed, and some of these bind as heterodimers, creating a potentially complex and extremely multifaceted regulatory network (Hai et al., 1989).

We are interested in the mechanisms by which the β-pol promoter is regulated and, therefore, want to understand how the members of the ATF/CREB family interact with this promoter. In this report, we examine the interaction of proteins with the palindrome element of the β-pol promoter and describe a purification procedure for specific binding proteins using bovine testes as the source. This procedure is relatively simple and inexpensive and provides sufficient protein to study the interaction with the β-pol promoter in detail. For simplicity, we call the palindromic sequence in the β-pol promoter the ATF/CREB element and the protein that binds to it the palindrome binding protein (PBP).

Materials and Methods

Buffers. Homogenization buffer was composed of 10 mM HEPES, pH 8.0, 1.5 mM $MgCl_2$, 10 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/mL pepstatin A, and 10 mM sodium metabisulfite. Buffer A was 20 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.1 mM DTT, 0.1 mM PMSF, 1 μg/mL pepstatin A, and 10 mM sodium metabisulfite while buffer B is equal to buffer A except that 100 mM KCl replaces the NaCl in buffer A. Buffer C consisted of 20 mM Tris-HCl, pH 7.7, 10% glycerol, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM PMSF, while buffer D was 20 mM HEPES, pH 7.9, 10% glycerol, 1 mM DTT, 0.1 mM EDTA, and 0.1% Nonidet P-40.

Purification. (A) Nuclear Extracts. Frozen bovine testes were obtained from J. Schmidt Co. (Baltimore, MD) or Pel-Freeze and held at −80 °C until use. Corticol tissue containing blood vessels and fibrous material was removed by crushing the frozen tissue with a hammer. Pieces of frozen tissue were processed by one of two alternate methods that gave similar results, but enabled processing of different amounts of tissue.

Method A. Frozen tissue (10–125 g) was allowed to partially thaw at room temperature. The tissue was minced with a razor blade and then with scissors. The minced tissue was mixed with 3 volumes of homogenization buffer at 4 °C and homogenized with 6 full strokes in a motor-driven glass–Teflon homogenizer. All procedures were at 4 °C. The homogenate was centrifuged at 10000*g* for 10 min, and the pellet fraction was mixed with 1.5 volumes, relative to the starting weight of tissue, of the homogenization buffer plus 1 M NaCl. The mixture was homogenized, as before, with 3 strokes and then centrifuged at 100000*g* for 1 h. The supernatant fraction was dialyzed against 20 volumes of buffer A, and the insoluble material was removed by centrifugation at 10000*g* for 10 min. The clear supernatant was mixed with saturated $(NH_2)_2SO_4$, pH 7.7, to 40% saturation, and the mixture was stirred for 30 min. The precipitated material was collected by centrifugation at 15000*g* for 20 min, dissolved in a minimal volume of buffer B, and dialyzed against buffer B. The insoluble material was removed by centrifugation, and the resulting clear solution of crude nuclear extract was called fraction I.

Method B. Frozen tissue pieces (400–500 g) were allowed to thaw at room temperature and then minced with scissors. The tissue was mixed with 3 volumes of homogenization buffer at 4 °C. The mixture was divided into two portions, and each was homogenized in a Waring blender with five bursts of 5 s each. All procedures were at 4 °C. The homogenate was centrifuged at 10000*g* for 10 min, and the pellet fraction was mixed with 1.5 volumes, relative to the tissue weight, of homogenization buffer plus 1 M NaCl. The mixture was homogenized in a Waring blender with three bursts of 5 s each, and the mixture was centrifuged at 100000*g* for 1 h. The supernatant fraction was adjusted to 40% saturation in $(NH_2)_2SO_4$ by gradual addition of solid $(NH_2)_2SO_4$ with stirring. The pH of the mixture was maintained above 7.7 by addition of solid NaOH. The mixture was stirred for 30 min. The precipitated material was collected by centrifugation at 15000*g* for 20 min and dissolved in a minimal volume of buffer B. The solution was dialyzed against buffer B, and the insoluble material was removed by centrifugation. The resulting clear solution of crude nuclear extract was called fraction I.

(B) Chromatography. Material from up to 200 g of tissue was applied to a 100-mL heparin–agarose column (Bio-Rad Affigel, 2.5 × 20 cm) at 4 mL/min and 4 °C. The column was washed with 1 volume of buffer C with 0.1 M KCl and eluted with 0.4 M KCl in buffer C. The fractions containing protein were pooled, typically about 80–100 mL total, and dialyzed against buffer C containing 0.05 M KCl for about 3 h so that the final salt concentration was less than 0.1 M. After centrifugation, the pooled material was filtered through a 0.22-μm Millipore GV low protein binding filter. This is fraction II and was applied to a Mono Q anion-exchange column (Pharmacia FPLC, 1 × 10 cm) at 2 mL/min at room temperature. The column was washed with 0.1 M KCl in buffer C, and protein was eluted with a 90-mL gradient from 0.1 to 0.5 M KCl in buffer C. Specific binding activity eluted from 0.2 to 0.3 M KCl in a volume of about 20 mL (fraction III). The active fractions from two runs were pooled, mixed with 8 mg of sonicated salmon sperm DNA, half of which was denatured by boiling, and applied to a 3-mL oligonucleotide affinity column. This column was prepared according to Wu et al. (1988). The oligonucleotides were not ligated before conjugating to the column. The amount of DNA bound to the column was estimated to be about 37 μg/mL or about 2 nmol of binding sites/mL. The pooled Mono Q fractions were loaded at 0.5 mL/min, washed with 10 mL of 0.4 M KCl in buffer D, and eluted with 8 mL of 1.0 M KCl in buffer D. The 1.0 M fraction was diluted to 0.4 M KCl, mixed with 4 mg of salmon sperm DNA (half of which was denatured), and reapplied to the 3-mL column. This was washed and eluted as before, and the second 1.0 M fraction was diluted to 0.4 M KCl and applied to a 1-mL affinity column without added carrier DNA. This third column was washed with 5 mL of 0.4 M KCl and eluted with 1.0 M KCl in buffer D. Fractions of 1 mL were collected and assayed for binding activity. The active fractions were pooled and called fraction IV. For some experiments, this final fraction was concentrated by using a Centricon concentration unit (Amicon, molecular weight cutoff = 30000) as directed by the manufacturer. Protein was determined by the Bradford method using Bio-Rad's protein assay solution and bovine γ-globulin as a standard. Proteins were analyzed by 10% SDS–PAGE and silver staining or Coomassie blue staining as indicated.

Glycerol Gradient Sedimentation and Gel Filtration. A 2-mL 10–40% glycerol gradient, containing 1 M KCl, 20 mM HEPES, pH 7.9, 1 mM DTT, and 0.1 mM EDTA, was poured. Fifty microliters of concentrated fraction IV was mixed with 50 μL of the above buffer with no glycerol and layered onto the top of the gradient. A parallel gradient with bovine γ-globulin, bovine serum albumin, and ovalbumin was prepared with 5 μL of fraction IV. The gradients were centrifuged at 55000 rpm in a Beckman TLS 55 rotor for 13 h

at 4 °C. Fractions were collected by pipetting 100 μL at a time from the top of the tubes. Activity in each gradient was measured by gel mobility shift analysis and filter binding assays, and the size standards were detected by SDS–PAGE. Gel filtration was performed on a Pharmacia FPLC system Superose 12 column (1 × 30 cm) in 0.5 M KCl, 10% glycerol, 20 mM HEPES, pH 7.9, 1 mM DTT, and 0.1 mM EDTA; 100–200-μL samples were injected and run at a flow rate of 0.3 mL/min. Standards were bovine γ-globulin, BSA, and ovalbumin.

Filter Binding Assay. The assays were based on methods described by Riggs et al. (1970a,b). Binding reactions were performed in binding buffer as described for the gel mobility shift assay. For some experiments, the volumes were increased to 100 or 200 μL, and the salt concentrations and incubation times were varied. The reaction mix was then diluted to 1 mL with wash buffer (5% glycerol, 50 mM KCl, 1 mM DTT, and 0.1 mM EDTA) and filtered at 4–5 mL/min through a 25-mm-diameter nitrocellulose filter (Millipore HA, pore size 0.45 μm) using a 12-position Millipore sampling manifold. The filter was washed with 2 mL of wash buffer, dried, and measured by scintillation counting. The concentration of binding activity in fraction IV was determined as described in Riggs et al. (1970b). The on and off rate determinations were performed in 0.2 M KCl. For the determination of the on rate, 5×10^{-12} M labeled DNA was mixed with 2.5×10^{-11} M binding activity, and samples were filtered at the indicated times. One hundred nanograms of βP-1 oligonucleotide was added with the wash buffer to prevent further binding of protein to the labeled probe. For the off rate, binding reactions were set up so that the DNA was completely bound by protein, then a 1000-fold excess of unlabeled DNA was added, and samples were filtered at several time points. In addition, the off rate was determined without the use of competitor DNA by diluting a binding reaction 200-fold and measuring the time required to reach a new equilibrium.

Gel Mobility Shift and DNase Footprinting Assays. Gel mobility shift assays were based on the method of Fried and Crothers (1981). Labeled probe DNA (0.1 or 0.2 ng) was mixed with protein samples in a final volume of 20 μL containing 0.2 M KCl, 20 mM HEPES, pH 7.9, 0.1 mM EDTA, 1 mM DTT, and 2 μg of salmon sperm DNA. Reactions with purified protein contained 100 μg/mL BSA and only 10 ng of salmon sperm DNA. The competitor DNAs were added to the reaction before addition of protein. Reactions were incubated at room temperature for 15 min and then applied directly to a 4% native polyacrylamide gel. The gels were electrophoresed with buffer circulation for 2 h at room temperature in 6.7 mM Tris, pH 7.9, 3.3 mM sodium acetate, and 1 mM EDTA, then dried, and exposed to Kodak XAR-5 film. Footprinting reactions were performed in the same binding buffer with 1 ng of labeled promoter fragment in a 50-μL volume. After 15 min, 50 μL of 10 mM $CaCl_2$/10 mM $MgCl_2$ was added, followed by 10 ng of DNase I. Digestion was for 2 min; the reaction was stopped by phenol extraction, and the products were separated on a sequencing gel.

Renaturation of Binding Activity. Concentrated fraction IV was applied to three adjacent lanes of a 10% SDS–polyacrylamide gel. After electrophoresis, the center lane was cut out in one strip and stored at 4 °C. The remaining gel was silver-stained and lined up with the unstained lane. Slices of this lane were cut; then the proteins were eluted and renatured as described in Hager and Burgess (1980).

In Vitro Transcription. Template DNA (1 μg of pβP8; Widen et al., 1988) was mixed with 1 μL of fraction IV and 9 μL of binding buffer; then 20 μL of HeLa whole cell extract (Manley et al., 1983) was added. Water and nucleotide triphosphates were added to a final volume of 50 μL and 500 μM each nucleotide. The reaction was incubated at 30 °C for 45 min. Then the template was digested with DNase I, phenol-extracted, and ethanol-precipitated. The RNA transcripts were analyzed by S1 nuclease mapping as described in Widen et al. (1988).

DNA Probes. The βP-1 oligonucleotide consisted of GGCTCAGGAGGACGCGTGACGTCACAACAAGCG and its complement, CCTGAGCCGCTTGTTGTGACGTCACGCGTCCT. The Sp1 oligonucleotide was TCGACTCTAGCCCCGCCCCGCCCCGCCCAGGA and its complement, CCTGAGCCTCCTGGGCGGGGCGGGGCGGGGCTAGAG. The other oligonucleotides were as described in Hoeffler et al. (1988) and are listed in Table II. The promoter fragment probe is the 183 bp *Hin*dIII–*Kpn*I fragment isolated from plasmid pβP8 (Widen et al., 1988). It has 113 bp of 5′-flanking sequence and 57 bp 3′ to the transcription start site. This insert was cut from the plasmid with *Hin*dIII and *Kpn*I and treated with calf intestinal phosphatase. After heat inactivation and phenol extraction, the insert was separated from the vector by chromatography on a 1-mL Mono Q anion-exchange column (Pharmacia FPLC system) using a NaCl gradient to elute the fragments. This method removed greater than 95% of the vector sequence and circumvented potential problems involved with isolation of the fragment from agarose gels. The eluted fragment was ethanol-precipitated, dissolved in TE (10 mM Tris, pH 7.5, and 1 mM EDTA), and quantified by UV absorbance. Either 100 or 200 ng of DNA was labeled using polynucleotide kinase (BRL) and [γ-^{32}P]ATP (ICN, 7000 Ci/mmol). The specific activity was determined by acid precipitation of a portion of the labeling reaction; then the fragment was separated from unincorporated nucleotides by using a Pharmacia Nick T gel filtration column. Recovery was determined by acid precipitation of the purified probe and typically was greater than 90%. This probe was used both to quantify the binding activity in purified protein fractions and in the DNase footprinting and in some gel mobility shift assays.

Results

ATF/CREB binding elements derived from the somatostatin (SMS) and chorionic gonadotropin (CG) promoters confer cAMP responsiveness (Deutsch et al., 1988) and bind to CREB proteins whereas similar elements from the parathyroid hormone (PTH) and glucagon (Glu) promoters do not bind to the particular CREB protein isolated by Hoeffler et al. (1988). We synthesized ATF/CREB binding site oligonucleotides identical with those used by Hoeffler et al. and compared their binding with that of an oligonucleotide containing the β-pol ATF/CREB palindrome and flanking sequence. A nuclear extract was prepared from 293 cells, a human kidney cell line (Graham et al., 1977), and a gel mobility shift assay was performed with the various probes. The SMS and CG probes gave similar results, with a predominant shifted band (Figure 1, lanes 2 and 4, "Bound B") and a minor band of lower mobility ("Bound A"). The β-pol probe (βP-1) gave a different pattern, with a predominant band of low mobility (lane 1). The PTH probe pattern is similar to that of the βP-1 probe (lane 3) whereas the Glu pattern is very faint (lane 5), but longer exposure shows it to be similar to the SMS and CG patterns. Hoeffler et al. reported that the PTH and Glu oligonucleotides do not bind proteins in a JEG-3 cell nuclear extract. The differences between our results may be due to different assay conditions. We see similar binding

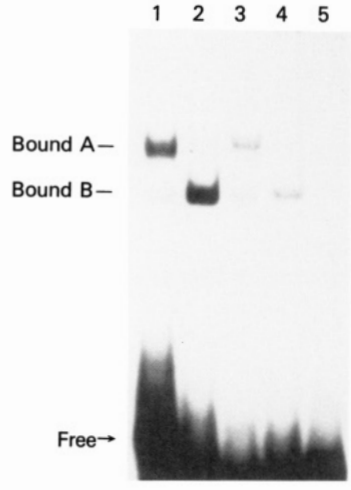

FIGURE 1: Gel mobility shift analysis with different ATF/CREB element probes. ^{32}P-Labeled oligonucleotides were incubated with 293 cell nuclear extracts, resolved on a 4% native polyacrylamide gel, and visualized by autoradiography. Protein–DNA complexes are indicated as Bound A and B. Free indicates unbound probe. Oligonucleotide probes are as follows: lane 1, β-polymerase; lane 2, somatostatin; lane 3, parathyroid hormone; lane 4, chorionic gonadotropin; lane 5, glucagon. Sequences of these are given in Table II.

patterns using HeLa cell extracts (data not shown). These differences in bandshift patterns with the various probes may indicate that a different protein or combination of proteins preferentially binds to the CG and SMS probes versus the PTH and βP-1 probes. However, from this experiment, it is not possible to rule out that the same proteins are responsible for results with all probes and that differences between the probes cause the different patterns.

We tested for the functional significance of the binding to the βP-1 oligonucleotide by competition with the complete promoter sequence (Figure 2a). A gel shift assay was performed using labeled βP-1 oligonucleotide and increasing amounts of unlabeled promoter fragment. A mutated promoter, pβP8*A, also was tested as competitor. This mutated promoter has the central ACGT of the palindrome changed to GGCC and has previously been shown to have reduced promoter activity in transient expression experiments in 293 cells (Widen et al., 1988). Figure 2a shows that the wild-type promoter effectively competes for binding to the oligonucleotide whereas the mutated promoter that is less active in vivo does not compete when added at a 150-fold molar excess. These results confirm that binding to the oligonucleotide probe used here correlates with binding to the biologically active promoter sequence.

The proteins binding to the βP-1 probe were fractionated over a Superose 12 gel filtration column to estimate their size in a crude nuclear extract (Figure 2b). The peak of binding activity eluted between BSA (66 kDA) and bovine IgG (155 kDa). The fractionation experiment shown was performed in the presence of 0.5 M KCl. The elution position did not change when the concentration of KCl in the column buffer was changed to 0.1 or 1.0 M, and the size of the binding activity was estimated to be 100–120 kDa. Interestingly, proteins responsible for the faster migrating band ("Bound B" in Figure 1) eluted before the main peak of binding activity, suggesting that the proteins responsible for the two bands can be separated by this gel filtration method (Figure 2b, fractions 10–11).

We wish to study binding properties of β-pol promoter-specific factors as they exist in the cell. In order to obtain enough protein to do this in detail, we set out to purify binding proteins from a tissue source rather than cultured cells. Bovine testes was selected because the highest level of β-pol mRNA of any tissue examined in rodent tissue surveys was found in testes (Hirose et al., 1989; Nowak et al., 1989). A nuclear extract was prepared and tested by gel shift assay for the presence of specific binding activity (Figure 3). The βP-1 oligonucleotide probe was bound by a factor that was competed

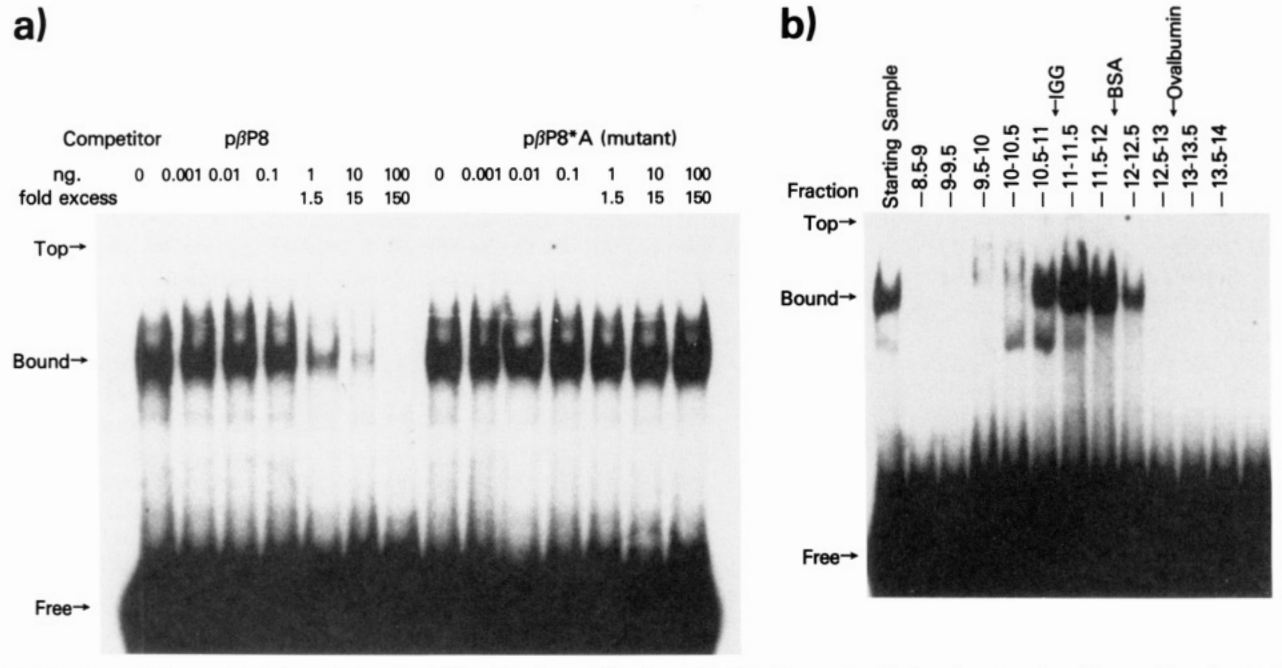

FIGURE 2: Competition analysis (panel a) and gel filtration (panel b) of proteins binding to the βP-1 probe. Panel a: Competition for binding with wild-type and mutated promoter fragments. Binding reactions were as indicated in Figure 1, except that the indicated amounts of competitor DNAs were added to the reactions. Panel b: Gel filtration analysis of the binding activity. Nuclear extract from 293 cells was applied to a Superose 12 column. Fractions of 0.5 mL were collected and assayed for binding activity by gel mobility shift analysis. Fractions are indicated above each lane, and the unlabeled lanes are trailing fractions. The elution positions of size markers are indicated. IGG, bovine γ-globulin, 155 kDa; BSA, bovine serum albumin, 66 kDa; ovalbumin, 43 kDa.

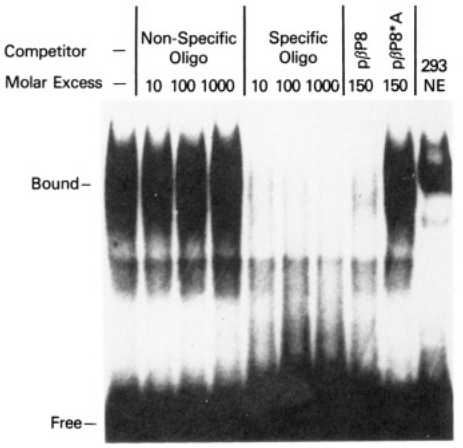

FIGURE 3: Competition analysis using bovine testes nuclear extract. Labeled βP-1 oligonucleotide was incubated with testes extract (fraction I, Table I) in the presence of the indicated amounts of competitor DNAs. Nonspecific DNA was the sequence AATTCGGGACCATGGCACCTGTGCACGGCGACG and its complement. Specific oligo was the βP-1 oligonucleotide, and pβP8 and pβP8*A were the normal and mutated polymer fragments. In the right-hand lane, complexes between 293 cell nuclear extract and the βP-1 oligonucleotide were analyzed as reference.

Table I: Summary of Purification of the Bovine Testes Palindrome Binding Protein

fraction	concn (mg/mL)	vol (mL)	total (mg)	total act. (pmol)	yield (%)
(I) crude nuclear extract[a]	20	100	2000	200	100
(II) heparin–agarose eluate	1.7	220	370	44	20
(III) Mono Q eluate	1.2	32	38	26	13
(IV) oligonucleotide affinity eluate	0.001	4	0.004	40	20

[a] 200 g of tissue used.

by the specific oligonucleotide but not by a nonspecific oligonucleotide. The wild-type promoter fragment (pβP8) competed for binding whereas the mutated promoter (pβP8*A) did not. The shifted band is similar in position to the band from 293 cell extract, but it is more diffuse. Subsequent preparations of extract gave more discrete bands, and we suspect that partial proteolysis or other modifications are responsible for these diffuse bands.

Several columns were tried for purification of the activity with varying degrees of success. Hydroxylapatite and phosphocellulose columns resulted in complete loss of activity, and DEAE-cellulose resulted in partial loss of activity and a change in the gel shift pattern (not shown). Heparin–agarose gave the best results and was used as the first column. The nuclear extract (fraction I) was passed over a heparin–agarose column at 0.1 M KCl, and the bound activity was eluted with 0.4 M KCl. The activity was monitored by gel shift assay. There is significant loss of activity, but this step was required to prepare the material for the next step. After dialysis to 0.1 M KCl, the heparin column eluate (fraction II) was loaded onto a Pharmacia FPLC Mono Q anionic exchange column. The activity bound to this column and was eluted with a linear gradient from 0.1 to 0.5 M KCl. The activity (fraction III) eluted in a broad peak from 0.2 to 0.3 M KCl. Some loss of activity was detected with this column (Table I). The active Mono Q fractions were pooled, mixed with sonicated salmon sperm DNA, and applied to a 3-mL oligonucleotide affinity column. The column was washed with 5 mL of 0.4 M KCl, and the activity was eluted with 1.0 M KCl. The 1.0 M

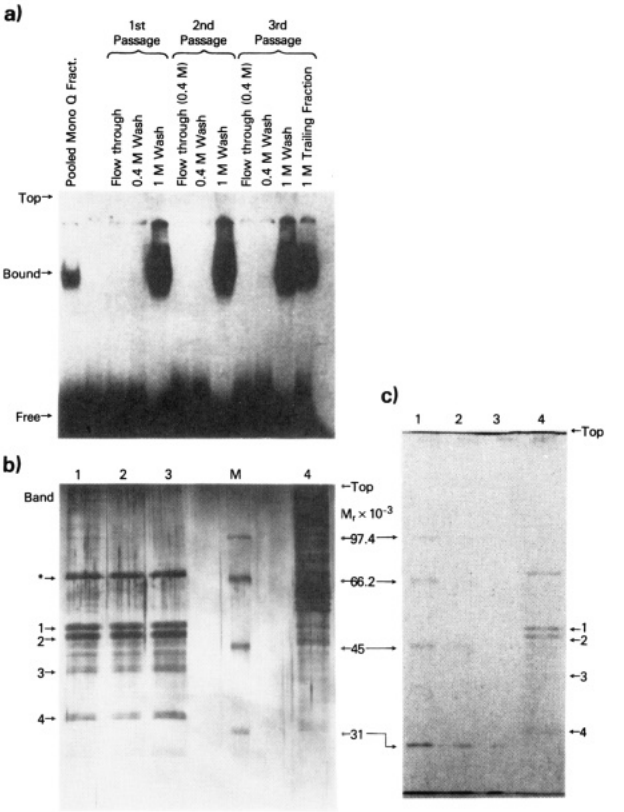

FIGURE 4: Oligonucleotide affinity purification of the palindrome binding proteins. Panel a: Gel mobility shift analysis of the fractions. Samples (1 μL) from each indicated fraction were tested for binding activity. Pooled Mono Q Fract. is fraction III from Table I. The 1 M KCl wash fractions from passages 1 and 2 are each 8-mL total volume. The 3rd passage 1 M KCl wash is fraction IV from Table I. Panel b: SDS–PAGE and silver staining of the final fractions. Samples (5 μL) from each of the 1 M wash fractions from passages 1, 2, and 3 were applied to a 10% SDS–polyacrylamide gel, lanes 1, 2, and 3, respectively, and the gel was eventually stained with silver. Sizes of the marker proteins (lane M) (Bio-Rad low molecular weight markers) are given × 10^{-3}. Lane 4 contained the Mono Q fraction. Bands 1–4 are the major proteins shown to be associated with binding activity in Figure 5. The asterisk indicates a protein doublet that routinely copurifies with the binding activity but does not exhibit detectable binding activity. Panel c: SDS–PAGE and Coomassie blue staining of the final fraction after oligonucleotide affinity purification. The fraction analyzed in lane 4 is the same as the passage 3, 1 M wash on the silver-stained gel (panel b, lane 3). Marker proteins were applied to lanes 1, 2, and 3 (0.4, 0.2, and 0.1 μg, respectively).

fraction was diluted to 0.4 M KCl, salmon sperm DNA was added, and the material was reapplied to the same column. After the column was washed with 0.4 M KCl, the activity was eluted with 1.0 M KCl, diluted to 0.4 M KCl, and applied to a 1-mL oligonucleotide affinity column with no salmon sperm DNA added. The column was washed with 0.4 M KCl and eluted with 1.0 M KCl. This third eluate is fraction IV. The activity was monitored by gel shift assay (Figure 4a). This affinity chromatography step gave an apparent increase in activity, possibly due to inhibitory material in the Mono Q fraction. The active fractions from each passage over the affinity column were analyzed by SDS–PAGE and silver staining (Figure 4b). The first oligonucleotide column provided most of the purification. The subsequent columns removed a few higher molecular weight minor bands but did not change the predominant bands (passages 2 and 3). All of the bands are present in similar amounts after each column, indicating that each cofractionates with the binding activity through this oligonucleotide affinity step. The relatively abundant polypeptides measured by silver staining (Figure 4b) are at M_r

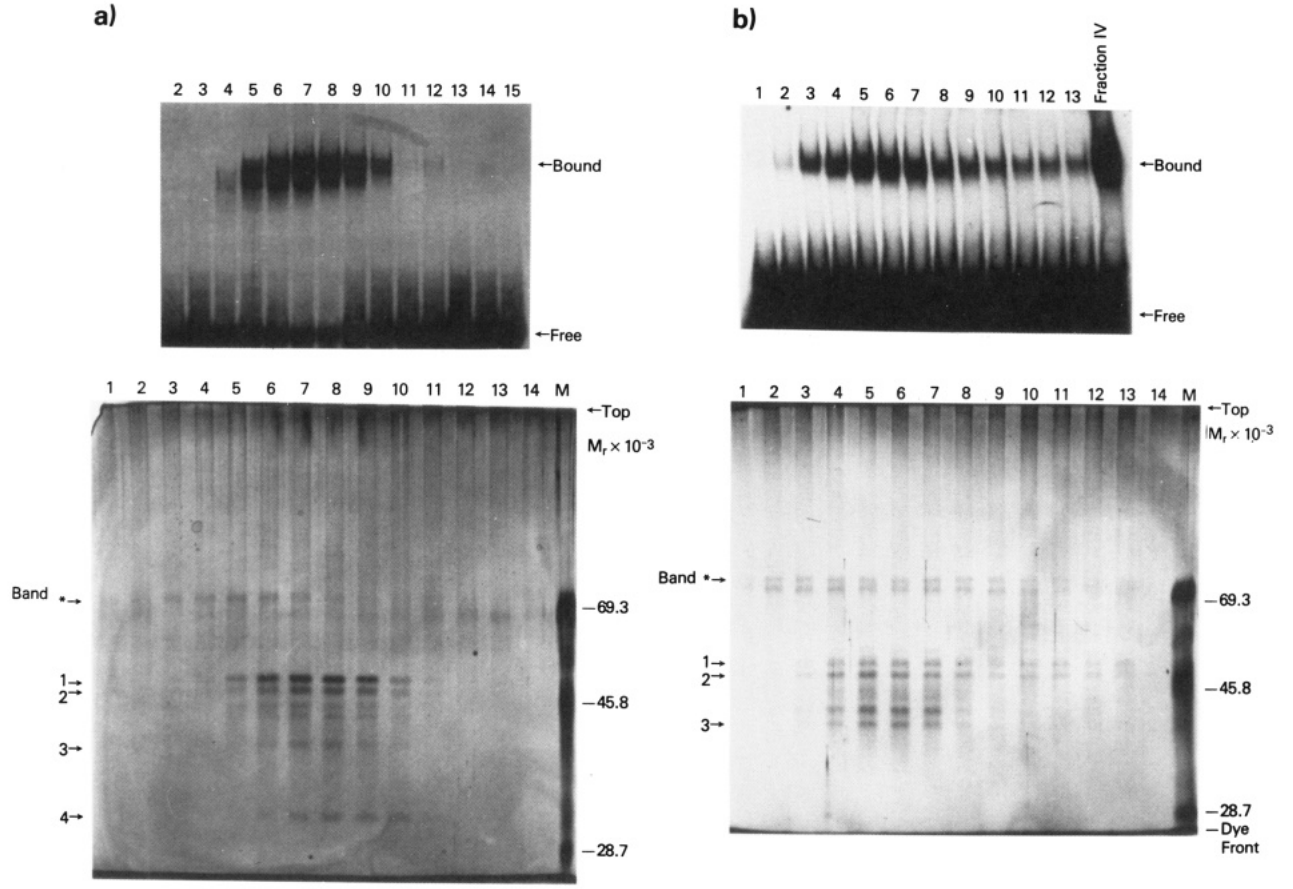

FIGURE 5: Cofractionation of fraction IV using glycerol gradient sedimentation (panel a) and gel filtration (panel b). For each panel, the top figure is a gel mobility shift assay of the fractions, and the bottom is a silver-stained SDS–polyacrylamide gel of the same fractions. Protein standards are prestained low molecular weight markers (BRL) and migrate slightly slower than unstained proteins. Panel a: Glycerol gradient sedimentation. The activity of each fraction is shown in the top figure. The proteins present in each fraction are visualized at the bottom. The proteins are numbered and designated as in Figure 5. Panel b: Gel filtration analysis. A portion of fraction IV was passed over a Pharmacia FPLC Superose 12 column and analyzed as above. The protein pattern is changed slightly from the starting material, presumably due to proteolysis occurring during chromatography, which was performed at room temperature.

32 000, 38 000, 49 000, 52 000, and 70 000. The relative abundance of each of the polypeptides was confirmed by Coomassie blue staining of the same fraction (Figure 4c).

In order to determine which of these proteins are associated with the DNA binding activity, we subjected fraction IV to several fractionation procedures. First, a portion of the final fraction was subjected to 10–40% glycerol gradient centrifugation, and as a control some of the final fraction was mixed with standard proteins and run in a parallel gradient. Sedimentation of the binding activity produced a peak between BSA and ovalbumin. All four relatively abundant polypeptides of M_r 32 000–52 000 cosedimented with the binding activity, whereas the peptides of M_r 70 000 were clearly separated from the binding activity. Therefore, the 70 000-kDa proteins have affinity for the oligonucleotide column and purify with the binding activity but fail to produce binding as measured by gel mobility shift assay. Another aliquot of fraction IV was passed over a Superose 12 gel filtration column. The binding activity eluted between BSA and IgG (Figure 5b), at the same position as the activity found in 293 cell extracts (Figure 2b) and in fraction I of the testes extract (not shown). The SDS–PAGE pattern of the fractions is shown in Figure 5b. The active fractions contain all of the proteins in the starting material. However, the 70-kDa proteins were found in similar levels in all the fractions whereas the smaller proteins, from 32 to 52 kDa, cofractionate with the binding activity. Some proteolysis apparently occurred on this column, resulting in slight changes in the protein pattern. This column was run at room temperature. There is a difference in the apparent size of the native binding activity obtained by glycerol gradient centrifugation and gel filtration. The gel filtration results were repeated with different salt and glycerol concentrations and with a Sephacryl S300 column with no differences in results (not shown). A portion of an active fraction lacking the 70-kDa protein (fraction 7) from the glycerol gradient was also run on the Superose 12 column, and the activity was found to elute at 100–120 kDa. The most likely explanation for these results seems to be that the native activity is a 100–120-kDa complex (e.g., dimer) of the peptides in fraction IV and that for some reason the complex dissociates under the conditions of glycerol gradient centrifugation.

To further explore the relationship between the peptides in the final fraction and the binding activity, we attempted to renature the activity from the various peptides after separation by SDS–PAGE. However, the recovery of activity by renaturation was low, less than 1% (data not shown). The proteins larger than 50 kDa did not give any activity. The 49-kDa band, however, gave a small amount of activity, resulting in a band shift in a similar position as the starting material. More activity was recovered from the region of about 35 kDa, but this resulted in a different band shift of higher mobility than the starting material. Since the amount of activity in this fraction was less than 1% of that in the starting material, we think this may be due to a minor contaminating activity that is able to renature more readily. It is possible that the low recovery of activity may indicate a requirement for dimer formation between peptides, consistent with the size estimate from gel filtration studies.

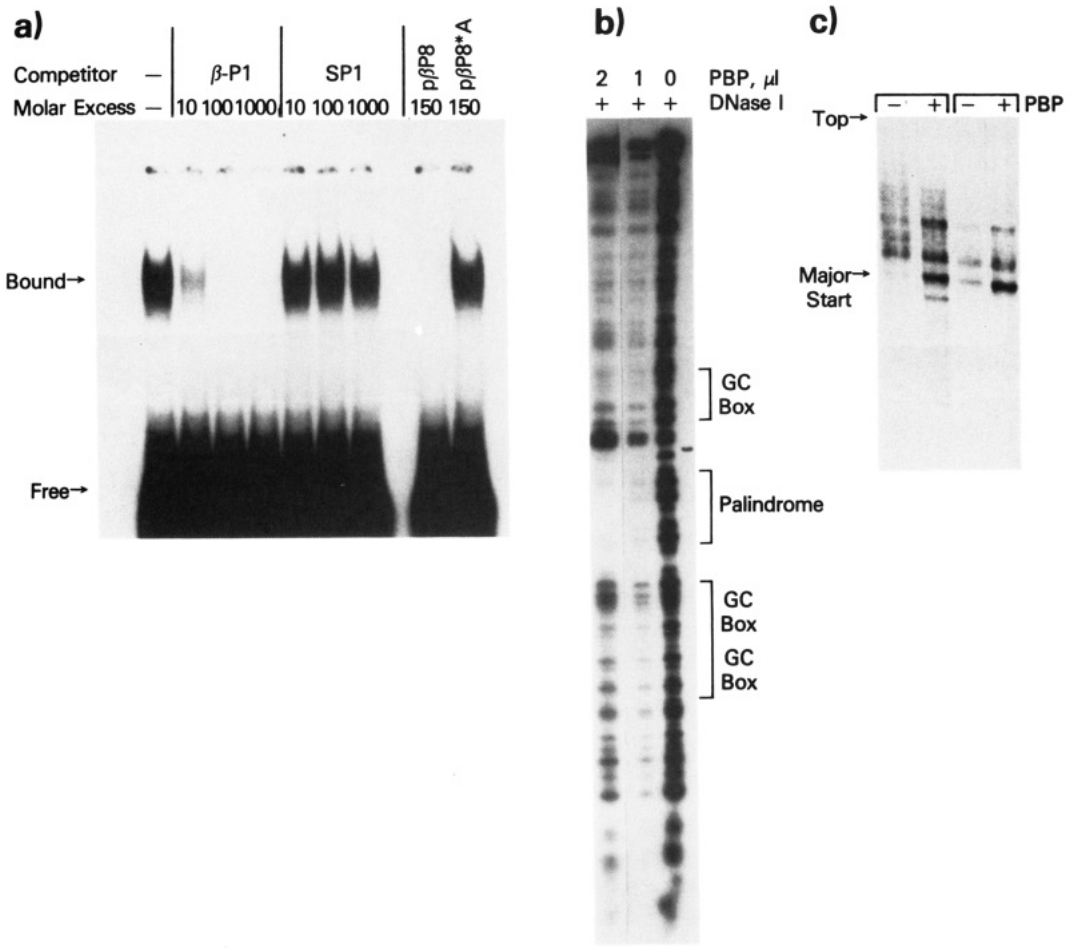

FIGURE 6: Characterization of the purified binding protein. Panel a: Specificity of binding of fraction IV. Labeled β-pol oligonucleotide was analyzed by gel mobility shift analysis. Competition for binding was with the indicated amounts of specific (βP-1) and nonspecific (Sp1) oligonucleotides and the normal (pβP8) and mutant (pβP8*A) β-pol promoter fragments. Panel b: DNase I footprinting. End-labeled β-pol promoter fragment was incubated with 1 or 2 μL of fraction IV, then digested with DNase I, and resolved on a DNA sequencing gel. A control lane with no added binding protein is shown with the positions of the palindrome sequence and the flanking Sp1 binding sites (GC boxes) indicated. Panel c: In vitro transcription. Plasmid pβP8 (Widen et al., 1988) was incubated in an in vitro transcription reaction using a whole cell extract. The plasmids were preincubated with fraction IV (lanes marked +PBP) or with buffer alone. RNA products were analyzed by S1 nuclease mapping. The two brackets indicate duplicate experiments.

The activity of the final fraction was characterized in more detail for binding specificity. Competition results with a gel shift assay show that the final fraction is competed by the same specific competitors as the activity found in the crude extracts (Figure 6a). The fraction gave a DNase I footprint on the β-pol promoter covering about 20 nt of the sense strand (Figure 6b), identical with the footprint seen with nuclear extracts (Englander & Wilson, 1990). The protein also causes a hypersensitive spot just 3′ to the binding site, between the palindrome and the single Sp1 site (labeled "GC Box"), which is also seen with nuclear extracts. Finally, the final fraction was added to an in vitro transcription assay using the wild-type promoter as a template. The added protein caused a severalfold increase in the mRNA initiated specifically from the β-pol promoter (Figure 6c). Thus, the final fraction binds specifically to the palindrome sequence of the β-pol promoter and is able to increase transcription from the promoter.

Filter binding assays have been used to study the binding kinetics of sequence-specific DNA binding proteins, notably the *lac* repressor (Riggs et al., 1970a,b). We used such an assay to study the properties of fraction IV. Initially, to test the effectiveness of this assay with our system, we performed parallel reactions and analyzed half by gel shift assay and half by filter binding. The results showed that for each assay, the same amount of protein was required to saturate binding (data not shown). When the probe was completely bound by protein, all of it was retained on the filter. In addition, we tested the glycerol gradient fractions from the Figure 6a by the filter binding assay and found that the activity peaks in the same fractions as measured by gel shift assay (data not shown), indicating that the 70-kDa proteins are not detected by the filter binding assay.

The filter binding assay was used to measure kinetic properties of binding by fraction IV. First, the precise protein concentration was confirmed by mixing increasing amounts of protein with a known amount of DNA probe, at a concentration several orders of magnitude greater than the K_D for binding. Under these conditions, when probe exceeds protein, the protein binding is essentially complete, and the amount of bound probe gives the concentration of binding activity (Riggs et al., 1970b). Next, the on rate, k_{on}, was determined by studying the time course of complex formation at a selected concentration of probe (5×10^{-12} M) and binding protein (2.5×10^{-11} M). These experiments were performed at 0.2 M KCl. The binding reaction reached equilibrium in about 20 min with an apparent single-exponential binding curve of $t_{1/2} = 30$ s (Figure 7a). The on rate calculated from this experiment is 7×10^8 M^{-1}, s^{-1}. Similar results were obtained by using different probes and concentrations. The off rate, k_{off}, of the complex also was measured in the experiment shown in Figure 7b. The binding reaction was allowed to go to equilibrium, and then a large excess of unlabeled

Table II: Summary of Binding of Bovine Testes PBP to Different Promoter Oligonucleotides

Promoter	ATF/CREB Element	Kinetics k_{off} $t_{1/2}$ (min.)	Kinetics k_{off} (x10^{-4}/sec.)
ß-pol	GCCCAGGACGCGTGACGTCACAACAA	13	9
Parathyroid Hormone	GATCCGGAGTGACGTCATCTGTAA	10	12
Somatostatin	GATCCTTGGCTGACGTCAGAGAGAGA	1	115
Chorionic Gonadotropin	GATCCAAATTGACGTCATGGTAA	1	115
Glucagon	GATCCGGCTCATTGACGTCAAAATTCAT	Not Detectable	

competitor DNA was added as a trap for dissociated protein. The loss of complex over time occurred by a single exponential as expected (Figure 7b) and corresponded to a half-life of about 9.5 min or a rate of 1.2×10^{-3} s^{-1}. The same rate was obtained when the experiment was performed by diluting the reaction rather than adding competitor DNA. The calculated K_A from these k_{on} and k_{off} values is 5.8×10^{11} M^{-1}, a value similar to the value reported for the *lac* repressor in the presence of 0.2 M KCl (Riggs et al., 1970a).

The effect of ionic strength on complex formation between the purified protein and promoter probe was evaluated (Figure 7c). Complex formation is very sensitive to the presence of KCl in the binding mixture at levels greater than 200 mM. Inhibition of complex formation follows a linear pattern when plotted against the square root of ionic strength in the usual fashion (data not shown), and from the slope of this plot, it is deduced that many charge–charge interactions are involved in the binding reaction. Analysis of the binding data by the log K_A vs log [KCl] method (Figure 7d) described by Record et al. (1976) revealed that K_A is substantial when the salt concentration is extrapolated to the reference state of 1 M, suggesting a nonelectrostatic contribution to the overall binding free energy at 200 mM KCl. The relationship between log K_A and log [KCl] was described by a linear plot with slope of about −7.4 (Figure 7d). We assume that this slope value is equal to the term $-m'$, where m' is the number of ion pairs formed between the protein and DNA and ψ is the fraction of counterions bound per DNA phosphate (i.e., about 0.88 in our calculations) (Record et al., 1976). Therefore, we estimate the palindrome binding protein forms about nine ion pairs upon DNA binding with an overall binding free energy value of about −16 kcal/mol in the presence of 0.2 M KCl. The value for the nonelectrostatic contribution to this binding energy, estimated by extrapolating the KCl titration data to 1 M, appears to be approximately 37%, or −6 kcal/mol, which is consistent with several high-energy contacts between the protein and DNA on the order of 1–2 kcal/mol each. These results are quite similar to results obtained by others on effects of NaCl or KCl on interactions between sequence-specific DNA binding proteins and their target sites. Typically, there is a large ionic component to the binding.

Finally, we used the filter binding assay to test if we could detect differences in binding with our purified protein to ATF/CREB sites from various other genes. To conduct such a comparison, we chose to examine k_{off} values. This approach has an advantage over measurement of K_d because k_{off} is independent of probe or protein concentration, thus eliminating the difficulty of accurately quantifying the specific activity

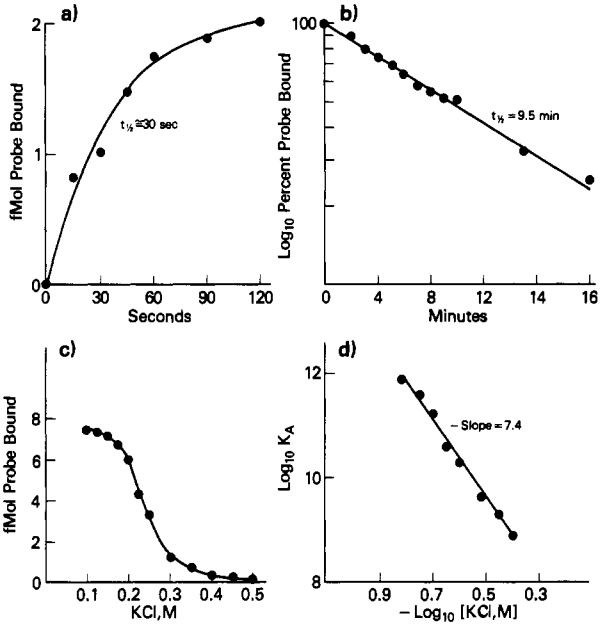

FIGURE 7: Binding properties of fraction IV measured by filter binding assays. Experimental procedures are described under Materials and Methods. (a) Determination of the on rate (k_{on}) using 5×10^{-12} M DNA probe and 2.5×10^{-11} M protein (binding activity). (b) Off rate determination. Binding reactions were allowed to go to completion; then unlabeled competitor DNA was added, and samples were assayed at the indicated time points. (c) Effect of salt concentration on the binding of protein to the β-pol promoter. Protein was mixed with DNA at the indicated salt concentrations and incubated until equilibrium was reached. (d) The data in panel c are plotted as log K_A vs −log [KCl] in order to determine the slope.

and annealing of oligonucleotides. In Table II, the k_{off} values and the particular sequences are listed. As expected, the βP-1 palindrome oligonucleotide gave a similar off rate as the complete promoter. The PTH oligonucleotide was virtually identical with βP-1, whereas the SMS and CG oligonucleotides had a $t_{1/2}$ about 10-fold less than the βP-1. These differences parallel those seen in Figure 1 and show that we can detect differences between sequences kinetically.

Discussion

The β-polymerase gene is one of many genes that contain an ATF/CREB-type sequence in the promoter region. It differs from the majority of well-characterized ATF/CREB element promoters in that the β-pol gene has a G+C-rich "housekeeping"-type promoter with no apparent TATA or CCAAT box regulatory elements. Thus, one might expect that the β-pol promoter could function differently than some other

promoters containing ATF/CREB sequences. In at least one case, this is true, because we found that the HTLV I *tax* protein represses transcription from the β-pol promoter whereas it appears to activate other promoters through ATF/CREB-containing elements (Jeang et al., 1990). In this report, we were able to show that proteins in human tissue culture cells bind differently to the β-pol promoter than to the CG and SMS sequences that are known to be CRE elements. Then we were able to purify an active fraction from bovine testes that binds tightly to the β-pol palindrome. We used a filter binding assay to determine kinetics of binding of the protein to DNA and found that overall the binding is highly dependent on ionic strength and is similar to that of the *lac* repressor to its operator. Using the same filter binding assays, we determined that the binding protein complexed with ATF/CREB elements from the SMS and CG promoters had a 10-fold faster off rate in 0.2 M KCl than the β-pol promoter complex. Thus, the binding properties of the β-pol ATF/CREB site are distinguishable from those of the SMS and CG promoters in the specific case of our purified testes protein. Interestingly, the PTH ATF/CREB element bound the purified protein with affinity similar to the β-pol sequence.

A comparison of the various probe sequences tested here (Table II) reveals no obvious reason for the differences in binding. All have the same 8 bp palindrome TGACGTCA, with different flanking sequences. The only similarity between the β-pol and PTH flanking sequences is the 5′ G of the 10 bp element. It is possible that if the protein makes a single contact at this point it would be sufficient to explain the differences in binding. It is interesting to note that this residue as well as the next three 5′ bp are conserved between the human and murine β-pol promoter sequences. We are currently cloning the β-pol promoter from bovine as well as other species to determine if these nucleotides are conserved in other species. In addition, direct mutagenesis of these nucleotides in the β-pol promoter will determine which are responsible for the differences in binding between different genes.

The purification procedure used here depends critically on the first oligonucleotide affinity column chromatography step and also upon Mono Q chromatography by FPLC. The tight binding of the protein to the palindrome sequence allowed the oligonucleotide column to be washed and loaded at 0.4 M KCl, which should remove most of the nonspecific DNA binding proteins. After the Mono Q step, the binding activity had been substantially purified and was stable during repeated oligonucleotide chromatography steps and also to concentration by membrane dialysis. However, we found that the binding activity in the crude nuclear extract was relatively unstable during conventional chromatography steps such as DEAE-cellulose, phosphocellulose, Sephacryl S-300 gel filtration, and hydroxyapatite, resulting in losses of specific activity for each. The heparin–agarose step gave a small increase in specific activity, but also caused the only major loss of activity in the procedure. The overall increase in specific activity was estimated to be about 100 000-fold, although it should be noted that calculation of the activity in crude fractions is difficult.

Analysis of the polypeptide composition of the final fraction revealed three major polypeptides of M_r 49 000, 52 000, and 70 000 and several smaller less abundant polypeptides. The M_r 70 000 protein sometimes resolved into a doublet depending on the gel conditions. Silver staining and Coomassie blue staining gave the same results for the relative abundance of each polypeptide. Hydrodynamic separation experiments, testing for comigration of peptides and binding activity, indicated that the M_r 70 000 species was not responsible for the binding activity, whereas the other peptides did comigrate with the binding activity. These results and the behavior of the binding activity during gel filtration are consistent with the idea that the native binding activity is a complex of the M_r 49 000 and 52 000 polypeptides. The other peptides may be proteolytic fragments of the active peptides or different members of the ATF/CREB family of proteins. Finally, many proteins are able to bind in vitro to ATF/CREB-related elements, and a particular cell type may have several of these ATF/CREB factors present. It is likely that these different proteins may have different functions and may not interact equally with the many genes that contain similar sequences. Thus, it is important to understand how the proteins can discriminate between different promoters. These proteins have been identified based on binding or transcriptional activity using various promoters. However, given the complexity of factors present, it is possible that the specific proteins that are obtained at the end of a purification scheme may not be the ones that regulate the gene of interest in vivo. A protein could bind to a promoter and activate transcription in vitro but still not be the one responsible for the regulation of that gene. Likewise, the inability of a purified factor to activate transcription in vitro does not necessarily mean that the factor does not regulate the gene but instead may indicate that some other factor is required for activation. We think that at this point we cannot determine whether or not the protein fraction we purified is important for regulation of the β-pol gene in vivo. One approach to answering this question will be analyzing the effect of point mutations in the promoter to correlate effects on protein binding with in vivo expression of the gene.

Registry No. DNA polymerase, 9012-90-2; somatostatin, 51110-01-1; parathyroid hormone, 9002-64-6; chorionic gonadotropin, 9002-61-3.

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Processivity of T7 RNA Polymerase Requires the C-Terminal Phe^{882}-Ala^{883}-COO$^-$ or "Foot"†

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ABSTRACT: The role of the C-terminal Phe^{882}-Ala^{883} residues of bacteriophage T7 RNA polymerase in specific transcription has been investigated by means of site-directed mutagenesis. A mutant enzyme that lacks the C-terminal Phe^{882}-Ala^{883} residues, denoted the "foot" mutant, has been cloned and overproduced, and the effects of the deletion on promoter recognition, initiation, and elongation have been determined. Gel retardation assays and DNase I footprinting show that the foot mutant specifically recognizes and binds to T7 promoters, although this binding appears to be approximately 30-fold weaker than that of the wild-type enzyme. Transcription assays using oligonucleotide templates that contain the consensus T7 promoter show a dramatic decrease in transcriptional activity for the foot mutant. With templates whose coding region begins CCC..., the mutant synthesizes poly(G) products even in the presence of all four nucleotides. The synthesis of poly(G) products from such templates has previously been observed for the wild-type enzyme when GTP is the sole nucleotide present in the reaction and is thought to occur by a novel mechanism involving slippage of the RNA chain 3′ to 5′ relative to the template [Martin, C. T., Muller, D. K., & Coleman, J. E. (1988) *Biochemistry 27*, 3966-3974]. These data suggest that the loss in transcriptional activity by the foot mutant results from a severe decrease in processivity as well as catalytic efficiency of the enzyme. Removal of the C-terminal Phe and Ala residues from the wild-type enzyme with carboxypeptidase A generates the phenotype of the mutant precisely, proving that all of the properties of the foot mutant derive from the loss of the Phe-Ala-COOH moiety. Protection of the C-terminal residues in the wild-type enzyme from hydrolysis by carboxypeptidase A in the presence of T7 promoter-containing DNA shows that these C-terminal residues may be intimately involved in the DNA–protein interactions, thereby explaining their crucial role in all phases of transcription.

The RNA polymerase from bacteriophage T7 consists of a single polypeptide chain of 883 amino acids that is capable of carrying out all the functions required for the initiation of specific transcription at the phage-specific promoters on the T7 genome. The enzyme recognizes and binds to a phage-specific promoter sequence from −1 to −17 relative to the transcription start site on one face of the DNA helix (Muller et al., 1989). During the early stages of transcription, there is a high probability of dissociation of the ternary enzyme–template–mRNA complex, which leads to abortive cycling (Martin et al., 1988). This dissociation of the ternary complex diminishes dramatically after the incorporation of about 8 bases into the message (Martin et al., 1988; Muller et al., 1988).

Extensive studies on the mechanism of action of T7 RNA polymerase in this laboratory have shown that the protein molecule can be divided into distinct functional domains. The C-terminal (~80K) domain, which can be prepared by partial proteolysis of the enzyme with trypsin, has been shown to contain the promoter recognition and binding function as well as the catalytic center of the enzyme, while the N-terminal

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