

Accurate Positioning of RNA Polymerase II on a Natural TATA-Less Promoter Is Independent of TATA-Binding-Protein-Associated Factors and Initiator-Binding Proteins

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Two promoter elements, the TATA element and initiator (Inr), are capable of directing specific transcription initiation of protein-encoding genes by RNA polymerase II (RNAPII). Although binding to the TATA element by the TATA-binding protein (TBP) has been shown to be the initial recognition step in transcription complex formation in vitro, the mechanism through which the basal machinery assembles into a functional complex on TATA-less promoters is controversial. Evidence supporting numerous models of Inr-mediated transcription complex formation exists, including the nucleation of a complex by Inr-binding proteins, a component of the TFIID complex, or a specific upstream activator common to many TATA-less promoters, Sp1. Using various techniques, we have undertaken a systematic analysis of the natural TATA-less human DNA polymerase β (β -pol) gene promoter. Although the β -pol promoter contains upstream Sp1 elements and a functional Inr that binds YY1, neither of these factors is essential for Inr-mediated transcription complex formation. A complex containing TBP, TFIIB, TFIIF, and RNAPII (DBPolF complex) is capable of forming on the promoter in an Inr-dependent manner. A single point mutation within the Inr that affects DBPolF complex formation diminishes β -pol transcriptional activity.

Transcription initiation of protein-encoding genes is a complex process requiring the precise positioning of RNA polymerase II (RNAPII) on promoter DNA. This is accomplished via a series of interactions between RNAPII and at least six accessory proteins, TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH, termed the general transcription factors (GTFs) (37, 70). Additionally, nucleation of an initiation complex occurs by recognition of a specific core element (31, 54, 66). In many genes, the TATA element is the primary core element responsible for positioning the basal transcription machinery on the promoter. TFIID, a multisubunit protein complex consisting of the TATA-binding protein (TBP) and TBP-associated factors (TAFs), nucleates the formation of the transcription initiation complex by binding to the TATA element (8, 22, 59). In vitro experiments have shown that preinitiation complex assembly proceeds by sequential recruitment of TFIIB followed by RNAPII together with TFIIF, TFIIE, and TFIIH to the promoter-bound TFIID. This multistep model has recently been challenged by the isolation of an RNAPII complex containing a subset of the GTFs, SRB (suppressor of RNAP B) proteins, and additional polypeptides capable of interacting with each other in the absence of promoter DNA (29). Under the appropriate conditions, this complex is capable of specifically initiating transcription and mediating a response to transcriptional activators in vitro (30, 38).

As more promoters of protein-encoding genes have been characterized, it has been found that many lack a TATA element (31, 54, 66). Studies using the TATA-less murine terminal deoxynucleotidyltransferase (TdT) promoter identified a

second core element, the initiator (Inr), which encompasses the transcription start site and is sufficient to position the basal transcription machinery in the absence of a TATA element (55). Various Inr elements have been characterized in numerous TATA-less and TATA-containing promoters, and a consensus Inr sequence has been defined by random mutagenesis and computer alignment of approximately 500 promoters transcribed by RNAPII (6, 24).

The mechanism through which RNAPII is recruited to the Inr has not been definitively elucidated; however, evidence from various laboratories has resulted in the proposal of several models. In agreement with the diverse nature of the Inr, distinct transcription factors, including TFII-I and YY1, have been shown to bind to different Inrs and recruit components of the basal transcription machinery. TFII-I is required for Inr-dependent transcription from the adenovirus major late promoter (AdMLP) in an in vitro reconstituted assay consisting of RNAPII, TBP, and the remaining GTFs except for TFIIA (47, 48). Recently, TFII-I has also been shown to play a role in transcription from the TATA-less T-cell variable region-derived ($V\beta$) and ribonucleotide reductase R1 promoters (26, 39). Transcription from the TATA-containing adeno-associated virus (AAV) p5 promoter and the TATA-less cytochrome oxidase (COX) Vb promoter requires YY1 (3, 50). YY1 binds to the AAV p5 Inr element and recruits TFIIB and RNAPII via protein-protein interaction (61). The resulting quaternary complex is capable of transcribing a supercoiled template containing the p5 promoter in a TBP-independent reaction (60). Interestingly, evidence suggesting that these Inr-binding proteins are not responsible for positioning the basal machinery also exists (24, 31).

It has also been postulated that the sequence-specific transcription factor Sp1 tethers the preinitiation complex to TATA-less promoters by interacting with a heat-labile component of TFIID (45, 56). Data from in vitro transcription,

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DNase I footprinting, and UV cross-linking experiments have led to the proposal that a component of the TFIID complex recognizes the Inr (27, 46, 49, 65, 73). In support of this model, it has been shown that TFIID contacts a downstream element conserved in many *Drosophila* TATA-less Inr-containing promoters (7). One of the components of *Drosophila* TFIID, TAF_{II}150, was shown to stimulate in an Inr-dependent manner transcription of a TATA-containing promoter *in vitro* (28). In agreement with this finding, a trimeric complex containing TBP, TAF_{II}250, and TAF_{II}150 stimulates transcription from an Inr-containing template (63).

A simpler model is that RNAPII itself has a weak affinity for the Inr and that this interaction is stabilized by the GTFs. Gel mobility shift experiments show that a specific, stable complex containing TBP, TFIIB, TFIIF, and highly purified RNAPII can form on the AdMLP, adenovirus IVa2 (AdIVa2), and TdT Inr elements as well as on the TATA-less dihydrofolate reductase (DHFR) promoter (1, 9). Template challenge experiments demonstrate that stable preinitiation complex formation on a wide variety of TATA-containing and TATA-less promoters requires TBP, TFIIB, and RNAPII and that TFIID and TFIIF increase and stabilize complex formation. In these experiments, a functional TBP-containing initiation complex can assemble on TATA-less promoters; however, complex formation is stabilized by TAFs (2). This finding is in agreement with the finding that a TAF binds a downstream promoter element and suggests that TAF recognition of sequences located downstream of +1 stabilizes rather than directs complex formation.

Most of the previous analyses of TATA-less promoters were performed with either a synthetic TATA-less promoter or a composite TATA⁺/Inr⁺ promoter; thus, it is not clear how RNAPII is recruited to a natural TATA-less promoter and which transcription factors are required for initiation. To understand how a preinitiation complex assembles on natural TATA-less promoters, the promoter of the human DNA polymerase β (β -pol) gene was analyzed. Expression of this promoter is basically constitutive; however, reduced levels are observed in the liver and increased amounts are found in the testes (67). Unlike other DNA polymerases, β -pol is not cell cycle regulated. Similar to other housekeeping promoters, the β -pol promoter lacks TATA and CAAT elements, but it contains multiple Sp1 binding sites located approximately 70, 60, and 20 nucleotides upstream from the major transcription start site. The β -pol promoter also contains a palindromic decanucleotide element located 41 nucleotides upstream from the transcription initiation site which resembles a recognition site for the ATF (activating transcription factor) family of proteins (67). Although transcription initiates at multiple positions, these are clustered around one major *in vivo* transcription initiation site designated +1. Interestingly, sequences surrounding the transcription initiation site resemble those of the AAV p5 Inr (Fig. 1). We have identified an Inr that is essential for transcription *in vitro* and *in vivo*. Although YY1 binds to this element, binding of YY1 is not required for Inr transcriptional activity. Furthermore, TBP, TFIIB, TFIIF, and RNAPII are able to form a stable complex on the β -pol promoter which is dependent on the presence of a functional Inr. Together with TFIIE and TFIIF, these factors are capable of initiating transcription from the β -pol Inr in the absence of TAFs; however, TAFs may enhance transcription by stabilizing the transcription complex on the promoter.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The plasmid containing the wild-type β -pol promoter, pBP8, was described previously (67). Inr and Sp1 mutant plasmids were constructed by oligonucleotide-directed mutagenesis using pBP8 as a tem-

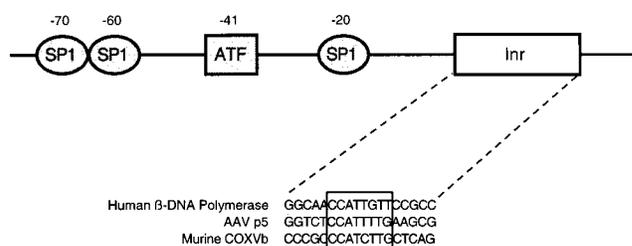


FIG. 1. Schematic diagram of elements present in the human β -pol promoter (top) and comparison of the nucleotide sequence of the β -pol promoter Inr element with sequences of previously described YY1 binding sites which overlap Inr elements (bottom). Conserved sequences are indicated by a box.

plate. The sequences of the oligonucleotides used for the Sp1 mutant constructions were as follows: -70, 5'-GTCTGGGCGGGCGCTGCAGGGCTAGAGGGGGAGC; -60, 5'-CGTCACGCGTCTGGTCTAGACGGGGCGGGGCTAGAGG; and -20, 5'-GCCCCGATGGGGGATCCCGCACGGGGCTTGTTG. TATA variants of the wild-type and Inr mutant β -pol promoters were constructed by oligonucleotide-directed mutagenesis using the -20 Sp1 mutant promoter as a template. The oligonucleotide used was 5'-GCCCCGATGGGGGATCCCGTTTATAGCGCTTGTTGTGACGTCAC. To form pG5 β polCAT, the *Bam*HI/*Kpn*I fragment of the -20 Sp1 mutant β -pol promoter was subcloned into pG5 Δ 38E4CAT (32), which had been digested with *Bam*HI and *Kpn*I to remove the adenovirus E4 promoter.

Plasmid pSP73 β Pol was constructed by ligating the *Hind*III/*Pvu*II fragment from pBP8 (containing β -pol sequences from -110 to +62) to pSP73 (Promega) which had been digested with *Hind*III and *Sma*I.

Oligonucleotides used in the electrophoretic mobility shift assays contained the minimal β -pol Inr and exogenous flanking sequences as follows: wild type, 5'-GATCCGGTCGCGGATCCCGCGTTCGGGGGGTACCATTGTTCCGCGGTCGGTAC; +2, 5'-GATCCGGTCGCGGATCCCGCGTTCGGGGGGTACCAGTGTCCCGCGTCCGGTAC; +3 to C, 5'-GATCCGGTCGCGGATCCCGCGTTCGGGGGGTACCAGTGTCCCGCGGATCCCGCGTTCGGGGGGTACCATTGTTCCGCGGTCGGTAC; +3 to A, 5'-GATCCGGTCGCGGATCCCGCGTTCGGGGGGTACCAGTGTCCCGCGGTCGGTAC; and their complementary oligonucleotides. These oligonucleotides were hybridized and either used for the electrophoretic mobility shift assay or ligated to pG5 Δ 38E4CAT which had been digested with *Bam*HI and *Kpn*I as described above. The β -pol promoter-containing *Eco*RI/*Psi*I fragments were isolated and used as templates in the abortive initiation experiments described below.

In vitro transcription experiments. Nuclear extracts were prepared from HeLa cells as described previously (13). *In vitro* transcription reactions which were to be analyzed by RNase protection and primer extension (final volume, 100 μ l) contained 20 mM HEPES buffer (pH 7.9), 8 mM MgCl₂, 60 mM KCl, 10 mM ammonium sulfate, 12% (vol/vol) glycerol, 4 mM dithiothreitol (DTT), 0.6 mM nucleoside triphosphates, 2.4% polyethylene glycol, 1.5 μ g of circular template, and 320 μ g of HeLa cell nuclear extract. Incubation was carried out at 28°C for 45 min. For primer extension analysis, products of transcription reactions were analyzed by using a primer that hybridized to the chloramphenicol acetyltransferase (CAT) gene (5'-CTCCATTTTACTTCTTAG-3') (9). For RNase protection analysis, transcription reactions were stopped by the addition of 2 M ammonium acetate-0.05% sodium dodecyl sulfate-0.05 mM EDTA, extracted with phenol-chloroform, and chloroform and ethanol precipitated. Samples were then treated with RNase-free DNase I (1 h at 37°C). Subsequently, samples were extracted with phenol-chloroform and chloroform and ethanol precipitated with an Sp6 antisense probe synthesized from pSP73 β Pol which had been digested with *Hind*III. Samples were then hybridized at 63°C and digested with RNase T₂ as previously described (62). Protected fragments and products from the primer extension reaction were denatured and fractionated by electrophoresis in 8 M urea-6% polyacrylamide sequencing gels.

Unless otherwise indicated in the figure legends, assays measuring abortive initiation were performed with 20 mM HEPES (pH 7.9), 5.2 mM MgCl₂, 2.5 mM ammonium sulfate, 45 mM KCl, 10 mM DTT, 12% (vol/vol) glycerol, 2.4% polyethylene glycol (molecular weight [MW], 20,000), 0.3 mM rifampin, 1 ng of linear template (prepared as described above), 1 mM ATP, 1 μ M UTP, 5 μ Ci of [α -³²P]UTP, 20 ng of recombinant yeast TBP, 20 ng of recombinant human TFIIB, 60 ng of recombinant human TFIIF, 25 ng of RNAPII (anti-C-terminal domain affinity purified), 65 ng of recombinant human TFIIF, 100 ng of recombinant human TFIIE, and 40 ng of TFIIF (anti-ERCC3 affinity purified). In reactions using pG5MLT (which contains five GAL4 binding sites upstream of the AdMLP fused to a G-less cassette), 1 μ M CTP and 5 μ Ci of [α -³²P]CTP were substituted for the UTP present in reactions analyzing β -pol transcription. Reactions were incubated at 28°C for 50 min and stopped by heating at 75°C for 10 min. Products of the reaction were treated with alkaline phosphatase for 1 h at 37°C, after which the phosphatase was heat inactivated and the RNA was resolved on an 8 M urea-15% polyacrylamide sequencing gel. At least two different

preparations of DNA were analyzed. Recombinant yeast TBP, human TFIIB, and the subunits of human TFIIE and human TFIIF were overexpressed in *Escherichia coli* and purified as described previously (35). RNAPII was purified with monoclonal antibodies against the C-terminal domain (CTD) as described previously (35). TFIIF was isolated by affinity purification using monoclonal antibodies directed against ERCC3 (XPB), the largest subunit of the TFIIF complex (31a). Briefly, anti-ERCC3 monoclonal antibodies were cross-linked to protein A-agarose at a concentration of 1 mg/ml as described previously (16). The resin (1 ml) was equilibrated with buffer H (20 mM HEPES [pH 7.5], 0.5 mM EDTA, 10% [vol/vol] glycerol, 2 mM DTT, 0.1% [vol/vol] Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride) containing 50 mM KCl. Crude TFIIF (DEAE-5PW fraction [16]; 6 ml; 0.5 mg/ml) was precleared with protein A-agarose. The flowthrough was incubated with the anti-ERCC3 resin in an Econo column (Bio-Rad) for 6 h at 4°C, after which the flowthrough was collected and reloaded onto the column three times. The column was washed at 4°C with 50 column volumes of buffer H containing 200 mM KCl followed by 50 column volumes of buffer H containing 50 mM KCl. The resin was then washed at 28°C with 10 column volumes of buffer H containing 50 mM KCl. TFIIF was eluted from the column by incubating (15 min at 28°C) the column with 1 column volume of buffer H containing 50 mM KCl and a peptide (2 mg/ml) containing the epitope recognized by the monoclonal antibodies. This elution procedure was repeated once. Additional fractions were collected by incubating the resin with buffer H containing 50 mM KCl without peptide. Fractions were analyzed for the ability to transcribe the AdMLP in a TFIIF-dependent assay and for the p62 and cyclin H subunits of TFIIF by Western blot analysis.

Activated transcription from pG5MLT was performed as previously described (34). Briefly, transcription reaction mixtures contained 20 mM HEPES (pH 7.9), 4 mM MgCl₂, 6.25 mM ammonium sulfate, 2.6% (vol/vol) polyethylene glycol (MW, 8,000), 10 mM DTT, 0.1 μ g of pG5MLT, 0.6 mM ATP, 0.6 mM CTP, 0.6 μ M UTP, 5 μ Ci of [α -³²P]UTP, 15 U of RNase T₁, 80 ng of recombinant human TFIIA, 20 ng of recombinant human TFIIB, 1.8 ng of recombinant human TFIIF, 30 ng of recombinant human TFIIE, 80 ng of RNAPII (alkyl superose fraction [33]), 65 ng of TFIIF (hydroxylapatite fraction [14]), 12% (vol/vol) glycerol, and 42.5 mM KCl. Recombinant human TBP (2.6 ng), epitope-tagged holo-TFIID (eTFIID) (2.6 ng of TBP), recombinant human PC4 (30 ng), and recombinant GAL4-VP16 (60 ng) were added as indicated in Fig. 9C. Transcription factors were preincubated with the template. After 30 min at 30°C, ribonucleotides were added as indicated above, and reactions were allowed to proceed for 50 min at 30°C. Reactions were stopped, and the RNA products were separated by electrophoresis on denaturing polyacrylamide gels as described previously (36). Equivalent amounts of TBP and eTFIID were used as determined by immunoblot analysis using polyclonal antibodies against TBP. Reactive materials were detected by using alkaline phosphatase conjugated anti-rabbit immunoglobulin G and visualized by using nitroblue tetrazolium and bromochloroindolylphosphate according to standard procedures (19).

Transfections. Human kidney 293 cells were grown in monolayer cultures (100-mm-diameter plates) in Dulbecco's modified Eagle medium containing 10% defined calf serum. Cells at 60 to 80% confluency were transfected with 30 μ g of reporter DNA by the calcium phosphate procedure (68). Medium was replenished 1 h before the precipitate and DNA were added to the cells. After an overnight incubation, medium was replenished. Cells were harvested 40 h posttransfection, in ice-cold phosphate-buffered saline, and resuspended in 0.25 M Tris-HCl buffer (pH 7.5). Cells were lysed by three freeze-thaw cycles. Soluble extracts were prepared and assayed quantitatively for CAT activity by using ³H-acetyl coenzyme A and chloramphenicol as described previously (53). CAT levels were normalized for protein concentration. Transfections were repeated at least three times with at least two different preparations of each plasmid DNA.

Electrophoretic mobility shift assays. Oligonucleotides used as probes were hybridized in 10 mM Tris-HCl buffer (pH 8.0)-0.1 mM EDTA-0.1 M NaCl and end labeled with the Klenow fragment. Alternatively, *Hind*III/*Kpn*I fragments were isolated from pBP8 and β -pol Inr mutant plasmids which were 3'-end labeled with the Klenow fragment. Nuclear extracts were prepared from HeLa cells as described previously (13). Bacterially expressed histidine-tagged recombinant human YY1 was purified under denaturing conditions, using nickel chelate columns as described previously (3). Purified recombinant YY1 was renatured by dialysis against buffer containing 50 mM HEPES (pH 7.9), 10% glycerol, 100 mM KCl, 0.2 mM EDTA, 10 μ M ZnSO₄, 2 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride. Recombinant yeast TBP, human TFIIE, and human TFIIF were purified as described above. TFIIF was purified from HeLa cell nuclear extract as described previously (15). Binding reactions were performed in 13 mM HEPES buffer (pH 7.9)-2.6% polyethylene glycol (MW, 2 \times 10⁴)-10% (vol/vol) glycerol-4 mM DTT-50 mM KCl-0.4 μ g of poly(dG-dC)-poly(dG-dC)-10 mM NaCl-8 \times 10³ cpm of probe (36). Proteins were added as indicated in the figure legends. Reaction mixtures were incubated for 30 min at 28°C and analyzed as described previously (36). Modifications are described in the figure legends.

RESULTS

A functional Inr is present in the β -pol promoter. Since the Inr element plays a critical role in transcription initiation from many TATA-less promoters, nucleotides surrounding the transcription start site (-6 to +12) were sequentially mutated in the context of the natural promoter in the hope of localizing the β -pol Inr (Fig. 2A). These constructs were analyzed in an in vitro transcription assay using HeLa cell nuclear extracts. Transcription was measured by using RNase protection (Fig. 2B) and primer extension (Fig. 2C) assays. As is observed in vivo, transcription from the wild-type promoter initiates at multiple sites which are clustered around one major in vivo transcription initiation site designated +1 (Fig. 2B, lane 1; Fig. 2C, lanes 1 and 3). Analysis of promoters containing mutant Inrs showed that there is a region extending from -2 to +6, that is important for transcription (Fig. 2B and C). Mutations within these residues (-2 to +1, +1, and +2 to +6), exhibited transcriptional levels markedly lower than that of the wild-type promoter (Fig. 2B; compare lanes 3 to 5 with lane 1; Fig. 2C, compare lanes 5 to 7 with lane 3). Mutating nucleotides flanking this region (from -6 to -3, +5 alone, or from +9 to +12) did not have such a severe effect on in vitro transcription (Fig. 2B, lanes 2, 6, and 7; Fig. 2C, lanes 4, 8, and 9). The transcription levels from the internal AdMLP control template remained constant (Fig. 2C). Interestingly, a mutation from -6 to -3 did not alter the levels of transcription initiation, but it did affect transcription start site usage (Fig. 2B, lane 2, asterisk; Fig. 2C, lane 4). The protected fragment arising from the +9/+12 template is smaller than the others, not because of differential start site usage but because all transcripts were hybridized to the same wild-type antisense probe for quantitation purposes and the RNase cleaves the nonhomologous region between +9 and +12 (Fig. 2B; compare lane 7 with lane 1). Primer extension analysis of the +9 to +12 mutation confirmed that the transcription initiation sites were the same as observed with the wild-type promoter (Fig. 2C, lane 9). These mutant promoters were also analyzed for the ability to direct transcription from a reporter gene, the CAT gene, by transient expression in human kidney 293 cells. These cells were used because expression of the β -pol promoter is higher in this cell line due to the constitutive expression of the adenovirus E1A and E1B proteins (67). The same mutations, from -2 to +6, that were defective in transcription in vitro had a dramatic negative effect in vivo (Fig. 2D).

These experiments indicate that residues extending from -2 to +6 are important for transcription from the β -pol promoter and may constitute a functional Inr element. Since Inr elements recruit the basal transcription machinery to TATA-less promoters, the Inr seems to be functionally analogous to the TATA element. We speculated that if residues -2 to +6 of the β -pol promoter constitute a functional Inr, then the placement of a consensus TATA element 30 nucleotides upstream from the transcription start site should abrogate the effect of the above-described mutations in vitro. The upstream TATA element would thus provide an alternate pathway for the basal machinery to assemble a functional preinitiation complex in vitro.

A TATA motif was inserted in the various Inr mutant promoter constructs (Fig. 3A). To eliminate the possibility of steric hindrance, the Sp1 element at -20 was removed in the TATA-containing promoters, which resulted in no effect on in vitro transcription (data not shown; see Fig. 6). Placement of a TATA motif at -30 increased transcription from the wild-type promoter. Placement of a TATA element upstream of the -2/+1 or +2/+6 mutant promoters restored transcriptional

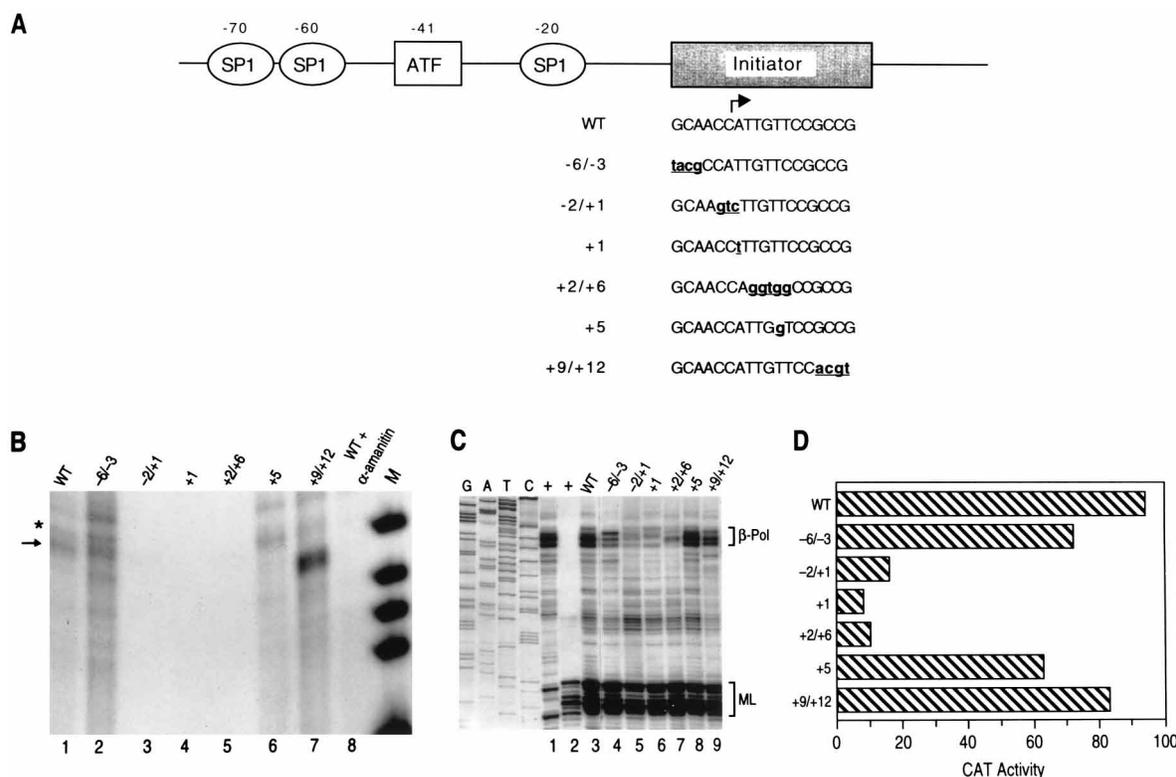


FIG. 2. An element important for transcription is located between -2 and $+6$ on the β -pol promoter. (A) Nucleotide substitutions of the various Inr mutations analyzed. (B) Transcripts from the various templates were analyzed by RNase protection using an antisense probe containing wild-type (WT) β -pol sequences from -110 to $+62$ and 156 nucleotides of vector sequences. The arrow denotes $+1$ as estimated by using a pBR322/*Hpa*II ladder (lane M). An alternate major start site is denoted by an asterisk. The strong faster-migrating band in lane 6 corresponds to the arrow, and the faint band above corresponds to the asterisk. The mobilities of the bands are slightly aberrant (curved) due to uneven heat distribution during electrophoresis. α -Amanitin was added at a final concentration of $2 \mu\text{g/ml}$ in lane 8. (C) Primer extension analysis of in vitro transcription reactions using templates illustrated in panel A. Assay conditions were as described in Materials and Methods except that to control for recovery, separate transcription reactions were performed with a template containing the AdMLP fused to the CAT gene. The AdMLP-containing reactions were mixed with the reactions containing the β -pol promoter and analyzed as described previously (9). (D) Transient-transfection analysis of the various Inr mutations. Promoter constructs in panel A were placed upstream of a bacterial CAT gene and transfected into 293 cells. CAT activity was measured and plotted as picomoles of incorporated ^3H /microgram of protein assayed. The results presented are representative of five independent transfections.

activity (Fig. 3B, lane 2, and data not shown). The β -pol promoter has at least two major transcription initiation sites (Fig. 2B and 3B, lanes 1). Placement of an upstream TATA in the context of a wild-type promoter had no effect on these two initiation sites (Fig. 3B; compare lane 2 with lane 1). Primer extension analysis of these templates shows that although the two major initiation sites are utilized by the TATA⁺/Inr⁻ β -pol promoter, other minor start sites are not utilized in the presence of an upstream TATA (data not shown).

The activity of each of these constructs was also analyzed by measuring the ability to drive a CAT reporter gene in transiently transfected 293 cells. Although the mutation from -2 to $+1$ resulted in a promoter which was 10% as active as the wild type, the presence of the TATA element restored transcription to over 80% of wild-type activity (Fig. 3C). Similar results were obtained with the $+2/+6$ mutant (data not shown). Interestingly, although placement of a TATA element upstream from the wild-type Inr stimulated transcription in vitro, the upstream TATA did not increase promoter activity of the wild-type promoter in transiently transfected cells. This observation is consistent with what has been observed when a consensus TATA element is placed upstream from a TATA-less ribosomal protein promoter (12).

Since the effect of mutating the residues between -2 and $+6$ can be overcome by the presence of an upstream TATA motif in vitro and in vivo, we concluded that residues between -2

and $+6$ constitute a functional Inr element that is important for basal transcription from the β -pol promoter.

The specific transcription factor YY1 binds to the β -pol Inr. The functional analyses suggested that perhaps a factor bound to residues -2 to $+6$ and nucleated the basal transcription machinery. Gel mobility shift experiments with HeLa cell nuclear extracts resulted in the formation of a strong complex and two weaker complexes on a probe containing the β -pol Inr (Fig. 4A, lane 1). Because of the homology between this element and a consensus binding site for the specific transcription factor YY1 (Fig. 1), it was possible that the complex observed contained YY1. To analyze this, HeLa cell nuclear extract was preincubated with either preimmune serum or serum containing antibodies against YY1. The antibodies against YY1 inhibited the formation of the strong complex, whereas the preimmune serum did not (Fig. 4A, lanes 2 and 3). The two weaker complexes were stimulated by both the anti-YY1 antibodies and the control serum, most likely due to the increase in protein concentration. Moreover, recombinant YY1 comigrates with the complex observed in nuclear extract that is affected by anti-YY1 antibodies (data not shown). To confirm that YY1 could bind to the β -pol Inr, the ability of recombinant YY1 to bind was analyzed by gel mobility shift assay. Although recombinant YY1 retarded the mobility of the wild-type β -pol promoter in the electrophoretic mobility shift assay, mutations from -2 to $+1$, $+1$ alone, and $+2$ to $+6$ abolished

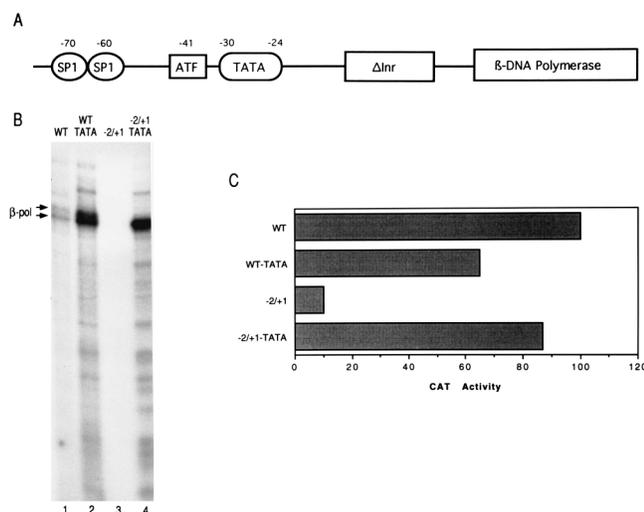


FIG. 3. The β -pol promoter $-2/+6$ element is an Inr element and is essential for recruiting the basal transcription machinery. (A) Schematic diagram of the β -pol constructs which had been mutated to contain a TATA element. The Sp1 element located at -20 in the wild-type promoter was mutated in these constructs. (B) In vitro transcription analysis of the wild-type (WT) and $-2/+1$ mutant β -pol promoter constructs in the presence and absence of a TATA element. Transcripts were analyzed by RNase protection as described in the legend to Fig. 2 and Materials and Methods. (C) Transient-transfection analysis of promoters analyzed in panel B fused to the CAT gene. CAT enzyme activity was analyzed from three independent transfections as described in Materials and Methods, and the average activity from each is illustrated as a percentage of wild-type activity, which is designated as 100%.

the ability of YY1 to bind to the promoter (Fig. 4B; compare lane 1 with lanes 3 through 5). Therefore, the identical residues that affected transcription (-2 to $+6$) were critical for binding of YY1 to the β -pol promoter.

The YY1 binding site overlapping the Inr is not required for transcription initiation from the β -pol promoter. Previous studies from the Smale and Farnham laboratories have shown that mutating the DHFR or the AAV p5 Inr at $+2$ affects the promoter recognition of various sequence specific Inr-binding proteins, and mutating the thymidine present in the consensus Inr element at $+3$ affects transcriptional activity (24, 31). To further analyze the possible role of the YY1 binding site overlapping the Inr in positioning the basal transcription machinery on the β -pol promoter, point mutations at $+2$ and $+3$ were made. Thus, $+2$ was changed from a thymidine to a guanosine residue and $+3$ was changed from a thymidine to a cytosine residue. These mutations were analyzed for the ability to be transcribed in nuclear extract in vitro. Primer extension analysis of the resulting transcripts shows that the $+2$ mutation had no effect on in vitro transcription (Fig. 5A; compare lanes 3 and 1); however, mutation of $+3$ to a C resulted in a dramatic decrease in transcriptional activity (Fig. 5A, lane 5). The $+3$ mutation affected the promoter's ability to recruit the basal transcription machinery, since placement of the AdMLP TATA element 30 nucleotides upstream from the transcription start site restored transcription in vitro (Fig. 5A, lane 7). The transcription observed from the $+2$ mutation as well as that of the template containing a TATA motif was directed by RNAPII because it was sensitive to $2 \mu\text{g}$ of α -amanitin per ml (Fig. 5A, lanes 4 and 8).

The activity of each of the mutant promoters was also analyzed in vivo. The promoters were fused to the bacterial CAT gene and transiently transfected into 293 cells for reasons previously described. Mutation at $+2$ had a small effect, if any, on

the promoter's CAT activity; however, mutation at the $+3$ nucleotide reduced CAT activity by at least 80% (Fig. 5B).

The ability of recombinant YY1 to bind to the $+2$ and $+3$ mutant promoters was analyzed by using the gel mobility shift assay. YY1 binding to the $+2$ mutant β -pol promoter was severely reduced; however, the $+3$ mutant promoter was able to bind a significant amount of YY1 in this assay (Fig. 5C). In agreement with this result, the strong complex which is observed on the wild-type β -pol Inr in an electrophoretic mobility shift assay using nuclear extract does not form on the $+2$ mutant Inr but does form on the $+3$ mutant Inr (Fig. 4A; data not shown). These results collectively demonstrate that the YY1 binding site overlapping the Inr is dispensable for promoter activity. The implication of these findings is that the formation of a transcription-competent complex at the β -pol promoter is able to proceed by a YY1-independent mechanism; however, these results do not rule out the possibility that YY1 may aid in positioning or stabilizing complex formation in vivo.

The upstream Sp1 elements of the β -pol promoter do not direct formation of, but aid in positioning, and stimulate transcription preinitiation complexes. In light of the findings described above and since it has been postulated that Sp1 may direct formation of the transcription complex and/or position the basal transcriptional machinery on TATA-less promoters, the functions of the three Sp1 elements present in the β -pol promoter were analyzed (Fig. 6A). Initially, each Sp1 element was mutated independently. Our rationale was that if Sp1 was directing the formation of a preinitiation complex at the Inr (if a particular Sp1 site directs preinitiation complexes to the Inr) or alter the transcription start site usage (if Sp1 positions the transcription start site). Replacement of each Sp1 site individually by an irrelevant sequence did not drastically alter the transcription levels or pattern of in vitro transcription ini-

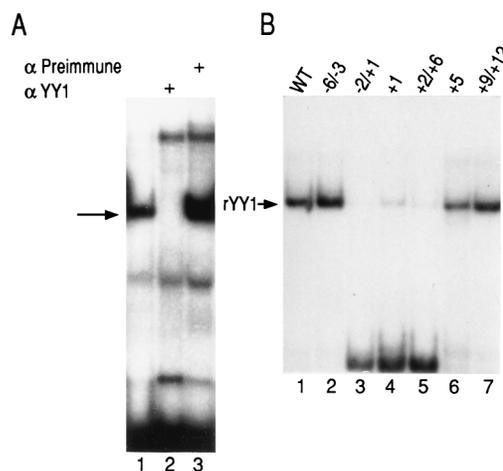


FIG. 4. The β -pol Inr binds the specific transcription factor YY1. (A) Electrophoretic mobility shift assay using HeLa cell nuclear extract and a double-stranded DNA fragment containing β -pol sequences from -15 to $+10$. Binding reactions were performed as described in Materials and Methods with the exception that ZnSO_4 was added to a final concentration of $10 \mu\text{M}$, poly(dI-dC) ($2 \mu\text{g}$) was used instead of poly(dG-dC)-poly(dG-dC), and reactions were separated by native electrophoresis at 4°C in a 4.5% polyacrylamide gel with $0.25 \times$ Tris-borate-EDTA at 120 V . (B) Electrophoretic mobility shift assay of recombinant YY1 (20 ng) and Inr mutant probes containing β -pol sequences from -110 to $+62$ and various mutations as described in the legend to Fig. 2A. Reaction conditions were as described for panel A except that the concentration of poly(dI-dC) was reduced from 2 to $0.05 \mu\text{g}$. WT, wild type.

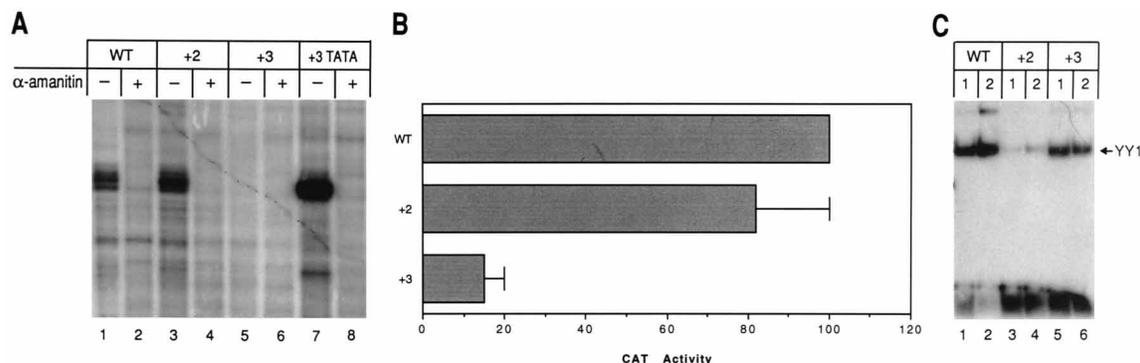


FIG. 5. YY1 binding is not required for transcription initiation from the β -pol promoter. (A) Point mutations at +2 and +3 were analyzed for the ability to be transcribed in vitro. The transcriptional activity of a TATA-containing +3 mutant promoter (Fig. 3A) was also analyzed. Transcription was analyzed by primer extension. α -Amanitin was added at a final concentration of 2 μ g/ml where indicated. WT, wild type. (B) Transient-transfection analysis of the +2 and +3 β -pol mutant promoters fused to the CAT gene. CAT activity from three independent transfections was determined, and the average was plotted as a percentage of wild-type activity (which was normalized to 100%). (C) Electrophoretic mobility shift assay of recombinant YY1 (2.5 and 5 ng) to probes containing mutations at +2 and +3.

tiation, although some minor differences in usage were observed, as measured by primer extension analysis (Fig. 6B; compare lanes 2 to with lane 1). To be certain that these differences were not artifacts of the primer extension assay, transcription was also analyzed by RNase protection (Fig. 6C). Although mutation of the Sp1 element at -20 resulted in the formation of new initiation sites, these sites were still clustered within the Inr (Fig. 6B, lane 2; Fig. 6C, lane 2). On the other hand, mutation of the Sp1 element at -60 slightly altered the preference of start site usage (Fig. 6B and C, lanes 3). To further investigate if Sp1 was required for the tethering of the basal machinery to the β -pol promoter and to address the possibility that the Sp1 sites were functionally redundant, all upstream elements (the three Sp1 sites and the one ATF element) were removed and replaced by five contiguous binding sites for the yeast transcription activator GAL4 (Fig. 6A). In the absence of the upstream elements, transcription was weak compared with the wild-type promoter, but the positions of the major transcription start sites were unaffected (Fig. 6D, lane 1). Additionally, activated levels of transcription in the presence of a GAL4-VP16 fusion protein were observed (Fig. 6D, lane 3). These initiation sites are derived from RNAPII since they are sensitive to 2 μ g of α -amanitin per ml (Fig. 6E, lanes 2 and 4). Stimulation was also observed, although to a much lesser extent, when only the GAL4 DNA binding domain was used (Fig. 6D, lane 2). This was presumably due to the removal of nonspecific DNA-binding proteins (repressors). Since specific transcription occurs in the absence of any upstream elements, the upstream elements on the β -pol promoter are not required to form a transcription preinitiation complex on the Inr, although they stimulate the level of transcription, most likely by stabilizing complex formation. In light of the results, we suggest that promoter binding by Sp1, or other activators, is important for increasing the efficiency of complex formation on the β -pol promoter, since promoter activity is decreased in the absence of upstream elements. Additionally, the results suggest that the Sp1 sites may help to correctly position the transcription start sites (see below). Moreover, the results presented in Fig. 6D demonstrate that the Inr element possesses all of the determinants required to direct formation of transcription-competent complexes.

The +3 mutation affects the formation of a stable preinitiation complex containing TBP, TFIIB, RNAPII, and TFIIF. The studies described thus far showed that the YY1 recognition site overlapping the Inr, and the Sp1 sites present upstream of the

Inr, do not nucleate the formation of a preinitiation complex on the Inr of the β -pol promoter. Previous studies have shown that a stable and specific complex containing TBP, TFIIB, RNAPII, and TFIIF (DBPolF) could be formed on the DHFR promoter and Inr elements of the TdT, AdMLP, and AdIVa2

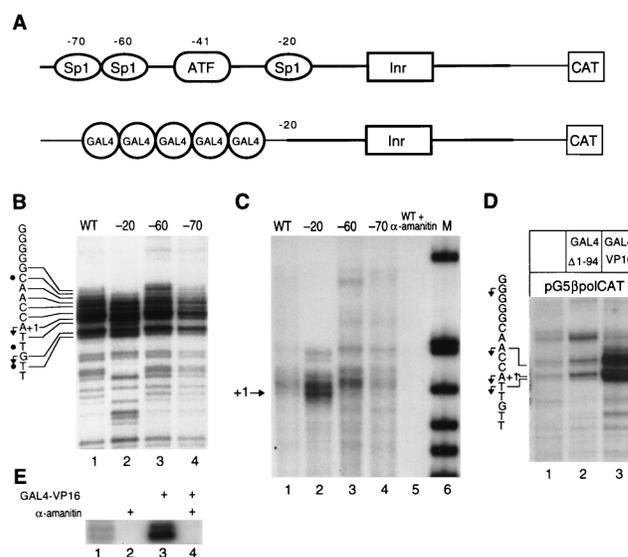


FIG. 6. The upstream Sp1 elements affect promoter strength but are not required to nucleate the formation of a preinitiation complex on the β -pol promoter. (A) Structure of the wild-type β -pol promoter (top) and mutant β -pol promoter (bottom) in which all upstream elements were replaced by binding sites for the yeast specific transcription factor, GAL4 (pG5 β polCAT). Heavy lines indicate natural β -pol sequences, and lighter lines denote vector sequences. (B) Primer extension analysis of in vitro transcription products from either the wild-type (WT) promoter or promoters containing mutations in each of the Sp1 elements. The sequence of the β -pol Inr is shown alongside. Major start sites are as indicated by an arrow, and minor start sites are depicted by a solid circle. (C) RNase protection analysis of products from an independent in vitro transcription assay of promoters analyzed in panel B. +1 is as indicated. Lane M, pBR322/*Hpa*II ladder. (D) Primer extension analysis of in vitro-transcribed products of pG5 β polCAT. Equal binding units of GAL4 Δ 1-94 and GAL4VP16, purified as described previously (42), were added where indicated. The β -pol promoter sequence is illustrated to the left, and major start sites are determined by comparison with a sequencing ladder are depicted with arrows. Assays in panels B and D were performed at the same time and were analyzed in the same polyacrylamide gel. (E) Primer extension analysis of in vitro-transcribed products of pG5 β polCAT. α -Amanitin (2 μ g/ml) and GAL4-VP16 were added as indicated.

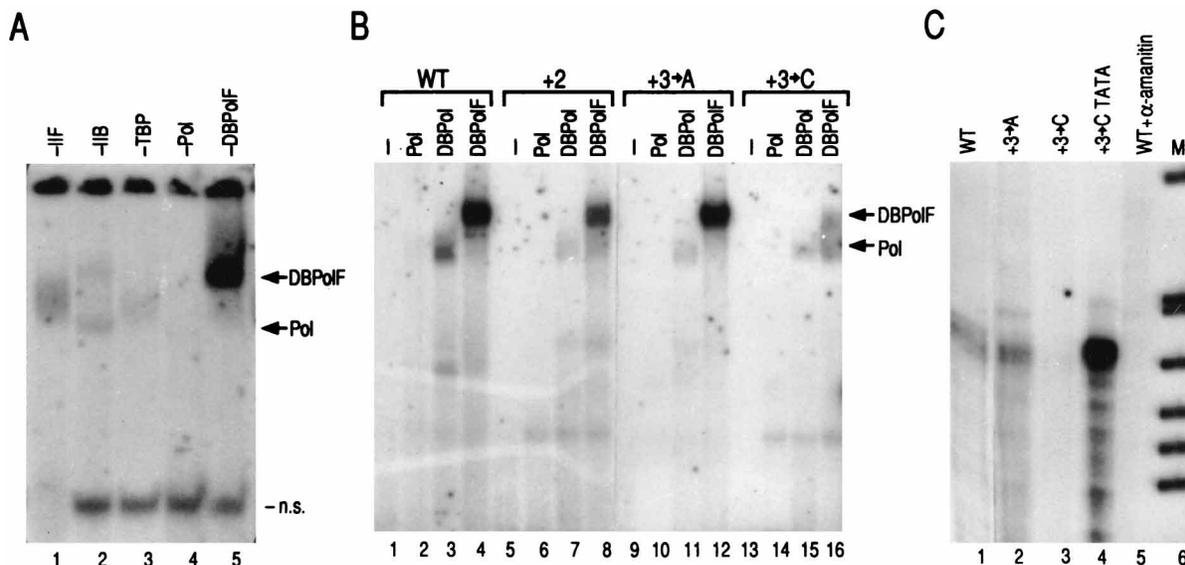


FIG. 7. The +3 T-to-C mutation is defective in transcription initiation because it is unable to nucleate a preinitiation complex containing TBP, TFIIB, TFIIF, and RNAPII. (A) Electrophoretic mobility shift assay using recombinant TBP, recombinant TFIIB, and HeLa cell-derived RNAPII (DEAE-5PW fraction) and TFIIF (phenyl-Superose fraction). Oligonucleotides containing the wild-type minimal β -pol Inr sequence were hybridized and labeled as described in Materials and Methods. In this experiment, the sequence of the wild-type β -pol oligonucleotide used as a probe was altered from the natural promoter to avoid complication from the multiple transcription initiation sites present in the promoter. n.s., nonspecific. (B) Electrophoretic mobility shift assay using recombinant TBP, TFIIB, and TFIIF and HeLa cell-derived RNAPII (DEAE-5PW fraction) was performed as described above and in Materials and Methods. WT, wild type. (C) RNase protection analysis of transcripts produced in vitro of templates containing mutations at +3 in the context of the wild-type promoter (lanes 2 and 3) and in the context of a TATA element (lane 4). Lane M, pBR322/HpaIII ladder.

promoters (1, 9). The electrophoretic mobility shift assay was used to examine if a similar complex could be formed on the β -pol promoter. In assays using recombinant TBP and TFIIB, and TFIIF and RNAPII purified from HeLa cells, a stable complex can assemble on the β -pol promoter (Fig. 7A, lane 5). Omission of any factor abolishes complex formation under these conditions (Fig. 7A, lanes 1 to 4). This result strongly suggests that this combination of GTFs and RNAPII has the ability to recognize the Inr. A stable complex containing recombinant TBP, TFIIB, TFIIF, and RNAPII purified from HeLa cells could also be formed on the minimal β -pol Inr (Fig. 7B, lane 4). We then speculated that the +3 mutation described above, which abolished transcription, should also eliminate complex formation. Furthermore, the +2 mutation, which affected YY1 binding but had no effect on transcription, should allow complex formation. Therefore, the ability of the complex to be formed on the +2 and +3 mutants was analyzed in the context of the minimal β -pol Inr. Under the conditions used, nonspecific binding of RNAPII alone and together with TBP and TFIIB (DBPol) was observed (Fig. 7B, lanes 3, 7, 11, and 15). When recombinant TFIIF was added to the DBPol complex, a slower-migrating complex (DBPolF) was formed on the wild-type and mutant +2 promoters (Fig. 7B, lanes 4 and 8). Complex formation on the +3 mutant promoter was not efficient (Fig. 7B; compare lane 16 with lane 4). The inefficiency of the +3 mutation in directing formation of the DBPolF complex specifically resulted from the basal transcription complex not recognizing the mutant Inr, since the formation of the specific DBPol complex was not affected. Moreover, to further scrutinize whether this mutation affected recognition of the Inr by the basal transcription machinery, the +3 nucleotide was changed to an A rather than a C residue. The +3 T-to-A mutant exhibits wild-type levels of transcription in the context of the TdT Inr (24) and the β -pol promoter (Fig. 7C). Consistent with this observation, the +3 T-to-A substitution resulted

in an Inr that was capable of directing DBPolF complex formation indistinguishably from the wild-type Inr (Fig. 7B, lane 12). Thus, we concluded that the Inr is directly recognized by the basal transcription machinery. To further analyze whether the formation of the DBPolF complex on the β -pol promoter correlated with transcription activity, we analyzed the GTFs required for transcription. This was of importance because studies using the AAV p5 promoter have shown that YY1 nucleates a transcriptionally active complex that is independent of GTFs other than TFIIB and RNAPII (60). In agreement with the concept that the same factors required for preinitiation complex formation on a TATA-containing promoter are also required for Inr-mediated transcription, we have observed that preincubation of nuclear extract (which contains YY1 activity [Fig. 4A]) with antibodies against TBP, the small subunit of TFIIF, the small subunit of TFIIE, or the large subunit of TFIIF specifically inhibits transcription from the β -pol promoter as measured by primer extension analysis (data not shown).

The basal factors are sufficient for transcription initiation on the β -pol Inr. Formation of the DBPolF complex is diminished on the +3 T-to-C mutation (Fig. 7B). To determine if this decrease was significant and correlates with transcription initiation, an in vitro reconstitution assay measuring abortive initiation of transcription was used. All recombinant factors were used with the exception of RNAPII and TFIIF, which were affinity purified as described in Materials and Methods. Abortive initiation on a linear template containing the wild-type β -pol Inr required all six GTFs as well as RNAPII (Fig. 8A). Furthermore, abortive initiation was dependent on the presence of a functional Inr, since activity from a template containing the previously characterized +3 T-to-C mutation was severely decreased; however, product formation from a template containing the +3 T-to-A mutation was similar to that in reactions using a wild-type template (Fig. 8B, lane 3;

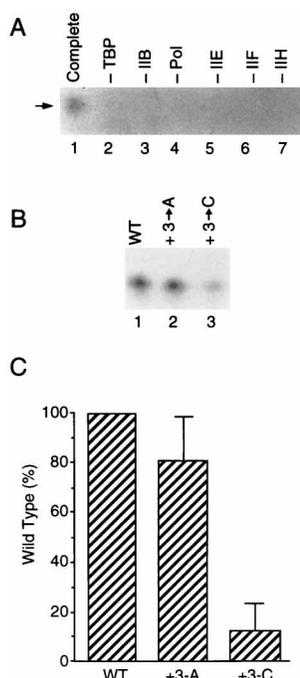


FIG. 8. The formation of the first phosphodiester bond requires RNAPII, all six GTFs, and a functional Inr. (A) Abortive initiation reaction performed using yeast TBP as described in Materials and Methods. Transcription factors were omitted from the assay as indicated. (B) Abortive initiation reaction analyzing templates containing mutations at +3. DNAs were quantitated by using the picogreen quantitation assay (Molecular Probes). WT, wild type. (C) Quantitation of three independent abortive initiation assays. Autoradiograms were scanned by using a Sharp 330 device. Results were quantitated by using Image-master software (Pharmacia). The average of three independent experiments is plotted as a percentage of wild-type activity (which is normalized to 100%), and error bars representing the standard error of the mean are shown.

Fig. 8C). Therefore, we concluded that the GTFs have the intrinsic activity to recognize the Inr. Previous studies have indicated that Inr recognition can be accomplished by TAF_{II}150. The reconstituted system used contained recombinant TBP, TFIIB, TFIIF, and TFIIE and highly purified RNAPII and TFIIH. Since human TAF_{II}150 has been reported not to be associated with the TFIID complex, we investigated whether TAF_{II}150 contaminated RNAPII or TFIIH by using antibodies against *Drosophila* TAF_{II}150 in Western blot analysis as previously described (28). Our analysis demonstrates that the HeLa-derived factors were free of contaminating TAF_{II}150 and YY1 (data not shown). Therefore, we concluded that the GTFs TFIIB, TBP, TFIIF, TFIIE, and TFIIH, together with RNAPII, can recognize the Inr. In accordance with this, we have compared the transcriptional activity of TBP and affinity-purified TFIID. We find, using equivalent units of TBP as determined by Western blot analysis (Fig. 9B), that TBP is almost as efficient as affinity-purified TFIID in initiating transcription from the β -pol Inr as measured by abortive initiation (Fig. 9A, lanes 2 and 3). Similar results were observed in assays using affinity-purified TFIIH (data not shown). The similar activities of TFIID and TBP observed were not due to the inactivation of the TAFs during purification of eTFIID since the eTFIID used was capable of supporting activated transcription from a TATA-containing promoter (Fig. 9C, lane 9). Activation was dependent on the presence of TAFs (lanes 2 to 5), activator (GAL4-VP16; lane 7), and the coactivator, PC4 (lane 8). In light of these results, we conclude that using the β -pol promoter and under the assay conditions

utilized, the TAFs do not play an important role in basal transcription. It is important to emphasize that our studies do not eliminate the possibility that TAFs are necessary under other in vitro conditions or in vivo, where the template is packed into chromatin and in the presence of factors that negatively regulate transcription. Our results, however, clearly demonstrate that the GTFs in the absence of interfering activities can nucleate formation of a transcription complex on the β -pol and other TATA-less promoters (9). It is likely that TAF_{II}150, or another subunit(s) of the TFIID complex, cooperates with the GTFs in the presence of the appropriate coactivator to produce a complex that is more efficient than TBP alone in recognizing the Inr.

DISCUSSION

The mechanism through which the basal factors assemble into a preinitiation complex and position transcription initiation on TATA-less promoters is not clear, although various hypotheses exist. To understand this phenomenon, we have systematically analyzed a natural human TATA-less promoter, that of the β -pol gene. Using site-directed mutagenesis, we have identified an Inr element in the β -pol promoter which is essential for promoter recognition by the basal transcription machinery. This element is homologous to the consensus Inr element [YYAN(T/A)YY] (24). Mutations within this element dramatically affect promoter activity. We then addressed how RNAPII is recruited to the β -pol Inr by analyzing the previously proposed models and showed that components of the basal transcriptional machinery recognize and specifically position RNAPII on the β -pol Inr, not a specific Inr-binding protein, Sp1 or TFIID. However, our studies do not rule out the possibility that such factors enhance recruitment of the GTFs to the Inr. Indeed, we demonstrated that the upstream elements stimulate transcription.

Inr elements with various strengths have been identified in numerous TATA-containing and TATA-less promoters, including the AdMLP, AdIVa2, murine and human DHFR, and AAV p5 promoters, among others (2, 9, 10, 50, 52, 55). Sequences surrounding the transcription initiation sites of the human β -pol promoter from -2 to +6 are required for the recruitment and precise positioning of the basal transcription machinery. The detrimental effect of mutating this element can be circumvented by the placement of an upstream TATA element. The upstream TATA element provides an alternative pathway to recruit the basal machinery in the absence of a functional Inr in vitro.

We found that a consensus binding site for the transcription factor YY1 is contained within the β -pol Inr. In agreement with our studies, a polypeptide similar in molecular weight to human YY1 has been purified from calf thymus and has been shown to bind to the β -pol Inr by gel mobility shift assay and UV cross-linking (20). Our functional studies, however, indicate that the YY1 binding site present in the β -pol promoter is dispensable for transcription in vitro and in transiently transfected cells. YY1 binding sites coincident with Inrs were first identified in the AAV p5 and COX Vb promoters (3, 50, 51). In the case of the TATA-containing AAV p5 promoter, YY1 was found to be required for transcription and nucleated a functional "mini" preinitiation complex containing RNAPII and TFIIB. This trimeric complex was found to be sufficient for transcription (60, 61). YY1 has also been shown to be required for transcription of the COX Vb promoter in vitro in assays using *Drosophila* nuclear extract, which lacks YY1 binding activity (3). In our studies, we found that a point mutation at +2 on the β -pol promoter, which decreases the affinity of YY1

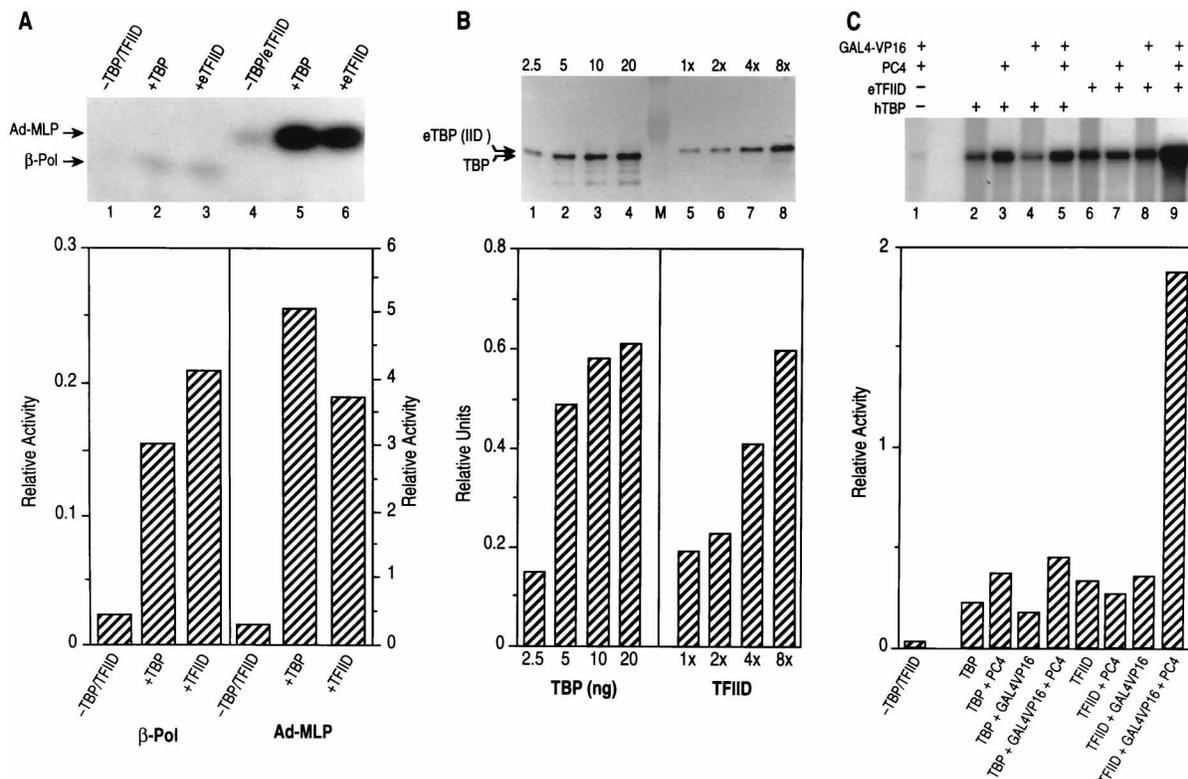


FIG. 9. Complex formation on the TATA-less β -pol promoter is TAF independent. (A) Abortive initiation reaction performed as described in Materials and Methods with the following modifications. Reactions were performed with the wild-type β -pol fragment digested with *EcoRI* and *PstI* (100 ng, 940 fmol) or pG5MLT linearized by digestion with *EcoRI* (50 ng, 24 fmol), TFIID (hydroxylapatite fraction, 33 ng), and recombinant human TBP or eTFIID. Transcription factors were preincubated with the templates for 20 min at 28°C prior to the addition of ribonucleotides. After addition of nucleotides, reactions were incubated for an additional 50 min at 28°C and analyzed as described in Materials and Methods. The autoradiogram was scanned by using a Sharp 330 device, and results were quantitated by using Imagemaster software (Pharmacia). The quantitation of the abortive RNA transcripts is depicted below the autoradiogram. (B) Quantitative immunoblot to determine the amount of TBP present in the purified eTFIID. Various concentrations of eTFIID and known amounts of human recombinant TBP were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Immunoblot analysis was performed as described in Materials and Methods. The immunoblot was scanned with a Sharp 330 scanner and was quantitated with Imagemaster software. The quantitation of the reactive material is illustrated below the immunoblot. (C) In vitro-reconstituted transcription assay of the G-less AdMLP-containing template, pG5MLT. Productive transcription was performed as described in Materials and Methods. Human recombinant TBP, eTFIID, the coactivator PC4, and the activator GAL4-VP16 were added as indicated. The transcripts were quantitated as for panel A, and results are graphed in the lower panel.

for its Inr-overlapping binding site, has no effect on transcription. These results are analogous to those from the Smale laboratory using the AAV p5, DHFR, and TdI Inrs (24). The transcripts derived from the β -pol +2 mutant promoter were transcribed by RNAPII, since they were sensitive to low concentrations of α -amanitin (2 μ g/ml). Another major difference between transcription directed by the β -pol promoter and that of the AAV p5 promoter is that transcription from the former promoter requires the different GTFs. Incubation of nuclear extracts with antibodies which recognize TBP, the small subunit of TFIIE, the small subunit of TFIIF, or the large subunit of TFIID resulted in inhibition of transcription from the β -pol promoter (data not shown). Furthermore, these results are in perfect agreement with our findings demonstrating that initiation of β -pol Inr-directed transcription measured by abortive initiation requires all six GTFs in addition to RNAPII. Our results strongly suggest that the YY1 binding site present in the β -pol promoter does not direct the formation of transcription complexes. It is possible that the YY1 binding site is important for proper positioning of the transcription complex in vivo, where the promoter is in the context of chromatin. Consistent with this hypothesis are the findings demonstrating that YY1 interacts with TBP, TFIIB, and RNAPII and that YY1 is a component of the human RNAPII holoenzyme complex (38,

61, 66a). Our results, also, do not eliminate the possibility that YY1 can mediate regulation of the β -pol promoter.

Sp1 has been proposed to be required to tether the basal transcription machinery to TATA-less promoters via interaction with the TFIID complex (45, 56). Our results demonstrate that Sp1 is not required for nucleation of the basal factors on the TATA-less β -pol promoter. Accurate initiation of transcription occurs in the absence of upstream elements; however, the levels of transcription were drastically decreased (Fig. 6D). These results suggest that the GTFs are sufficient to recognize the Inr and that the upstream elements function to increase the levels of transcription. Since the ATF and Sp1 elements were removed simultaneously, the contribution of each transcription factor to the level of transcription is not clear. It is likely that the decrease is due to the removal of the Sp1 elements, since the ATF binding site has been shown to mediate responses to stimuli (42). Thus, Sp1 may aid in positioning transcription initiation by further stabilizing a preexisting interaction between the basal factors and the Inr and may play a more critical role in targeting the basal machinery to the TATA-less β -pol promoter in vivo. In addition, slight alterations in start site usage were observed when the Sp1 elements were mutated individually. Similar observations have been made for the DHFR promoter, where individual mutations in three of the four

upstream Sp1 elements affect levels and usage of the major and minor transcription initiation sites *in vivo* and *in vitro* (5).

It has also been proposed that TFIID itself mediates preinitiation complex assembly on TATA-less promoters. As has been previously described, TFIID makes multiple contacts with the promoter; TBP has been shown to bind to the TATA element located approximately 30 nucleotides upstream from the transcription start site, and TAFs have been shown to contact regions downstream of the transcription start site. Gel mobility shift assays have also shown that TBP binds to the -30 regions from the TATA-less simian virus 40 major late, TdT, DHFR, interferon regulatory factor, and mouse ribosomal protein L32 promoters, although it does so with a much lower affinity than it binds to a consensus TATA element (69). This is in agreement with studies that correlate the similarity between a promoter's TATA element and the TATA consensus element with promoter strength (71). These results suggest that TBP contacts the -30 region of promoters and that the strength of the interaction aids in stabilizing preinitiation complex formation, thus resulting in increased levels of transcription. Recently it has been shown that a TFIID complex containing a TBP defective in TATA recognition is capable of supporting Inr-dependent transcription (41). Thus, sequence-specific binding of TBP to the -30 element is not required to direct the formation of a transcription-competent complex. It has also been demonstrated by using partially purified transcription systems that Inr-mediated transcription has similar factor requirements as TATA-mediated transcription and that later steps in transcription initiation proceed by similar mechanisms regardless of the presence of a TATA element (25, 40, 45, 72). Consequently, it is possible that TBP is positioned at the -30 promoter region through interaction with other components of the basal machinery, such as RNAPII, which has been shown to have a weak affinity for the Inr (9).

Several lines of evidence support the hypothesis that a TAF nucleates preinitiation complex formation on the Inr. DNase I footprinting experiments demonstrated that TFIID footprints extend downstream of the TATA and Inr motifs to approximately $+30$, whereas TBP protects only the TATA region (see references 11 and 73 and references therein). The downstream protection was found to be absent on promoters that apparently lack an Inr (7, 27, 65). DNase I footprinting experiments from the Smale and Gilmour laboratories have shown that binding of TFIID to a promoter containing TATA and Inr elements produces a hypersensitive site at $+5$ (27, 46). Although the appearance of this hypersensitive site was sensitive to mutations in the Inr, including a point mutation at $+3$, close examination of the data reveals that TFIID actually binds more strongly to sequences surrounding the Inr, rather than to the Inr itself. Recent studies from the Smale laboratory have shown that *Drosophila* TAF_{II}150 stimulates transcription from a TATA-containing promoter in an Inr-dependent fashion (28). Interestingly, human TAF_{II}150 does not appear to be associated with the TFIID complex (27, 28, 40, 73). Burke and Kadonaga (7) have identified a downstream element conserved in many TATA-less *Drosophila* promoters which is (i) protected by TFIID from cleavage in DNase I footprinting experiments, (ii) required for transcriptional activity of the promoter, (iii) able to compensate for mutations of the TATA element, and (iv) capable of functioning together with the Inr to facilitate TFIID interaction with the promoter (7). The TdT promoter has also been shown to contain a downstream element that stimulates transcription from the promoter in a TAF-dependent fashion (40, 55). Consequently, the primary function of TAFs with respect to Inr-mediated transcription may be to stabilize the preinitiation complex via interaction

with a downstream element, not nucleate complex formation on the Inr.

Results from our laboratory suggest that TAFs are not essential for nucleation of the basal factors to the Inr. Recombinant TBP can reconstitute transcription in an *in vitro* assay devoid of TFIID from the AdIVa2 promoter, an AdMLP containing a mutant, nonfunctional TATA element, and the TATA-less DHFR promoter (1, 2, 10). Also, a stable and specific complex observed in the electrophoresis mobility shift assay can be detected on the AdIVa2, AdMLP, TdT, and DHFR Inrs in the presence of recombinant TBP, TFIIB, and TFIIF and highly purified RNAPII (1, 9). We have extended these studies to show that a similar complex is formed on the β -pol Inr and that this complex is dependent on the presence of all four factors, since omission of TBP, TFIIB, TFIIF, or RNAPII abolishes complex formation. Importantly, a point mutation which abolishes Inr function results in decreased specific complex formation, although point mutations which do not affect transcriptional activity of the Inr also do not affect DBPolF complex formation as dramatically. We do observe a slight reduction in the amount of DBPolF complex being formed on the $+2$ mutant Inr which exhibits wild-type levels of transcription (Fig. 5A). This apparent dichotomy may be explained by the stabilization of the complex by the binding of another factor, such as TFIIE, TFIIF, or a TAF, which results in the $+2$ mutant being as transcriptionally active as the wild type. Simultaneous mutation of the minimal β -pol Inr at $+1$, $+2$, and $+3$ abolishes DBPolF complex formation, thus strengthening our observation that a mutation at $+3$ reduces DBPolF complex formation (data not shown and Fig. 7). Furthermore, abortive initiation experiments show that the basal machinery is sufficient for initial phosphodiester bond formation on the β -pol Inr. The abortive transcript observed is specific, since a mutation in the Inr results in decreased activity. TBP-mediated abortive initiation is almost as efficient as TFIID-mediated initiation (Fig. 9A). Collectively, our results strongly suggest that the basal factors together nucleate a complex on the Inr and position transcription initiation. However, we do not rule out the possibility that additional factors such as TAFs or specific transcription factors aid in Inr recognition and efficiency of complex formation.

Studies performed *in vivo* support the idea that components of the basal transcription machinery other than TFIID position transcription initiation. Genetic analysis has identified mutations in TFIIB, and three subunits of RNAPII, RPB1, RPB2, and RPB9, which alter transcription start site selection in yeast cells *in vivo* (4, 17, 21, 23, 44). Interestingly, mutations in the large subunit of TFIIF are able to compensate for mutations in TFIIB and restore the correct position of transcription initiation (58). These results further illustrate the critical role that the protein-protein interactions between the basal transcription factors play in the nucleation and positioning of the preinitiation complex on promoters recognized by RNAPII.

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