Purification and Characterization of a DNA Polymerase β Promoter Initiator Element-Binding Transcription Factor from Bovine Testis†

Feng He, Satya Narayan, and Samuel H. Wilson*

Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, Texas 77555-1068

Received November 1, 1995⊗

ABSTRACT: A low-abundance DNA-binding protein for the DNA polymerase β (β-pol) promoter initiator element was purified from bovine testis. The transcriptional initiator element (Inr) of the mammalian β-pol promoters characterized is highly conserved, and the bovine β-pol promoter Inr has the sequence $^{-11}$CAGAGGCGGCCATTGTT^{+6}. The purified initiator element-binding protein (Inr-BP) binds with high affinity to an oligonucleotide corresponding to the β-pol promoter Inr ($K_d = 5$ pM), and increasing ionic strength decreases stability of the protein−DNA complex. Mutational analysis of the Inr shows that the purified Inr-BP binds with sequence specificity to the sequence CCAT at −2 to +2 of the Inr, but that seven residues on the 5′ side and three residues on the 3′ side of the CCAT sequence are required also. Using an *in vitro* transcription assay with HeLa cell nuclear extract, we find that the endogenous Inr-BP is required for transcriptional activity of the β-pol promoter; addition of purified Inr-BP restores activity to the nuclear extract depleted in Inr-BP by affinity chromatography. These results, based upon the sequence specificity for DNA binding, indicate that Inr-BP is a YY1-like protein and suggest that it is a required transcription factor in β-pol gene expression.

DNA polymerase β (β-pol)[1] is one of the required components of the mammalian DNA repair pathway known as base excision repair, conducting the short gap-filling DNA synthesis step prior to DNA ligation (Singhal et al., 1995; Sobol et al., 1996). After treatment of Chinese hamster ovary (CHO) cells with monofunctional DNA alkylating agents, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) or methyl methanesulfonate (MMS), β-pol gene transcription and the cellular β-pol level are up-regulated, and the mechanism of this up-regulation through the protein kinase A pathway is under investigation (Englander & Wilson, 1992; Kedar et al., 1991; Narayan et al., 1995; Srivastava et al., 1995; Wilson, 1990). Several mammalian β-pol promoters, including human, rodent, and bovine, have been cloned and characterized (Widen et al., 1988; Yamaguchi et al., 1989; Chen et al., 1995). TATA elements located just upstream of the transcriptional start sites have not been found in these β-pol promoters, and β-pol promoters are considered members of the family of "TATA-less promoters". These mammalian β-pol promoters share many similarities, including multiple GC boxes and a lone CRE site, to which transcriptional activators of the ATF/CREB family bind, especially ATF-1 and CREB-1 (unpublished data). These proteins are known to be important regulators of β-pol promoter transcriptional activity (Widen et al., 1988; Narayan et al., 1994, 1995). The β-pol promoters also share a conserved sequence at and around the transcriptional start site, termed the Inr.

A model for assembly of the RNA polymerase II closed preinitiation complex (RP_c) with TATA-containing promoters has been developed. The TATA element is generally present ~30 bp upstream of the start site, and assembly of RP_c consists of multiple sequential steps after the initial binding of TFIID at the TATA element; binding of several other basal transcription factors, and eventually RNA pol II, proceeds in an ordered sequential fashion leading to the assembly of RP_c (Maldonado & Reinberg, 1995). However, with TATA-less promoters, such as mammalian β-pol, TFIID does not bind directly to the promoter, and RP_c assembly in these promoters is thought to hinge instead on Inr-BP (Usheva & Shenk, 1994). The TATA-less promoters have been divided into five subgroups according to the DNA sequence of the respective Inr. These subgroups are termed AAV p5, TdT, PBGD, DHFR, and ribosomal, respectively, after the various promoters with which the subgroups were characterized (Weis & Reinberg, 1992). In all TATA-less promoters, the Inr is considered critical in positioning the RP_c for initiation of pre-mRNA synthesis, and it appears that the role of the Inr is to act as a DNA-binding site for Inr-BP; after Inr-BP binds at the Inr, assembly of general transcription factors occurs to form the RP_c (Weis & Reinberg, 1992). Several different Inr-BPs have been identified, including TFII-I (Roy et al., 1991, 1993), E2F (Blake & Azizkhan, 1989; Means & Farnham, 1990; Means et al., 1992), CBF (Garfinkel et al., 1990; Safer et al., 1991), and YY1 (Seto et al., 1991; Shi et al., 1991; Hariharan et al., 1991; Usheva & Shenk,

† This work was supported by NIH Grant ES06492.

* Address correspondence to this author at the Sealy Center for Molecular Science, 5.104 Medical Research Building, Route 1068, 301 University Blvd., University of Texas Medical Branch, Galveston, TX 77555-1068. Telephone: (409)-772-3367. Fax: (409)-772-6334.

⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1996.

[1] Abbreviations: ATF, activating transcription factor; CREB, cAMP response element-binding protein; β-pol, DNA polymerase β; RNA pol II, RNA polymerase II; YY1, cellular ying-yang-one transcription repressor/activator; Inr, initiator element; Inr-BP, initiator element-binding protein; RP_c, closed preinitiation complex; NE, nuclear extract; NEd, initiator element-binding protein depleted nuclear extract; AAV p5, adeno-associated virus p5; DHFR, dihydrofolate reductase; MLP, major late promoter; PBGD, porphobilinogen deaminase; TdT, terminal deoxynucleotidyl transferase; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MMS, methyl methanesulfonate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

0006-2960/96/0435-1775$12.00/0

1994). All of these Inr-BPs specifically bind to the Inr of respective TATA-less promoters and appear to facilitate assembly of the RP_c.

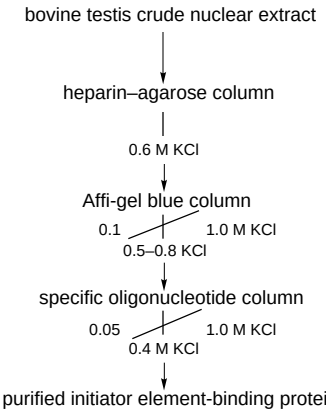
Previously, we found that mammalian nuclear extract proteins bind to the Inr of the human β-pol promoter, as revealed by the DNase I footprinting assay (Englander & Wilson, 1990), and that purified TFIID could not bind to the core β-pol promoter. The Inrs of the bovine and human β-pol promoters are similar and have sequence homology with the Inr of the adeno-associated virus (AAV) p5 promoter, which is known to bind the Inr-BP termed YY1 or ying-yang-one. Therefore, the β-pol promoter Inr is a member of the AAV p5 subgroup. Transcriptionally, YY1 has been proposed as the Inr-BP for this subgroup (Weis & Reinberg, 1992). In addition, YY1 appears to be capable of either up-regulation or down-regulation of several cellular and viral promoters. In the present study, we purified a bovine testis YY1-like Inr-BP. Characterization of the DNA-binding properties of this protein showed that it has very high affinity for the Inr of both the β-pol promoter and the AAV p5 promoter, but not for other TATA-less promoter Inrs or for the model TATA-containing promoter, the adenovirus major late promoter (MLP). Our results also indicate that the purified bovine Inr-BP is a transcription factor required for β-pol promoter transcriptional activity *in vitro*.

MATERIALS AND METHODS

Purification of Inr-BP from Bovine Testis. Nuclear extract was prepared from frozen bovine testis (Pel-freez Biologicals, Rogers, AR) as described by Widen and Wilson (1991). All procedures were done at 4 °C. In brief, 400 g of tissue was minced in 3 volumes of buffer A (10 mM HEPES, pH 8.0, 1.5 mM $MgCl_2$, 100 mM NaCl, 10 mM sodium metabisulfite, 0.5 mM DTT, 1 mM PMSF, and 1 μg/mL pepstatin A) and homogenized with a blender. The homogenate was pelleted by centrifugation at 10000*g* for 20 min. The pellet was resuspended in 1.5 volumes of buffer B (buffer A plus 1 M NaCl) and blended for 30 s. The homogenate was centrifuged at 100000*g* for 1 h. The clear supernatant fraction was brought to 50% saturation by adding solid $(NH_4)_2SO_4$ with stirring. The precipitate was recovered by centrifugation for 20 min at 15000*g*. The pellet was resuspended in buffer C [10 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM DTT, 1 mM PMSF, 1 μg/mL pepstatin A, and 10% (v/v) glycerol], and the solution was dialyzed against the same buffer for 14 h. Nuclear extract was then clarified by centrifugation at 10000*g* for 20 min. The method of purification of Inr-BP is summarized in Scheme 1.

The crude nuclear extract fraction was applied on a 100 mL heparin−agarose column (Sigma) with a flow rate of 2 mL/min. The column was washed with 4−5 volumes of buffer C containing 400 mM KCl. The Inr-BP-binding activity was step-eluted from the column with 0.6 M KCl in buffer C. The 0.6 M KCl fraction was diluted to bring the KCl concentration to 0.1 M and applied to a 50 mL Affi-gel blue column (BioRad) with a flow rate of 0.5 mL/min. Bound protein was eluted with a linear gradient of KCl (0.1−1 M) in buffer C. The specific activity of Inr-BP was recovered between the 0.5 and 0.8 M KCl gradient fractions. The active fractions were pooled and dialyzed against buffer D [10 mM HEPES, pH 8.0, 50 mM KCl, 10% (v/v) glycerol,

Scheme 1



1 mM DTT, 1 mM PMSF, and 1 μg/mL Pepstatin A]. The pooled fractions were mixed with 4 mg each of sonicated and heat-denatured salmon sperm DNA. This mixture was applied to a 3 mL β-pol initiator element-specific oligonucleotide affinity column with a flow rate of 0.5 mL/min. The column was prepared as described by Wu et al. (1988), except that the oligonucleotide was not ligated before coupling to CNBr-activated Sepharose 4B resin (Pharmacia Biotech). The column was washed with 10 mL of 50 mM KCl in buffer D, and the bound protein was eluted with a gradient of KCl (50 mM − 1 M) in buffer D. Most of the binding activity appeared in the 0.2−0.4 M gradient fractions. The active fractions were pooled and reapplied to the oligonucleotide affinity column, and the process was repeated 3 times. The purity of the protein was determined by resolution with SDS−PAGE and staining with silver stain.

UV Cross-Linking of Purified Inr-BP to ^{32}P-Labeled β-pol Initiator Element-Specific Oligonucleotide. The β-pol initiator element-specific oligonucleotide (5′-GGCGGCCATT-3′) was 5′-end-labeled with [γ-^{32}P]ATP, annealed to its complementary strand, and then purified using a Nensorb-20 column (DuPont). The labeled oligonucleotide (2 ng) was incubated for 30 min at room temperature with 10 μL of purified protein in a final volume of 20 μL containing 20 mM HEPES, pH 8.0, 50 mM KCl, 1 mM DTT, 100 μg/mL BSA, 10 ng of salmon sperm DNA, and 10% (v/v) glycerol. The samples were spotted on parafilm and irradiated at 254 nm using a UV-stratalinker (Stratagene) for various times. The samples were boiled for 5 min in SDS−PAGE sample buffer and separated by 10% SDS−PAGE. The gel was dried and exposed to X-ray film for autoradiography.

In Vitro Runoff Transcription Assay. HeLa cell nuclear extract (NE) was prepared as described (Shapiro et al., 1988). The Inr-BP-depleted NE was prepared by passing NE through a β-pol initiator element-specific oligonucleotide affinity column 3 times. The flow-through was collected and used as Inr-BP-depleted NE. The extent of Inr-BP depletion was determined by the electrophoretic mobility shift assay (EMSA) as described below. The wild-type β-pol promoter (pβP8) was linearized with *Pvu*II to generate the 210 nt runoff transcript (Narayan et al., 1994). The reaction mixture in a final volume of 25 μL contained 20 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 2 mM DTT, 65 mM KCl, 10% (v/v) glycerol, 1% (w/v) polyvinyl alcohol, 20 units of RNasin (Promega), 1.5 μg of plasmid, and 40 ng of Inr-BP-depleted NE, supplemented with increasing amounts of purified Inr-BP. The reaction mixture was preincubated at

22 °C for 30 min. The NTPs solution (500 μM each of ATP, GTP, and UTP and 25 μM [α-^{32}P]CTP) was added, and the incubation was continued for an additional 60 min at 22 °C. RNA transcripts were extracted with chloroform/phenol, precipitated with ethanol, and resolved on a 6% polyacrylamide/8 M urea gel.

Filter-Binding Assay. The filter-binding assay was performed as described previously (Widen & Wilson, 1991; DasGupta et al., 1993). The concentration of 19-mer double-stranded initiator element-specific oligonucleotide used in the binding assay was determined from its UV absorbance at 260 nm. One hundred nanograms of the oligonucleotide was 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The specific activity and the recovery of purified labeled oligonucleotides from the Sephadex G-25 column were determined by TCA precipitation (Beard & Wilson, 1995). The specific binding activity of Inr-BP was determined by the filter-binding assay. The concentration of oligonucleotide was fixed, and the concentration of Inr-BP was varied. The amount of Inr-BP at saturating oligonucleotide concentration was used to determine the specific binding activity (Riggs et al., 1970). For the filter-binding assay, the binding buffer was the same as in the EMSA, as described below. The k_{on} and k_{off} rates of Inr-BP were determined in a final volume of 160 μL. For k_{on} rate determinations, 10×10^{-12} M ^{32}P-labeled oligonucleotide and the same amount of purified Inr-BP were incubated for 30 min at room temperature, and 3 50 μL portions of the reaction mixture were filtered through 3 individual nitrocellulose membranes (Millipore HA, 25 mm, 0.45 μm) in a 12-position Millipore sampling manifold without washing. The data at each time point were gathered in triplicate. The background on the filter was determined with the same mixture without the addition of protein. For k_{off} rate determinations, the DNA−protein mixture was incubated for 30 min, and a 500-fold excess of unlabled specific initiator element oligonucleotide was added to the reaction. Aliquots of the reaction were filtered at different time points. The calculation of k_{on}, k_{off}, and K_d was performed according to equations described by Lehninger (1978). To determine the salt effect on protein−DNA complex formation, equal amounts of DNA and Inr-BP were equilibrated for 30 min in a reaction buffer containing various KCl concentrations and then filtered through a nitrocellulose membrane.

Electrophoretic Mobility Shift Assay (EMSA). The assay was based on the method described by Fried (1989) and modified by Widen et al. (1991). The initiator element-specific oligonucleotide of the bovine β-pol promoter (CAGAGGCGGCCATTGTTCA) and other promoters (AAV p5, TdT, PBDG, DHFR, ribosomal, and MLP) were labeled with [γ-^{32}P]ATP. Purified Inr-BP (1−2 μL) was mixed with 0.25 ng ^{32}P-labeled double-stranded oligonucleotide in 20 μL of reaction mixture containing 20 mM HEPES, pH 8.0, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 100 μg/mL BSA, 0.5 μg/mL sonicated salmon sperm DNA, and 10% (v/v) glycerol and incubated for 30 min at room temperature. For crude nuclear extract and partially purified Inr-BP, the concentration of sonicated salmon sperm DNA was increased to 100 μg/mL, and BSA was omitted. In competition experiments, different concentrations of unlabeled specific or nonspecific oligonucleotide (GTGCCTGATCCTTACGGTA) were added in excess to the reaction mixture before addition of protein samples. The protein−DNA complexes were separated on a 4% native polyacrylamide gel. Electrophoresis was run in 0.5× TBE buffer (45 mM Tris−borate, 1 mM EDTA, pH 8.0) at 100 V for 2.5 h at room temperature.

Table 1: Purification of Inr-BP from Bovine Testis[a]

fraction	volume (mL)	protein concn (mg) per mL	protein concn (mg) total	total act. (pmol)
nuclear extract	200	11	2200	131.7
heparin−agarose	70	0.73	51.1	175.5
Affi-gel blue	15	0.33	4.95	46.2
DNA affinity	5	0.0005	0.0025	125

[a] 400 g of bovine testis used.

RESULTS

Purification of Inr-BP from Bovine Testis. In preliminary gel mobility shift experiments with crude nuclear extract (NE) from bovine testis and a β-pol Inr oligonucleotide as probe, we observed a protein−DNA complex that was specific for the β-pol Inr (data not shown). Therefore, this assay was used to follow the purification of Inr-BP. Bovine testis was used as the protein source because it is known to contain a high level of β-pol mRNA and is used to purify a ATF/CREB family member that is important for the transcriptional regulation of the β-pol promoter (Widen & Wilson, 1991). A NE was prepared and applied to a heparin−agarose column, as described for bovine ATF/CREB (Widen & Wilson, 1991). Bound proteins were eluted in a stepwise fashion with serial addition of buffer with increasing concentrations of KCl. Most of the Inr-BP DNA-binding activity was present in the 0.6 M KCl fraction. This fraction was diluted to 0.1 M KCl and loaded onto an Affi-gel blue column. The Inr-BP activity was eluted with a linear gradient of KCl, in the 0.5−0.8 M KCl range (data not shown). Use of these two column steps yielded ∼150-fold purification of the Inr-BP. Oligonucleotide affinity column chromatography was used in the final step of purification; active fractions from the Affi-gel blue column were pooled, mixed with salmon sperm DNA, and applied to a β-pol Inr-specific oligonucleotide affinity column. A linear gradient of KCl was used to elute the protein between 0.05 and 1.0 M KCl. Overall purification of Inr-BP is summarized in Table 1. Recovery of activity from the initial NE was >50% and probably reflected removal of an inhibitor in the last step; the specific activity increased over 100 000-fold, suggesting that the quantity of Inr-BP per cell is relatively low.

To confirm the specificity of the purified Inr-BP for the β-pol promoter initiator element, an oligonucleotide competition EMSA was performed (Figure 1). Inr oligonucleotide, but not nonspecific oligonucleotide, blocked Inr-BP binding to the ^{32}P-labeled β-pol initiator element oligonucleotide. To examine the polypeptide composition of the purified Inr-BP, SDS−PAGE analysis was conducted on the final fraction; proteins were visualized with silver stain. The purified fraction contained one major polypeptide of ∼62 kDa, and a minor amount of 40 and 34 kDa polypeptides (Figure 2A). UV cross-linking and Southwestern blotting were used to further examine Inr binding by these polypeptides. Typical results from UV cross-linking are shown in Figure 2B. A single cross-linked polypeptide migrating at ∼70 kDa was observed. The migration of this material was

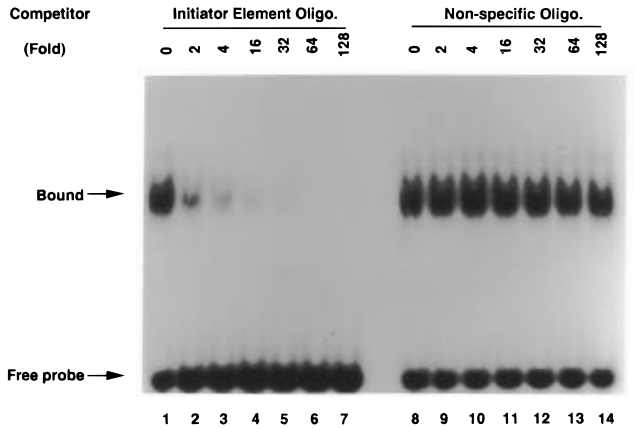


FIGURE 1: Competition analysis of purified Inr-BP binding to the initiator element oligonucleotide by electrophoretic mobility shift assay. ^{32}P-Labeled initiator element oligonucleotide was used as a probe for binding assays. Purified Inr-BP (1−2 μL) was mixed with 0.25 ng of ^{32}P-labeled probe in a 20 μL reaction mixture containing 20 mM HEPES, pH 8.0, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 100 μg/mL BSA, and 10% (v/v) glycerol and incubated for 30 min at room temperature. The competitor oligonucleotide was added to the reaction mixture before addition of purified Inr-BP. Numbers above the lanes indicate the molar excess of unlabeled specific Inr oligonucleotide (lanes 1−7) or unlabeled nonspecific oligonucleotide (lanes 8−14) as competitors. After preincubation, the protein−DNA complexes were separated on a 4% native polyacrylamide gel.

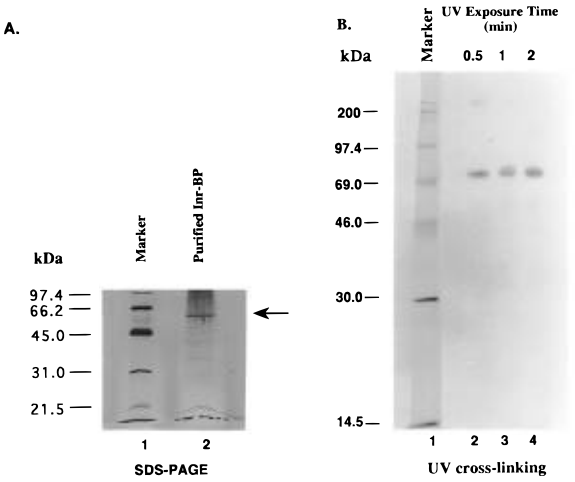


FIGURE 2: Identification of Inr-BP purified from bovine testis by silver-staining and UV cross-linking with ^{32}P-labeled Inr oligonucleotides. Panel A: Inr-BP purified from three passages on an Inr-specific oligonucleotide affinity column was concentrated with Centricon-10 (Amicon) and analyzed by resolving on a 10% SDS−PAGE and silver staining: lane 1, 250 ng of BioRad low molecular mass protein marker; lane 2, 10 μL of purified and concentrated bovine Inr-BP. The arrow indicates a major peptide of ~62 kDa. Panel B: UV cross-linking of purified Inr-BP with specific initiator element oligonucleotides. ^{32}P-Labeled oligonucleotide (2 ng) spanning from the −7 to +3 region of the β-pol promoter (GGCGGCCATT) was incubated with 10 μL of purified Inr-BP in reaction buffer for 30 min at room temperature; the mixture was pipetted on parafilm and exposed to UV light at 254 nm in a UV-stratalinker (Stratagene) at the indicated times (lanes 2−4 for 30 s, 1 min, and 2 min, respectively). Samples were transferred into test tubes and boiled for 5 min in SDS sample buffer. Proteins were separated by 10% SDS−PAGE. The gel was dried and subjected to autoradiography. The arrow indicates the predicted complex of ~62 kDa peptide and 6.5 kDa oligonucleotide to make the ~70 kDa protein−DNA complex.

consistent with the 62 kDa polypeptide cross-linked to one molecule of Inr oligonucleotide. Similarly, the 62 kDa polypeptide was found to be Inr-BP in Southwestern analysis (data not shown).

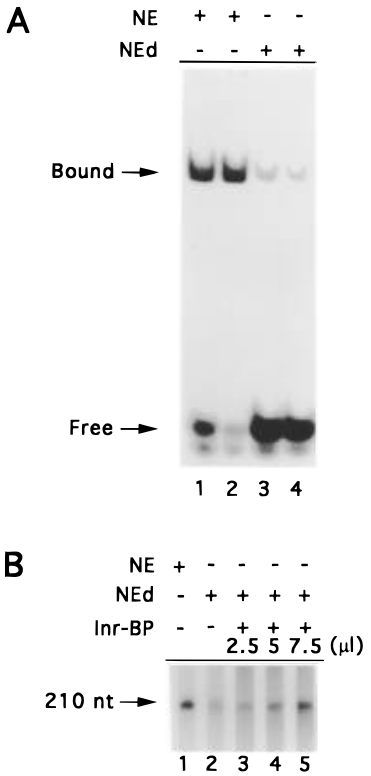


FIGURE 3: Purified Inr-BP stimulates transcriptional activity of the β-pol promoter in an *in vitro* transcription assay system. Panel A: Electrophoretic mobility shift assay to examine the depletion of Inr-BP from HeLa cell nuclear extract (NE). Inr-BP-depleted NE (NEd) was prepared by passing NE 3 times through a β-pol initiator element-specific oligonucleotide column. The flow-through was collected as NEd. The extent of Inr-BP removal in the NEd was determined by electrophoretic mobility shift assay: lanes 1 and 2, undepleted NE (as control); lanes 3 and 4, NEd. Panel B: *In vitro* runoff transcription assay. pβP8 plasmid (1.5 μg) containing wild-type β-pol promoter as template was mixed with NE (lane 1) or NEd, and the indicated amount of purified Inr-BP was added in a standard *in vitro* runoff transcription reaction mixture. Runoff product of 210 nt with NEd alone (lane 2) and with NEd supplemented with 2.5, 5, and 7.5 μL of purified Inr-BP, respectively (lanes 3−5), is shown with an arrow. RNA transcripts were resolved on a 6% polyacrylamide/8 M urea gel.

Purified Inr-BP Activates in Vitro Transcription from the β-pol Promoter. To examine transcriptional activity of the purified Inr-BP, *in vitro* transcription assays were performed. An Inr-BP-depleted HeLa cell NE was prepared by passage of the NE through an Inr-specific oligonucleotide affinity column; the Inr-BP depletion was first verified by EMSA, where Inr-BP-binding activity in the NE was undetectable after the depletion (Figure 3A). The *in vitro* transcription mixture was assembled with β-pol promoter plasmid pβP8 and the Inr-BP-depleted NE, either alone or with purified Inr-BP. The Inr-BP-depleted NE had much lower transcriptional activity than the initial undepleted NE extract (Figure 3B, lanes 1 and 2), indicating that Inr-BP is required for activity. The activity of the depleted extract was restored by addition of the purified Inr-BP (Figure 3B, lanes 3−5). The results indicate that this purified Inr-BP is a required transcription factor for the β-pol promoter.

DNA-Binding Properties of Inr-BP. A quantitative DNA-binding assay was used to characterize properties of the interaction between the purified Inr-BP and Inr oligonucle-

otide. In preliminary studies, we observed that most of the Inr-BP/DNA complex was retained on the nitrocellulose membrane. Yet, the membrane-bound complex was extremely sensitive to washing with binding buffer. Therefore, instead of washing the membrane to reduce background, we used the method described by DasGupta et al. (1993) to correct for background as follows: observed counts $= X + (100 - X)Y$, where $X =$ percent of counts due to DNA−protein complexes and $Y =$ the counts retained on the membrane in the absence of Inr-BP. To determine the rate constant of disassociation (k_{off}), the Inr-BP and 5′-end-labeled Inr oligonucleotide were first preincubated, and then an excess of unlabeled specific oligonucleotide was added. The dissociation of complex corresponded to a first-order decay with a $t_{1/2}$ of ∼10 min and a k_{off} of 1×10^{-3} s^{-1} (Figure 4B). To determine the rate constant of association (k_{on}), 10 pM each of both 5′-end-labeled Inr oligonucleotide and Inr-BP was incubated in binding buffer containing 50 mM KCl for various times at 25 °C. The $t_{1/2}$ for complex formation was ∼25 s (Figure 4A), corresponding to a k_{on} of 4×10^8 M^{-1} s^{-1}. The calculated K_d value from k_{on} and k_{off} was 5×10^{-12} M. The effect of KCl concentration on the Inr-BP/DNA complex was examined also. The amount of complex formed was sensitive to increasing KCl concentrations of the binding buffer (Figure 4C).

DNA Sequence Specificity for the Inr-BP/DNA Interaction. Based upon the initiator element sequences, the TATA-less promoters are divided into five-subgroups: AAV p5, TdT, PBGD, DHFR, and ribosomal. Comparison of the Inr of the bovine β-pol and the AAV p5 promoters reveals homology in the 5 bp sequence (CCATT), and some similarity is seen with Inrs of other TATA-less promoters (Figure 5B). Inr-BP affinity for the bovine β-pol promoter Inr was compared with that of Inrs from these five TATA-less promoter subgroups and also with the Inr of the adenovirus major late promoter (MLP), a TATA-containing promoter. The purified Inr-BP showed binding affinity only with the β-pol and the AAV p5 promoter Inrs (Figure 5A). Thus, the initiator element of the β-pol promoter appears to be functionally similar to that of Inr-BP binding of the AAV p5 promoter. The binding specificity of purified Inr-BP for both human and bovine β-pol Inr oligonucleotides was observed (Figure 7A, lanes 1 and 10). Therefore, these results indicate our purified bovine Inr-BP is a YY1-like protein, binding to the Inr of the β-pol/AAV p5 promoter family. Based upon this similarity, it appears that the bovine Inr-BP is related to the human YY1 protein, which is known to bind human β-pol and AAV p5 promoter Inrs [see for review Weis and Reinberg (1992)].

Various modified β-pol promoter Inr oligonucleotides were used to examine base pair residues involved in binding to the purified Inr-BP. Oligonucleotides designated M1−M4 (Figure 6B) were sequentially truncated in the 5′-flanking region of the initiator element, whereas oligonucleotides designated M5−M10 were truncated in the 3′-flanking region. Binding of these oligonucleotides to Inr-BP as measured by EMSA is illustrated in Figure 6A. The binding efficiency was much less with oligonucleotides having deletions from −11 to −7 as compared with wild-type oligonucleotide. Oligonucleotides with 6 or fewer residues on the 5′-side of +1 failed to show binding activity (Figure 6, lanes 4 and 5). Oligonucleotides deleted from +2 (M5) or +3 (M6) in the 3′ side also failed to show the binding

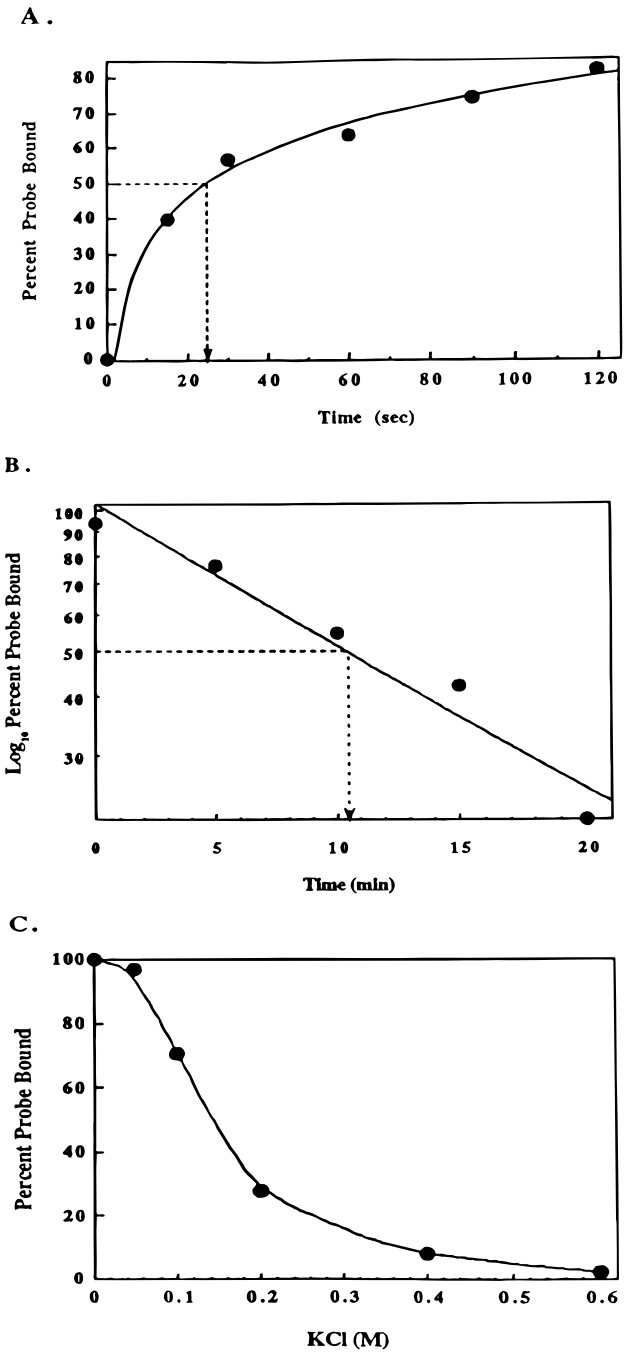
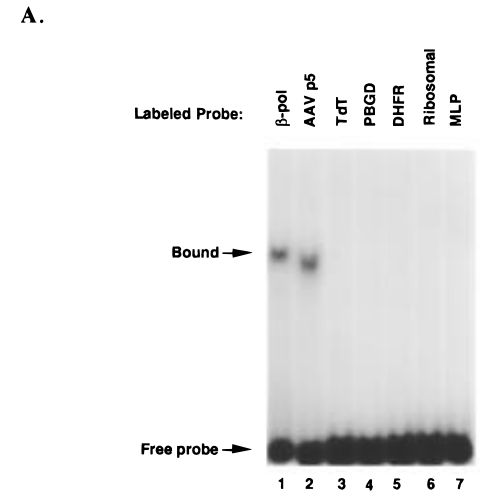


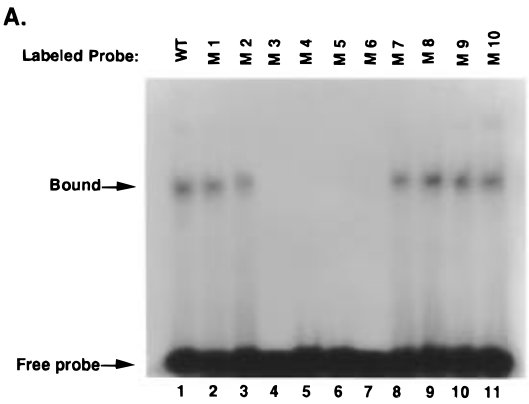
FIGURE 4: Kinetic properties of Inr-BP binding with β-pol promoter initiator element oligonucleotides. Panel A: The rate of association (k_{on}) of purified Inr-BP with Inr oligonucleotides. Equal amounts of ^{32}P-labeled oligonucleotide and purified Inr-BP (10×10^{-12} M) were used in the filter-binding assay. Labeled oligonucleotides and Inr-BP were mixed in 160 μL reaction mixture at various times. Three 50 μL aliquots of the reaction mixture were filtered through three individual nitrocellulose membranes by using 12-position Millipore sampling manifold without further washing. Data in each time point are in triplicate. Values of bound oligonucleotides were corrected for background using the relationship: $X + (100 - X)Y =$ observed counts ($X =$ counts due to protein−DNA complex; $Y =$ counts retained on the filter in the absence of the protein). Panel B: Determination of the rate of dissociation (k_{off}) of Inr-BP−DNA complexes. After DNA was bound with the same amount of Inr-BP-binding activity, dissociation of the protein/DNA complex was performed in the presence of a 500-fold excess of unlabeled competitor DNA in the reaction to trap the free protein. The mixture was incubated at the indicated times and filtered through the membrane. Panel C: The effect of ionic strength on protein−DNA complex formation. Purified Inr-BP and DNA were incubated with different concentrations of KCl in the reaction buffer, equilibrate for 30 min at room temperature, and passed through the filters. The bound radioactivity on the filter was counted and is presented as the percent of bound labeled probe.



B. Inr Oligonucleotide Sequences:

	Promoter	Binding Activity
CAGAGGCGGCCATTGTTCA (+1)	β-pol	+
AGGGTCTCCATTTTGAAGC (+1)	AAVp5	+
AGAGCCCTCATTCTGGAGA (+1)	TdT	-
GCTCAGTGTCCTGGTTACT (+1)	PBGD	-
CGATTTCGCGCCAAACTTG (+1)	DHFR	-
CCGCGCTTCCCTTTTCCGG (+1)	Ribosomal	-
CTCGTCCTCACTCTCTTCC (+1)	MLP	-

FIGURE 5: Purified Inr-BP-binding activity with the β-pol promoter and its comparison with other TATA-less promoters and a TATA-containing promoter (MLP). Inr oligonucleotides were designed corresponding to five TATA-less promoters terms AAV p5, TdT, PBGD, DHFR, and ribosomal. The sequences are shown in panel B. These oligonucleotides were 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled oligonucleotides were incubated with purified Inr-BP as described above for the electrophoretic mobility shift assay. The data are shown in panel A. Binding affinities of Inr-BP with the β-pol promoter, other TATA-less promoters, and MLP (TATA-containing promoter) were quantified with a computerized Bioimager system (Millipore); the results are shown in panel B.



B. Mutant Inr Oligonucleotides

		Binding Activity
CAGAGGCGGCCATTGTTCA (-11 … +1 … +8)	WT	++++
AGGCGGCCATTGTTCA	M1	+++
GGCGGCCATTGTTCA	M2	+
GCGGCCATTGTTCA	M3	-
CGGCCATTGTTCA	M4	-
CAGAGGCGGCCA	M5	-
CAGAGGCGGCCAT	M6	-
CAGAGGCGGCCATT	M7	+
CAGAGGCGGCCATTG	M8	+++
CAGAGGCGGCCATTGT	M9	+++
CAGAGGCGGCCATTGTT	M10	++++

FIGURE 6: Determination of the range of β-pol promoter initiator element oligonucleotides required for Inr-BP-binding activity. Panel A: Wild-type and mutated Inr oligonucleotides (shown in panel B) were used as substrates for electrophoretic mobility shift assays. ^{32}P-labeled wild-type or various truncated Inr oligonucleotides were incubated with 1 μL of purified Inr-BP in a binding buffer for 30 min and applied to a 4% native polyacrylamide gel. The migration of bound and free probes is indicated with arrows. Panel B: Oligonucleotide sequences of truncated bovine β-pol Inr. The truncated Inr oligonucleotides in the 5′-flanking regions are designated as M1−M4, and those in the 3′-flanking regions are designated as M5−M10. The size of each oligonucleotide was the same. The binding activity was determined with a computerized Bioimager system, and the results are shown on the right-hand side of the oligonucleotide sequences as the relative binding activity.

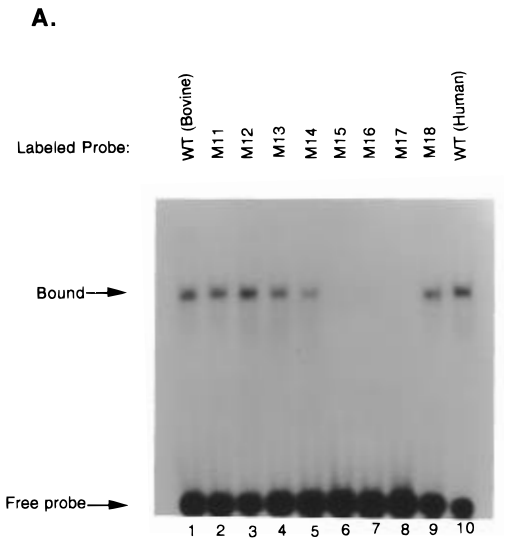
activity (Figure 6, lanes 6 and 7); truncation to +4 (M7) showed some binding activity; and truncation to residues further downstream (M8 to M10) showed full binding activity. These results indicate that residues flanking on either side of +1 are important for full Inr-BP DNA, especially residues −7 through +3.

To further examine the binding of Inr-BP with the bovine β-pol promoter Inr, a series of modified oligonucleotides with single nucleotide differences in the −7 to +3 region were used (Figure 7). Binding analysis showed that nucleotides (bp) at −2, −1, +1, and +2 play an important role in Inr-BP DNA binding. Mutation in the latter three positions eliminated binding, and the effect of a point mutation at −2 resulted in a 72% decrease in binding (Figure 7, lanes 6−8). By contrast, point mutations in the region of −7 to −3, or to +3, did not significantly alter binding of Inr-BP. The binding with either bovine or human β-pol wild-type Inr oligonucleotides was similar, indicating that the substitution at A at −3 and −4 and the substitutions at −8 and −10 had no effect on Inr-BP binding. From these results, we concluded that Inr-BP binding to the β-pol promoter Inr requires specific residues CCAT at −2 to +2 and that additional residues on both the 5′ and 3′ sides contribute to optimal binding.

DISCUSSION

In TATA-containing promoters, formation of a closed preinitiation complex (RP_c) with RNA pol II appears to be initiated by the transcription factor TFIID, which binds to the TATA element and nucleates the assembly of RP_c. About half of the so-called "housekeeping" gene promoters, such as that for β-pol, do not have a TATA element, and, therefore, TFIID probably does not bind directly to these promoters. Instead, initiator element-binding proteins, such as YY1, recognize specific sequences at the start site and may play a role in the initiation of RP_c assembly. In the studies described here, we purified a protein from bovine testis that specifically binds to the bovine or human β-pol promoter initiator region. The 5 bp sequences at −2 to +3 of the start sites of the two β-pol promoters and the AAV p5 promoter are identical (CCATT) and, as shown in this study, are critical for binding the purified Inr-BP. Our results showed similar DNA-binding affinity of the purified Inr-BP for the Inrs of the β-pol and AAV p5 promoters, all of which share primarily the CCATT sequence.

Human YY1 is a known transcription factor that can act either as a transcriptional repressor or as an activator. For example, YY1 has been shown to down-regulate the actin (Lee et al., 1994), β-casein (Raught et al., 1994), and HIV-1 (Margolis et al., 1994) promoters, but to active the c-myc



B. Point Mutant Inr Oligonucleotides — Binding Activity

-11 +1		
CAGAGGCGGCCATT	Bovine	++++
CAGA<u>T</u>GCGGCCATT	M11	++++
CAGAG<u>A</u>CGGCCATT	M12	++++
CAGAGG<u>T</u>GGCCATT	M13	++++
CAGAGGCGG<u>A</u>CATT	M14	++
CAGAGGCGGC<u>A</u>ATT	M15	-
CAGAGGCGGCC<u>C</u>TT	M16	-
CAGAGGCGGCCA<u>G</u>T	M17	-
CAGAGGCGGCCAT<u>C</u>	M18	++++
CGGGGGCAACCATT	Human	++++

FIGURE 7: Identification of specific nucleotides of the β-pol promoter initiator element that are required for Inr-BP-binding. Panel A: ^{32}P-Labeled mutant and wild-type initiator element oligonucleotides were incubated with 1 μL of purified Inr-BP in the binding buffer for 30 min at room temperature. The protein−DNA complexes were resolved on a 4% native polyacrylamide gel. Various oligonucleotides containing a point mutation at around the Inr were designed as shown in panel B. These oligonucleotides were used as probes for electrophoretic mobility shift assays. Results in lanes 1 and 10 are with wild-type initiator element oligonucleotides of bovine and human β-pol promoters, respectively. Lanes 2 through 9 represent the results with various point mutant oligonucleotides of the bovine β-pol promoter. Panel B: Sequences of point mutant oligonucleotides. Underlined nucleotides are the mutant residues. Binding activities of Inr-BP with modified initiator element oligonucleotides are shown on the right-hand side of panel B.

promoter (Lee et al., 1994). This differential effect of YY1 *in vitro* is dependent on the concentration of the protein and is determined within a specific promoter sequence context. The mechanism of YY1 regulation of promoter activity is not completely understood. YY1 is a Zn-finger DNA-binding protein, specifically binding to the initiator element of certain TATA-less promoters. YY1 also interacts directly with some cellular transcription proteins, such as Sp1 (Seto et al., 1993; Lee et al., 1993), TFIID (Chiang & Roeder, 1995), and E1A (Lewis et al., 1995). The role of YY1 in preinitiation complex assembly may occur by interaction with these or other transcription factors. Usheva and Shenk (1994) found that a YY1-binding element can direct initiation of basal transcription on supercoiled AAV p5 template in the presence of a minimum set of transcription factors, such as TFIIB and RNA pol II. In our experiments, we found that the purified YY1-like Inr-BP from bovine testis can activate transcription of the β-pol core promoter, which is TATA-less and contains three Sp1 and one ATF/CREB sites. In our *in vitro* runoff transcription assay with HeLa cell nuclear extract, a YY1 antibody inhibited activity of the β-pol promoter (our unpublished data), and when Inr-BP was depleted from the nuclear extract, β-pol promoter activity was lost. Thus, Inr-BP is required for β-pol promoter transcriptional activity in this *in vitro* transcription system.

Our data indicate that Inr-BP binds with high affinity to the Inr in the β-pol promoter. The K_d value of $\sim 5 \times 10^{-12}$ M obtained in the presence of 50 mM KCl is similar to that reported for other high-affinity sequence-specific DNA-binding proteins, such as lac repressor (Riggs et al., 1970) and bovine ATF/CREB (Widen & Wilson, 1991). We found that the "core" sequence CCAT at −2 to +2 of the initiation start site of the β-pol promoter is critical for binding activity of the purified Inr-BP. This core element is the same as the YY1-binding consensus sequence, i.e., CCAT (Javahery et al., 1994). The TdT and DHFR initiator element sequences, in comparison with β-pol initiator element sequences, are similar except for a single residue difference: TCAT in the TdT promoter; and CCAA in the DHFR promoter. However, purified Inr-BP did not bind to Inrs of the TdT and DHFR promoters. A similar result was obtained with point mutations in the CCAT sequence at the initiator element of the β-pol promoter: mutation of only one of the residues in the core sequence CCAT remarkably reduced binding activity, i.e., C to A at −2, C to A at −1, A to C at +1, or T to G at +2. Nevertheless, the consensus sequence CCAT alone was not sufficient to confer protein binding; residues adjacent to the core sequence also were important for binding, yet point mutants in these residues did not change Inr-BP binding affinity. We suggest that the purified Inr-BP specifically recognizes the core CCAT sequence, and that the 5′- and 3′-flanking sequences act to stabilize the complex in a sequence-nonspecific manner.

In summary, we purified and characterized binding properties of Inr-BP for the β-pol promoter. The interaction of this Inr-BP with general transcription factors and activators appears to play a required role in regulation of β-pol promoter activity. Further studies will be required to examine interactions between Inr-BP and other transcription factors in the assembly of RP_c at the β-pol promoter.

ACKNOWLEDGMENT

We thank Teresa Y. Rawson and Rajendra Prasad for the critical reading of the manuscript.

REFERENCES

Beard, W. A., & Wilson, S. H. (1995) *HIV Volume 2: A Practical Approach* (Karn, J., Ed.) pp 15−36, Oxford University Press. Oxford, U.K.

Blake, M. C., & Azizkhan, J. C. (1989) *Mol. Cell. Biol. 9*, 4994−5002.

Chen, K., Wood, T., He, F., Narayan, S., & Wilson, S. H. (1995) *Gene 164*, 323−327.

Chiang, C., & Roeder, R. G. (1995) *Science 267*, 531−536.

DasGupta, S., Mukhopadhyay, G., Papp, P. P., Lewis, M. S., & Chattoraj, D. K. (1993) *J. Mol. Biol. 232*, 23−34.

Englander, E. W., & Wilson, S. H. (1990) *Nucleic Acids Res. 18*, 919−928.

Englander, E. W., & Wilson, S. H. (1992) *Nucleic Acids Res. 20*, 5527−5531.

Fried, M. G. (1989) *Electrophoresis 10*, 366−376.

Garfinkel, S., Thompson, J. A., Jacob, W. F., Cohen, R., & Safer, B. (1990) *J. Biol. Chem. 265*, 10309−10319.

Hariharan, N., Kelley, D. E., & Perry, R. P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9799−9803.

Javahery, R., Khachi, A., Lo, K., Zenzie-Gregory, B., & Smale, S. T. (1994) *Mol. Cell. Biol. 14*, 116−127.
Kedar, P. S., Widen, S. G., Englander, E. W., Fornace, A. J., Jr., & Wilson, S. H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3729−3733.
Lee, J., Galvin, K. M., & Shi, Y. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6145−6149.
Lee, T., Zhang, Y., & Schwartz, R. J. (1994) *Oncogene 9*, 1047−1052.
Lehninger, A. (1978) *Biochemistry*, pp 186−189, Worth Publishers Inc., New York.
Lewis, B. A., Tullis, G., Seto, E., Horikoshi, N., Weinmann, R., & Shenk, T. (1995) *J. Virol. 69*, 1628−1636.
Maldonado, E., & Reinberg, D. (1995) *Curr. Opin. Cell Biol. 7*, 352−361.
Margolis, D. M., Somasundaran, M., & Green, M. R. (1994) *J. Virol. 68*, 905−910.
Means, A. L., & Farnham, P. J. (1990) *Mol. Cell. Biol. 10*, 653−661.
Means, A. L., Slansky, J. E., McMahon, S. L., Knuth, M. W., & Farnham, P. J. (1992) *Mol. Cell. Biol. 12*, 1054−1063.
Narayan, S., Widen, S. G., Beard, W. A., & Wilson, S. H. (1994) *J. Biol. Chem. 269*, 12755−12763.
Narayan, S., Beard, W. A., & Wilson, S. H. (1995) *Biochemistry 34*, 73−80.
Raught, B., Khursheed, B., Kazansky, A., & Rosen, J. (1994) *Mol. Cell. Boil. 14*, 1752−1763.
Riggs, A. D., Suzuki, H., & Bourgeois, S. (1970) *J. Mol. Biol. 48*, 67−83.
Roy, A. L., Meisterernst, M., Pognonec, P., & Roeder, R. G. (1991) *Nature 354*, 245−248.
Roy, A. L., Malik, S., Meisterernst, M., & Roeder, R. G. (1993) *Nature 365*, 355−359.
Safer, B., Reinberg, D., Jacob, W. F., Maldonado, E., Carcamo, J., Garfinkel, S., & Cohen, R. (1991) *J. Biol. Chem. 266*, 10989−10994.
Seto, E., Shi, Y., & Shenk, T. (1991) *Nature 354*, 241−245.
Seto, E., Lewis, B., & Shenk, T. (1993) *Nature 365*, 462−464.
Shapiro, D. J., Sharp, P. A., Wahli, W. W., & Keller, M. J. (1988) *DNA 7*, 47−55.
Shi, Y., Seto, E., Chang, L., & Shenk, T. (1991) *Cell 67*, 377−388.
Singhal, R. K., Prasad, R., & Wilson, S. H. (1995) *J. Biol. Chem. 270*, 949−957.
Sobol, R. W., Horton, J. K., Kühn, R., Gu, H., Singhal, R. K., Prasad, R., Rajewsky, K., & Wilson, S. H. (1996) *Nature 379*, 183−186.
Srivastava, D. K., Rawson, T. Y., Showalter, S. D., & Wilson, S. H. (1995) *J. Biol. Chem. 270*, 16402−16408.
Usheva, A., & Shenk, T. (1994) *Cell 76*, 1115−1121.
Weis, L., & Reinberg, D. (1992) *FASEB J. 6*, 3300−3309.
Widen, S. G., & Wilson, S. H. (1991) *Biochemistry 30*, 6296−6305.
Widen, S. G., Kedar, P. S., & Wilson, S. H. (1988) *J. Biol. Chem. 263*, 16992−16998.
Wilson, S. H. (1990) *The Eukaryotic Nucleus* (Strauss, P. R., & Wilson, S. H., Eds.) Vol. I, pp 199−233, The Telford Press Inc., Caldwell, NJ.
Wu, C., Tsai, C., & Wilson, S. (1988) *Genet. Eng. 10*, 67−74.
Yamaguchi, M., Hayashi, Y., & Matsukage, A. (1989) *J. Biochem. 105*, 79−83.

BI9525987