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A Mouse Mammary Tumor Virus Promoter Element near the Transcription Initiation Site

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Transcription from the promoter of mouse mammary tumor virus is subject to both positive and negative control by cellular factors, and proviral promoter elements that mediate a basal level of transcription must in some way respond to these cellular regulatory signals. Several such elements, including a TATA box, a region containing three octamer-related sequences, and a binding site for nuclear factor 1, have been previously defined. Additional promoter mutations have allowed a fourth basal promoter element to be identified near the transcription initiation site between +2 and +10. Sequence alterations within this element affect transcription both in vivo and in vitro. Gel electrophoresis mobility shift and DNase I footprinting assays define a nuclear protein, termed initiation site-binding protein, that specifically recognizes this region of the promoter. Optimal levels of transcription from the mouse mammary tumor virus promoter require initiation site-binding protein, as demonstrated by a correlation between protein affinity and transcriptional activity and by specific inhibition of transcription in vitro by an oligonucleotide capable of titrating the protein from transcriptionally active fractions.

Appropriate expression of eukaryotic genes transcribed by RNA polymerase II is dependent on the interaction of *cis*-acting DNA elements with *trans*-acting, sequence-specific DNA-binding proteins (for reviews, see references 28, 37, and 53). These *cis*-acting elements can, in general, be divided into two nonmutually exclusive classes: promoter elements that function to define a basal level of transcription and regulatory elements that function to modulate this basal activity in either a positive or negative manner. Promoters of genes transcribed by RNA polymerase II typically include an A-T-rich element termed the TATA box, located at approximately −30 to −25 with respect to the start of transcription (+1). This sequence is recognized by transcription factor IID (TFIID), and this interaction appears to play the major role in fixing the site of transcription initiation (41). Other promoter elements are generally located 5′ of the TATA box within about 100 bp of the transcription start site. However, there are exceptions to this general organization (12, 27, 38, 56).

The interaction of basal promoter elements with both positive and negative regulatory elements has been studied in several systems. For example, transcription from the promoter within the long terminal repeat (LTR) of the proviral DNA of mouse mammary tumor virus (MMTV) is subject to both positive and negative regulation. Transcription is induced by several classes of steroid hormones, including glucocorticoids (49, 62), progestins (10, 11), and androgens (10), and the hormone response element that mediates induction by all of these hormones is located between about −200 and −80 (25, 32, 36, 48). In addition, the MMTV LTR contains a distal negative regulatory element, located between −427 and −364, which selectively represses basal activity of the promoter, thus increasing the ratio of gene expression in the presence and absence of hormone (24, 33, 40). This element functions in cooperation with a more promoter-proximal negative regulatory element centered around −150 and thus located within the hormone response element (33). Understanding the molecular details by which such regulatory elements modulate transcription depends, ultimately, on defining the mechanisms of basal, unregulated transcription and elucidating how this basal transcription responds to both positive and negative regulatory signals. To this end, we have recently performed a detailed mutational analysis of the MMTV promoter, including construction of a series of linker-scanning mutations across the entire promoter region from −100 to +1 (59). This analysis revealed several elements that are required for both basal and glucocorticoid-induced transcription from the promoter. One element contains a TATA box centered at about −30. A second set of elements is composed of two tandem 10-bp repeats between −60 and −39, within which are three sequences related to the octamer (16, 46, 59); these octamer-related sequences were shown to be recognized by a nuclear protein. A third element contains a recognition sequence for nuclear factor 1 (NF-1) (13, 14, 44) between −77 and −63.

Here we identify an additional MMTV promoter element immediately 3′ of the transcription initiation site. This element is defined by promoter mutations that alter transcription from the MMTV promoter in vivo and in vitro. Furthermore, we identify a nuclear protein, which we term the initiation site-binding protein (ISBP), that recognizes this element. Correlation of transcriptional activity and ISBP binding with mutated promoters and inhibition of in vitro transcription by an oligonucleotide capable of titrating ISBP from transcriptionally active fractions indicate that ISBP is required for optimal levels of MMTV promoter activity.

MATERIALS AND METHODS

Plasmids and DNA templates. The construction of pLSwt has been described elsewhere (59). Plasmid pLS(−2/+6) was constructed in the same manner as were other linker-scanning plasmids that we have previously described (59). Both pLSwt and pLS(−2/+6) contain wild-type MMTV sequences between −363 and +133, the only difference being that pLS(−2/+6) contains a synthetic *Bgl*II linker which replaces nucleotides between −2 and +6 relative to the

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transcription initiation site; these MMTV sequences are linked to coding sequences for chloramphenicol acetyltransferase (CAT) and the simian virus (SV40) small-t intron and early poly(A) addition signal. A region of pBR322 DNA shown to inhibit gene expression in transient transfection assays (47) has been deleted from the vector sequences in these plasmids.

Plasmid pT7Δ5 contains MMTV-CAT coding sequences inserted into the polylinker of pT7/T3-19 (Promega). The starting plasmid for this construction was a 5′ deletion of the MMTV promoter with the deletion endpoint at +34 (pΔ5-58) (59). This plasmid was digested with *Sma*I, which cuts immediately 5′ to the MMTV sequences, a *Hin*dIII linker was added at this site, and the plasmid was religated to give pHΔ5. This plasmid was digested with *Hin*dIII and *Eco*RI to give a 350-bp fragment of MMTV-CAT DNA, which was isolated by gel electrophoresis and ligated into pT7/T3-19 digested with the same enzymes to give pT7Δ5. This construct allows MMTV-CAT RNA to be synthesized in vitro by using T7 RNA polymerase under conditions previously described (60).

Templates for in vitro transcription consisted of isolated restriction fragments as described in the figure legends. These template fragments were isolated from 5% polyacrylamide gels and quantitated by A_{260} ($\varepsilon = 20$ ml · mg^{-1} · cm^{-1}).

Transfections and CAT assays. Mouse Ltk$^-$ cells were transfected by the DEAE-dextran-dimethyl sulfoxide shock method (35) as previously described (60) except that 5×10^5 cells per dish were plated the day before transfection. CAT assays were performed as previously described (60); 40 μg of protein was assayed for 2 h when the extracts were from cells grown in the absence of hormone, and 5 μg was assayed for 1 h when extracts were from cells treated with 0.1 μM dexamethasone.

Nuclear extracts. Nuclear extracts of tissue culture cells were prepared by the method of Dignam et al. (15), with a few previously described modifications (31).

Nuclear extracts of liver cells were prepared from livers obtained from normal adult cows (Meat Science and Technology Center, Texas A&M University). Nuclei were prepared from the freshly removed livers by the method of Lichtsteiner et al. (34), with the following modifications. All operations were performed at 0 to 4°C. Liver (200 g) was minced and combined with 1 liter of homogenization buffer in a Waring blender. The blender was filled to the brim with buffer and sealed with Parafilm to exclude air and prevent the generation of foam. Leupeptin (1 μg/ml), pepstatin A (1.7 μg/ml), and phenylmethylsulfonyl fluoride (0.5 mM) were added to the nuclear lysis buffer. After isolation of the nuclei and extraction of the nuclear proteins, the protein pellet was resuspended in a minimal volume of buffer TM0.1 M (15) (50 mM Tris-HCl [pH 7.9], 20% glycerol, 1.0 mM EDTA, 1.0 mM dithiothreitol [DTT], 0.1 M KCl). The extract was dialyzed against two changes of 600 ml of the same buffer for at least 2 h for each change. The precipitate that formed during dialysis was removed by centrifugation. Protein concentration was determined by the method of Bradford (7) (Bio-Rad), with bovine serum albumin as the standard. The extract was divided into aliquots, quick-frozen in liquid nitrogen, and stored at −70°C.

Fractionation of nuclear extracts. Liver nuclear extracts were fractionated by two different protocols, both of which were originally developed for HeLa cell nuclear extracts. The first protocol was adapted from Tsai et al. (61). Nuclear extract was dialyzed against buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 20% glycerol, 0.1 mM EDTA, 5 mM $MgCl_2$, 2 mM DTT) containing 50 mM $(NH_4)_2SO_4$ and loaded onto a DEAE-Sephadex column. The flowthrough fraction (DE50) was collected, and two additional fractions were obtained by step elution with buffer A containing 175 mM $(NH_4)_2SO_4$ (DE175) and then with buffer A containing 500 mM $(NH_4)_2SO_4$ (DE500). All three fractions were concentrated by $(NH_4)_2SO_4$ precipitation; the DE175 and DE500 fractions were redissolved in buffer A, dialyzed, and aliquoted for storage at −70°C. The DE50 fraction was redissolved and dialyzed against buffer B (20 mM HEPES [pH 7.9], 20% glycerol, 0.2 mM EDTA, 2 mM DTT) containing 100 mM NaCl and loaded onto a phosphocellulose column (Whatman P-11). The column was washed with the same buffer to collect a flowthrough fraction (DE50.PC100), and three additional fractions were obtained by step elution with buffer A containing 250 mM KCl (DE50.PC250), 600 mM KCl (DE50.PC600), and 1 M KCl (DE50.PC1000). These fractions were concentrated by $(NH_4)_2SO_4$ precipitation; protein was redissolved in buffer A containing 100 mM KCl, dialyzed, and aliquoted for storage at −70°C. All procedures were carried out at 0 to 4°C. Transcription from the MMTV promoter was reconstituted with the DE175, DE500, and DE50.PC600 fractions. The relative amounts of these fractions required for optimal MMTV promoter activity varied somewhat among different preparations.

The second fractionation protocol was based on that described by Dignam et al. (15). Liver nuclear extracts in buffer TM0.1M were loaded on a phosphocellulose column (Whatman P-11) at a ratio of 15 to 20 mg of protein per ml of packed resin. The column was washed with the same buffer to collect a flowthrough fraction (PC100), and three additional fractions were obtained by step elution with buffer TM containing total KCl concentrations of 300 mM (PC300), 500 mM (PC500), and 1.0 M (PC1000). These fractions were concentrated by $(NH_4)_2SO_4$ precipitation; protein was redissolved in buffer TM0.1M, dialyzed, and aliquoted for storage at −70°C. All procedures were carried out at 0 to 4°C. Transcription from the MMTV promoter was reconstituted with the PC100, PC500, and PC1000 fractions.

Further purification of ISBP from the PC1000 fraction was carried out by sequence-specific DNA affinity chromatography (29). Approximately 300 μg of complementary 33-bp oligonucleotides encompassing MMTV sequences from −16 to +17 (Fig. 1A) were phosphorylated with ATP and T4 polynucleotide kinase (Bethesda Research Laboratories) and allowed to hybridize. The double-stranded oligonucleotides were concatamerized (T4 ligase; 20,000 U/μl; New England Biolabs) by way of *Bgl*II-*Bam*HI overhangs to give unidirectional oligomers (50) with an average size of 200 bp (6-mer), as determined on a 4% polyacrylamide gel. The DNA was covalently attached to CNBr-activated Sepharose CL-2B resin (Pharmacia) as described previously (1). The PC1000 fraction [containing poly(dI · dC) as nonspecific DNA in an amount determined by the method of Kadonaga and Tjian (29)] was loaded on this column, which was washed with TM0.1 M containing 0.1% Nonidet P-40 (NP-40) and then with 1 M KCl in TM buffer containing 0.1% NP-40. Fractions (0.2 ml) were collected, and aliquots were tested for ISBP activity by gel electrophoresis mobility shift assay. Active fractions were pooled and applied to a second DNA affinity column, which was run identically to the first. Active fractions from the second column were concentrated and dialyzed against TM0.1 M containing 0.1% NP-40, and aliquots were frozen and stored at −70°C.

In vitro transcription. The transcription procedure in-

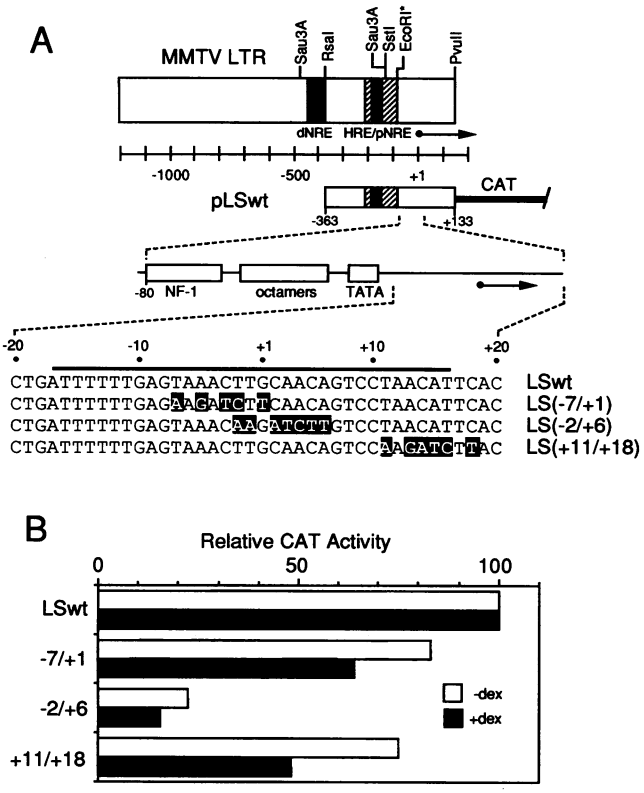

FIG. 1. (A) Sequence of MMTV promoter mutations near the transcription initiation site. The MMTV LTR is depicted as a box with several restriction sites and the locations of the HRE, proximal negative regulatory element (pNRE), and distal negative regulatory element (dNRE) indicated. Plasmid pLSwt (59) contains LTR sequences from −363 (*Rsa*I) to +133 (*Pvu*II) linked to a CAT reporter gene. The MMTV promoter contains a binding site for NF-1, three octamer-related sequences, and a TATA element (59). Promoter mutations near the initiation site are shown as shaded letters. The black bar (−16 to +17) defines the oligonucleotide used for gel electrophoresis mobility shift and transcription competition experiments. (B) CAT expression from mutated promoters. Plasmids containing MMTV promoter mutations were transfected into mouse Ltk$^-$ cells, and CAT expression was determined in the presence and absence of the synthetic glucocorticoid dexamethansone (dex). CAT expression from the mutated promoters was normalized to that obtained from LSwt, and the normalizations were performed separately for expression in the presence and absence of hormone. Each bar reflects an average of three to six independent transfections with a standard error of less than 20% of the mean. Absolute levels of CAT expression in extracts of cells transfected with LSwt were 0.13 and 5.38 nmol · min^{-1} · mg^{-1} in the absence and presence of hormone, respectively.

cluded a 30-min preincubation of DNA with nuclear extract fractions at 30°C followed by the addition of ribonucleoside triphosphates and a second 30-min incubation to allow for runoff transcription (21, 22). The preincubation was performed in 20 μl containing extract fractions as indicated in the figure legends and 100 ng of template DNA unless otherwise indicated. Buffer conditions were 12 mM Tris-HCl (pH 7.9), 12% glycerol, 60 mM KCl, 0.1 mM EDTA, and 10 mM $MgCl_2$ (including contributions made by the extract). After 30 min at 30°C, 10 U of RNasin (Promega) was added along with nucleotides to final concentrations of 600 μM ATP, CTP, and GTP and 50 μM [α-^{32}P]UTP (8 Ci/mmol, final specific activity), bringing the final volume to 25 μl. Reactions were terminated by the addition of 50 μl of 10 mM EDTA, 0.1 M sodium acetate (pH 5.5), 0.5% sodium dodecyl sulfate (SDS), and 1 mg of yeast tRNA per ml and then extracted with 75 μl of buffered phenol-chloroform (2:1). The organic phase was reextracted with 25 μl of transcription stop buffer, and the aqueous phases were combined and precipitated with 2.5 volumes of ethanol. Pellets were washed with 70% ethanol and resuspended in 5 μl of 98% formamide plus tracking dyes. Transcripts were subjected to electrophoresis on a 6% polyacrylamide gel containing 7 M urea. Running buffer contained 0.09 M Tris base, 0.09 M borate, and 2.5 mM EDTA. Gels were exposed to Kodak XAR-5 film (Eastman Kodak Co.), at −70°C with an intensifying screen. RNA generated by T7 RNA polymerase from pT7Δ5 in the presence of [α-^{32}P]UTP (0.1 Ci/mmol) was added to the transcription reactions with the stop buffer and served to standardize recoveries of the specific transcript during preparation for electrophoresis. Transcription was quantitated by excising bands from the dried gel and scintillation counting, by film densitometry, or by way of a Betagen blot analyzer.

The sequence of the double-stranded, nonspecific oligonucleotide used in the competition experiment described in Fig. 7 was 5′-GATCCAGTCTGATCAGACTGGATC-3′.

Gel electrophoresis mobility shift assay. Complementary 33-bp oligonucleotides spanning the MMTV transcription initiation site (−16 to +17) were synthesized on an Applied Biosystems DNA synthesizer (Oligonucleotide Synthesis Services, Department of Biochemistry and Biophysics, Texas A&M University). Equal molar amounts of the complementary strands were mixed and annealed by heating at 100°C for 2 min and cooling slowly to room temperature. Binding experiments were performed by incubating probe (0.5 to 1.0 ng, 5,000 to 10,000 Cerenkov cpm) labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear) with the indicated amount of protein for 15 min on ice in 15 μl of buffer containing 50 mM Tris-HCl (pH 7.9), 1.0 mM EDTA, 12.5 mM $MgCl_2$, 20% glycerol, 1.0 mM DTT, 1 μg of poly(dI · dC), and 0.1 M KCl. The mixture was then loaded on a 4% polyacrylamide gel containing 0.1 M Tris-borate buffer (pH 8.3), 2 mM EDTA, and 10% glycerol and electrophoresed at 4°C at 20 V/cm in the same buffer without glycerol. Gels were exposed to Kodak XAR-5 film for 24 h at −70°C with an intensifying screen. When a competitor was used, the competitor DNA was added to the extract-buffer mixture and incubated on ice for 10 min, the labeled DNA was then added, and incubation continued for an additional 15 min.

DNase I footprinting. Radioactively labeled DNA probes contained MMTV sequences from position −69 to +105 and were generated by the polymerase chain reaction, using plasmid pLSwt (59) as a template. For each footprinting probe, one oligonucleotide primer was 5′ end labeled with [γ-^{32}P]ATP (6,000 Ci/mmol) and T4 polynucleotide kinase. Size markers were prepared by subjecting a portion of the probe to Maxam and Gilbert DNA sequencing (39) as modified by Bencini et al. (4).

DNase I footprinting reactions were done in a total volume of 20 μl in a mixture containing 50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 100 mM KCl, and 20% (vol/vol) glycerol, with 1 μg poly(dI · dC), 9 fmol of polymerase chain reaction-generated probe (1,000 to 5,000 cpm/fmol), and ISBP that had been purified over two sequential sequence-specific DNA affinity columns. Binding reaction mixtures were incubated for 40 min at room temperature and then for 10 min on ice. Two units of pancreatic

DNase I (RNase free; U.S. Biochemical) was added, and the reaction mixtures were incubated for 2 min on ice and then stopped by adding 110 μl of a solution containing 0.1 M sodium acetate (pH 5.2), 10 mM EDTA, 0.1% (wt/vol) SDS, and 20 μg of tRNA per ml. The samples were phenol-chloroform extracted, and the DNA was precipitated with ethanol. Samples were resuspended in a mixture of 80% (vol/vol) formamide, 10 mM NaOH, 1 mM EDTA, and 0.1% (wt/vol) each xylene cyanol and bromphenol blue. Samples were fractionated on a 6% polyacrylamide gel containing 7 M urea, 90 mM Tris-borate, and 2 mM EDTA. The gel was fixed in 5% (vol/vol) methanol and 5% (vol/vol) acetic acid for 20 min, dried, and exposed to XAR-5 film with an intensifying screen for 2 to 4 days.

RESULTS

Mutations near the transcription start site decrease the activity of the MMTV promoter. Our previous linker-scanning analysis of the MMTV promoter extended from approximately −100 to the transcription initiation site (59). This analysis revealed no functional promoter elements 3′ of the TATA box. Subsequent experiments, in which we observed decreased transcription from the MMTV promoter when sequences at or immediately 3′ of the transcription initiation site were altered, led us to investigate this region of the MMTV LTR more systematically. To this end, we constructed two additional linker-scanning mutations in the MMTV promoter by recombining 5′ and 3′ deletions such that 8 bp of MMTV DNA were replaced by a synthetic *Bgl*II linker (AAGATCTT) as we have previously described (59). These mutated promoters were designated LS(−2/+6) and LS(+11/+18), where the numbers in parentheses indicate the nucleotides replaced by the *Bgl*II linker (Fig. 1A). As in our previous linker-scanning mutations, these constructions contain MMTV sequences from −363 (*Rsa*I) to +133 (*Pvu*II) linked to the coding sequences for CAT followed by the SV40 small-t intron and sequences required for poly(A) addition (59). The vector sequences contained a deletion of a region of pBR322 which has been shown to inhibit transient gene expression (47). These plasmids were transfected into mouse Ltk$^-$ cells by the DEAE-dextran-dimethyl sulfoxide shock procedure (35) as described in detail elsewhere (60). Sixty hours after shock, cells were harvested and crude extracts were assayed for CAT activity (60) and total protein (7). CAT expression from LS(−2/+6) was about six times lower than that from the wild-type promoter in LSwt (Fig. 1B), while adjacent linker-scanning mutations LS(−7/+1) (59) and LS(+11/+18) had comparatively little effect.

Since the LS(−2/+6) promoter contains five mutated bases downstream of the normal transcription start site, the mRNA transcribed from this promoter would be expected to be altered relative to the wild-type transcript. Thus, the observed decrease in CAT activity may not be due solely to changes in transcriptional activity but may also reflect altered RNA stability or efficiency of CAT translation relative to LSwt.

To determine whether the MMTV promoter mutation in LS(−2/+6) did, indeed, alter transcriptional efficiency of the promoter, we turned to in vitro transcription assays. An efficient in vitro assay for MMTV promoter activity was developed from fractionated bovine liver nuclear extracts. Extracts were prepared from fresh liver by a procedure slightly modified from that of Lichtsteiner et al. (34). The MMTV promoter was not transcribed in these crude extracts, but transcription could be obtained after minimal fractionation and reconstitution by either of two procedures involving fractionation by DEAE-Sephadex and phosphocellulose or by phosphocellulose alone (see Materials and Methods).

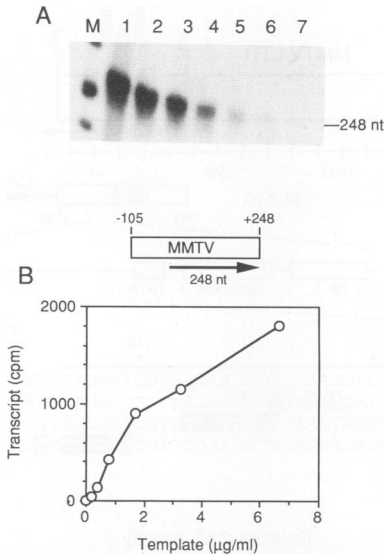

FIG. 2. In vitro transcription from the MMTV promoter. An isolated MMTV fragment (−105 to +248) was used as a template in a transcription system reconstituted from nuclear extracts of bovine liver. Transcription reaction mixtures contained the following amounts of protein from extract fractions (see Materials and Methods): 1.5 μg of DE175, 9 μg of DE500, and 27 μg of DE50.PC600. (A) Electrophoretic analysis of transcription products. Template concentrations were 6.6 (lane 1), 3.3 (lane 2), 1.7 (lane 3), 0.8 (lane 4), 0.4 (lane 5), 0.2 (lane 6), and 0.1 (lane 7) μg/ml. Lane M contained size markers consisting of ^{32}P-labeled fragments from a *Msp*I digestion of pBR322 DNA. (B) Template dependence of transcription. Bands were excised from the dried gel shown in panel A and counted. nt, nucleotides.

With the transcription system reconstituted from DEAE-Sephadex and phosphocellulose fractions and a transcription template composed of an isolated DNA fragment extending from −105 to +248 of the MMTV LTR, a runoff transcript of the appropriate size which was dependent on template concentration was detected (Fig. 2). Appearance of this transcript was sensitive to low levels of α-amanitin (0.2 μg/ml), and its synthesis was dependent on each of the three fractions used in the reconstitution (data not shown). A similar template fragment extending only to +133 yielded the expected shorter runoff transcript (Fig. 3), confirming the specificity of transcription initiation. As further confirmation that the observed in vitro transcription accurately reflects activity of the MMTV promoter, we assayed several transcription templates containing linker-scanning mutations in the MMTV promoter. Mutations in the binding site for NF-1 [LS(−65/−58)] and the TATA box [LS(−36/−29)] decreased in vitro transcription to 23 and 7%, respectively, of the level obtained with the wild-type promoter (Fig. 4); these activities roughly correspond to the relative in vivo transcriptional activities of promoters with these mutations (59). Quantitative assessments of transcription in vitro were made by normalizing results to those for a ^{32}P-labeled internal standard RNA of 370 nucleotides added immediately after completion of the transcription reactions; this standard RNA served to normalize for differential recovery of samples

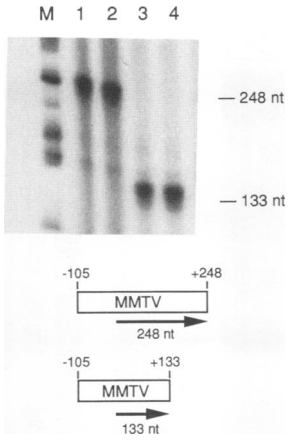

FIG. 3. Synthesis of appropriately sized transcripts in the reconstituted transcription system. Two isolated MMTV DNA fragments, one containing sequences from −105 to +248 (lanes 1 and 2) and the other containing sequences from −105 to +133 (lanes 3 and 4), were used as templates in the in vitro transcription system reconstituted from bovine liver nuclear extracts. Template concentrations were 1.8 μg/ml (lanes 1 and 3) and 3.6 μg/ml (lanes 2 and 4). Lane M contained size markers as described in legend to Fig. 1. Transcription reaction mixtures contained the following amounts of protein from extract fractions (see Materials and Methods): 13 μg of DE175, 3.5 μg of DE500, and 6 μg of DE50.PC600. nt, nucleotides.

during preparation for electrophoretic analysis (see Materials and Methods).

To determine whether MMTV sequences near the transcription initiation site contribute to promoter activity, in vitro transcription from the LS(−2/+6) mutant promoter was assessed (Fig. 4). Transcript accumulation from the mutated template was decreased to approximately 50% of the wild-type level. This decreased accumulation could be the result of decreased transcriptional activity of the mutated promoter or to differential stabilities of the RNAs derived from the wild-type and mutated templates. The contribution of differential RNA stability was determined by comparing the stabilities of transcripts from the wild-type and LS(−2/+6) templates in the reconstituted extract fractions. After

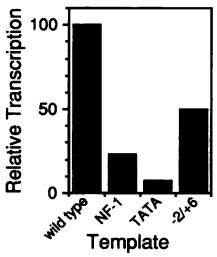

FIG. 4. In vitro transcription of mutated MMTV promoters. MMTV promoter templates (3.6 μg/ml) containing linker-scanning mutations were transcribed in vitro, and transcripts were quantitated by densitometry of exposed films after gel electrophoresis and autoradiography. A labeled internal standard RNA generated by T7 RNA polymerase was added to each sample to normalize recovery. Template activity is presented relative to that of the MMTV wild-type promoter. Transcription of templates containing mutations in the NF-1 binding site [LS(−65/−58)] and TATA box [LS(−31/−24)] (59) as well as the transcription initiation site [LS(−2/+6); Fig. 1] are shown. Reaction mixtures contained extract fractions as described in the legend to Fig. 3.

synthesis of transcripts in vitro, α-amanitin (2 μg/ml) was added to prevent further RNA synthesis. At 10-min intervals, aliquots were removed from each reaction mixture and processed. After 40 min, there was no preferential degradation of the RNA from the LS(−2/+6) template (data not shown). The decreased transcript accumulation observed with the LS(−2/+6) template is therefore due to altered transcriptional activity of the mutated promoter.

A nuclear protein recognizes sequences near the MMTV transcription initiation site. Decreased transcription from the MMTV promoter in LS(−2/+6) could be the result of altered recognition by a sequence-specific DNA binding protein. As initial experiments to identify protein(s) that specifically recognize sequences near the MMTV transcription initiation site, we performed a series of gel electrophoresis mobility shift assays (17, 19). A 33-bp synthetic oligonucleotide that contained MMTV sequences from −16 to +17 was synthesized (Fig. 1A); this DNA was 5′ end labeled with ^{32}P and incubated with nuclear extracts derived from various sources. These experiments revealed a specific binding activity in nuclear extracts from bovine liver as well as HeLa and Ltk$^-$ cells, all sources in which the MMTV promoter is transcriptionally active. An example of our results is shown in Fig. 5, in which HeLa cell nuclear extracts generate three bands of retarded electrophoretic mobility indicative of protein-DNA complexes. At least one of these complexes (labeled C2 in Fig. 5) is specific, as determined by the criteria of susceptibility to competition by excess unlabeled probe oligonucleotide (Fig. 5A, lanes 3 to 7) but relative resistance to competition by an oligonucleotide of similar length containing a binding site for NF-1 (lanes 8 to 10). Comparable results have been observed with nuclear extracts from bovine liver and Ltk$^-$ cells (data not shown). In more purified fractions containing binding activity specific for this oligonucleotide, only a single shifted band is observed with a mobility comparable to that of complex C2 (data not shown).

If any of the complexes detected by the mobility shift assay are relevant in transcription from the MMTV promoter, the ability of the proteins present in such complexes to recognize mutated promoters with decreased transcriptional activity such as LS(−2/+6) would be expected to be altered. To test this prediction, we used LSwt or LS(−2/+6) as a competitor in gel mobility shift experiments (Fig. 5B). The wild-type sequences in LSwt (lanes 3 to 6) were much more efficient in competing for complexes with the 33-bp labeled probe than were the mutated sequences in LS(−2/+6) (lanes 7 to 10). Interestingly, the linearized LSwt plasmid competitor (Fig. 5B) was apparently more efficient than the initiation site oligonucleotide competitor (Fig. 5A); this observation may reflect differences in the binding of ISBP to its recognition site in these different sequence contexts. Overall, these results establish a correlation between protein binding to sequences near the MMTV transcription initiation site and promoter activity. For ease of discussion, we term any such protein(s) ISBP.

To further localize the MMTV sequences recognized by ISBP, we used DNase I footprint analysis. These experiments were performed with ISBP that was partially purified from bovine liver nuclear extracts by phosphocellulose and sequence-specific DNA affinity chromatography (29). Protection by ISBP was observed surrounding the start of transcription on both the template and nontemplate DNA strands (Fig. 6). The footprint extended from −2 to +10 on the template strand and from −5 to +11 on the nontemplate strand. A DNase I-hypersensitive site was observed at −10

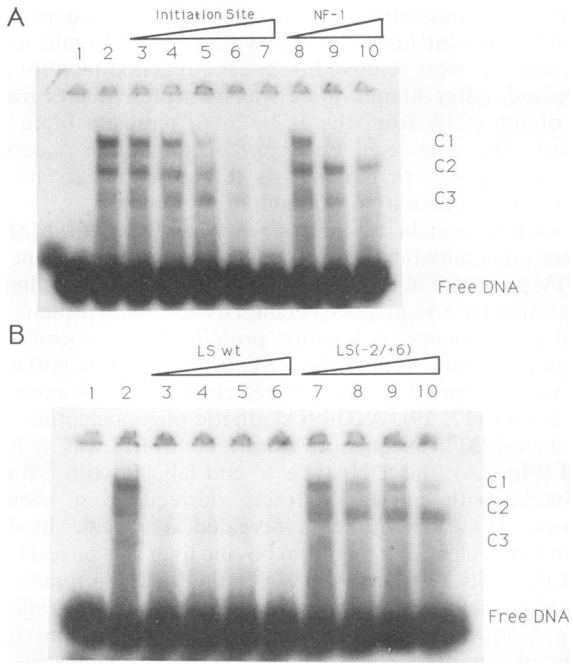

FIG. 5. Binding of a nuclear protein to the MMTV promoter near the transcription initiation site. (A) Specific binding assessed by competition. A double-stranded oligonucleotide containing MMTV sequences from −16 to +17 was ^{32}P labeled and used as a probe in a gel electrophoresis mobility shift assay with protein from a nuclear extract of HeLa cells (1 μg). Unlabeled, double-stranded competitor DNAs used to assess the specificity of protein-DNA complexes were the MMTV initiation site (−16 to +17) oligonucleotide (lanes 3 to 7) and an oligonucleotide containing a binding site for NF-1 (lanes 8 to 10). Competitor concentrations were 10 (lanes 3 and 8)- 25 (lane 4)-, 100 (lanes 5 and 9)-, 250 (lane 6)-, and 1,000 (lanes 7 and 10)-fold molar excess over the labeled probe. Lane 2 had no competitor, and lane 1 contained no extract. (B) Alteration of protein recognition by MMTV promoter mutations in LS(−2/+6). The MMTV initiation site oligonucleotide (−16 to +17) was used as a probe in a mobility shift assay with protein from a nuclear extract of HeLa cells (1 μg). Unlabeled competitor DNAs were linearized plasmids LSwt (lanes 3 to 6) and LS(−2/+6) (lanes 7 to 10). Competitor concentrations were 50 (lanes 3 and 7)-, 100 (lanes 4 and 8)-, 150 (lanes 5 and 9)-, and 250 (lanes 6 and 10)-fold molar excess over the labeled probe. Lane 2 had no competitor, and lane 1 contained no extract.

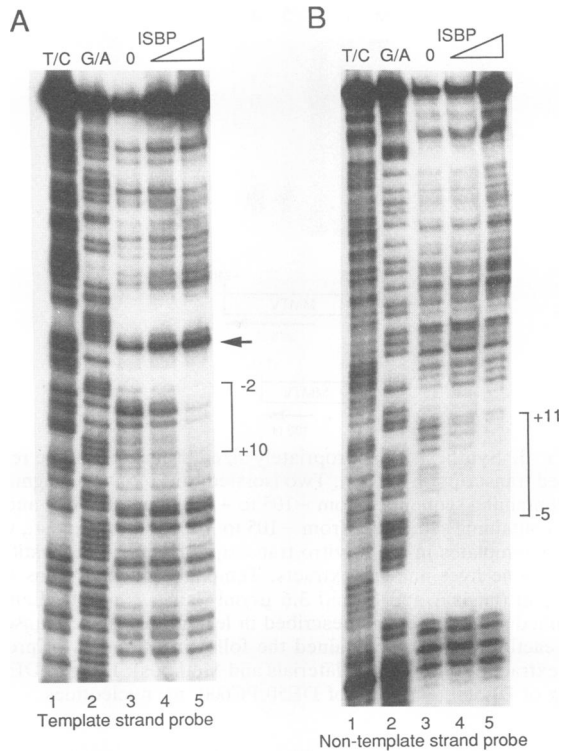

FIG. 6. DNase I footprint of ISBP. The footprinting probes contained MMTV sequences from −69 to +105, with the 5′ end label on the template strand at +105 (A) or on the nontemplate strand at −69 (B). Probe DNA was incubated with no protein (lanes 3) or with partially purified ISBP isolated by phosphocellulose and sequence-specific DNA affinity chromatography (0.1 μl [lanes 4] or 1 μl [lanes 5]). DNase I digestions were performed, and the products are shown after fractionation by denaturing polyacrylamide gel electrophoresis and autoradiography. Alignment of the cleavage pattern with the MMTV promoter sequence was accomplished with chemical sequencing reactions of the probe DNAs (lanes 1 and 2). Protected sequences are indicated by brackets. The arrow in panel A indicates a DNase I-hypersensitive site at −10.

on the template strand (arrow in Fig. 6A). No protection was seen over the TATA box on either strand.

ISBP is necessary for efficient MMTV transcription in vitro. Our results thus far indicate that MMTV promoter sequences near the transcription initiation site are required for efficient promoter activity in vitro and in vivo and that one or more nuclear proteins which we have termed ISBP recognize these sequences. To further test the idea that ISBP is required for MMTV promoter activity, the 33-bp double-stranded oligonucleotide (−16 to +17 of the MMTV LTR) used as a probe in the mobility shift assays (Fig. 5) was used as a competitor in in vitro transcription experiments. The premise of these experiments is that if ISBP is important for transcription, adding increasing amounts of the oligonucleotide will titrate out ISBP and transcription will decrease.

Preincubation of transcription assays with the 33-bp initiation site oligonucleotide (−16 to +17) resulted in a sequence-specific decrease in transcription (Fig. 7). Inclusion of a comparably sized oligonucleotide with no known protein binding site had little effect on transcription at the concentrations tested. We conclude from these experiments that titration of ISBP leads to a significant loss of MMTV promoter activity in vitro.

DISCUSSION

Efficient transcription from the MMTV promoter requires sequences near the transcription initiation site. Previous mutational analysis of the MMTV promoter defined a TATA element, several octamer-related sequences, and a binding site for NF-1 as critical for both basal and steroid hormone-induced transcription (59). We have shown here that in addition to these elements, efficient transcription from the MMTV promoter requires sequences near the transcription initiation site. Linker-scanning mutations in this region of proviral DNA were shown to affect expression of a linked CAT reporter gene (Fig. 1), and comparison of mutant and wild-type templates in an in vitro transcription assay verified that sequences near the transcription start site are necessary for optimal promoter activity (Fig. 4). This element appears to play a role in basal as well as hormone-induced transcription, since both were affected by these mutations in vivo.

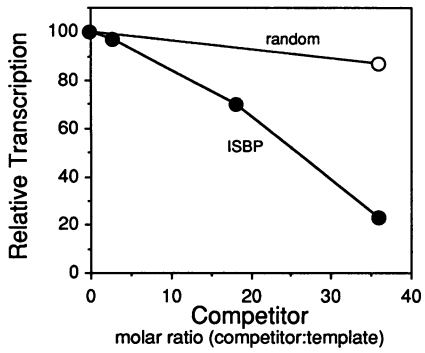

FIG. 7. Oligonucleotide titration of ISBP from the MMTV promoter. Competitor DNAs were added to transcription reactions during the preincubation of transcription factors with an MMTV template containing sequences from −105 to +248. Competitors consisted of oligonucleotides containing a binding site for ISBP (MMTV sequences from −16 to +17) (solid symbols) or a comparably sized oligonucleotide containing no known protein binding site (open symbols). Transcriptional activity is shown relative to that obtained in the absence of competitor. A labeled internal standard RNA generated by T7 RNA polymerase was added to each sample to normalize recovery. Transcription reaction mixtures contained the following amounts of protein from extract fractions (see Materials and Methods): 12 μg of PC100, 7 μg of PC500, and 1 μg of PC1000.

The linker-scanning analysis demonstrated that mutations in LS(−7/+1) and LS(+11/+18) had relatively little effect on transcription, while those in LS(−2/+6) decreased promoter activity. These experiments localized the functional promoter element to nucleotides between +2 and +10.

ISBP is required for efficient MMTV promoter activity. Electrophoresis mobility shift and DNase I footprinting experiments identified a protein (ISBP) that specifically protects MMTV promoter sequences between −5 and +11 from DNase I cleavage (Fig. 5 and 6). The binding of ISBP to the MMTV promoter is decreased in LS(−2/+6), thus correlating the transcriptional effect with the observed binding of ISBP (Fig. 5B). In addition, a requirement for ISBP binding for efficient MMTV promoter activity was demonstrated in a competition assay in which a synthetic oligonucleotide containing MMTV sequences from −16 to +17 was shown to specifically inhibit promoter activity in vitro, presumably by titrating out ISBP and making it unavailable to interact with the transcription template (Fig. 7).

A number of studies have shown that binding of TFIID and other basal transcription factors to the promoter can result in nuclease protection of sequences spanning the transcription initiation site. For example, studies on the histone H4 (20, 41) and several heat shock (20, 41) gene promoters, as well as the adenovirus major late (20, 54) and E4 (23) promoters, have shown that DNase I protection resulting from TFIID binding extends through the initiation site as far downstream as +35 (23, 41, 54). This downstream protection has been ascribed to weak, nonspecific interactions of TFIID which are stabilized once other transcription factors, along with RNA polymerase, are incorporated into the preinitiation complex (20, 63). Related observations have been made with MMTV; a phosphocellulose column fraction, presumably containing TFIID, has been shown to bind to a region of the MMTV promoter containing the TATA element by exonuclease III and DNase I protection assays and to extend weak DNase I protection as far downstream as +37 (13). This protection is similar to what we observe with use of an unfractionated nuclear extract (data not shown). However, there are several lines of evidence indicating that our results defining ISBP are not manifestations of TFIID binding at the MMTV TATA box. First, ISBP binding activity in crude nuclear extracts is specific for MMTV sequences between −16 and +17 (Fig. 5), a region which does not contain the MMTV TATA box, and our most purified fraction containing ISBP does not footprint over the TATA element. In addition, a direct competition experiment with an oligonucleotide containing the MMTV TATA region (−42 to −18) in a mobility shift assay showed that these sequences had no effect on the ability of ISBP to recognize sequences near the MMTV transcription initiation site (data not shown). Moreover, we have demonstrated that ISBP binding can be disrupted by introducing a clustered set of point mutations near the initiation site (Fig. 5B). Finally, exonuclease III protection experiments by Cordingley et al. (14) in isolated nuclei revealed a broad protected region in the MMTV promoter extending to +12, while exonuclease III protection of a TFIID-containing phosphocellulose column fraction from a nuclear extract could be demonstrated only to −4 (13). It is possible that the discrepancy between the results with isolated nuclei and fractionated nuclear extracts reflects the loss of ISBP in the tested extract fractions.

Relationship of MMTV ISBP binding site to other promoter elements. The location of the MMTV promoter element identified here is reminiscent of the promoter element termed the initiator (Inr), originally defined in the TATA-less promoter of the terminal deoxynucleotidyltransferase (TdT) gene (56). The Inr element is able to specify accurate but inefficient transcription initiation in the absence of any additional promoter element and is able to direct a high level of accurately initiated transcription in conjunction with appropriately located binding sites for transcription factors (45, 56, 57, 64). A functionally homologous element of similar sequence has also been identified at the transcription initiation site of the adenovirus major late promoter (56), indicating that Inr sequences are not restricted to TATA-less promoters. Within the context of the TdT gene, the Inr has been defined as CTCANTCT, with transcription initiation occurring at the adenine (57). However, the C at position −3 can be changed to an A without seriously destroying Inr function, and the TCT at positions +3 to +5 can be changed to AGA with retention of at least partial Inr function (57). A protein termed TFII-I has been shown to bind to Inr sequences in the adenovirus major late, TdT, and human immunodeficiency virus type 1 promoters (51). TFII-I also recognizes a binding site for the transcription factor USF, and the two proteins bind cooperatively to sites on the adenovirus major late promoter (51).

A number of genes have been shown to contain functionally defined promoter elements at or near their transcription initiation sites which bear some sequence similarity to the Inr. These include the *Drosophila* gypsy retrotransposon (26) as well as the genes encoding porphobilinogen deaminase (3), glial fibrillary acidic protein (42, 43), and myelin basic protein (58). In a number of these promoters, proteins that bind specifically to the Inr-related sequences have been identified. The extent to which the elements in these promoters are mechanistically homologous to the Inr element has not yet been determined.

The relationship of the MMTV initiation site element to the Inr is not clear. Sequences immediately surrounding +1 of the MMTV promoter bear no similarity to the Inr, but an

Inr-related sequence (AACAGTCC) is located between +3 and +10, overlapping both the functionally defined MMTV promoter element and the ISBP binding site.

Several proteins, in addition to TFII-I, have been shown to recognize sequences near the transcription initiation sites of one or more promoters. For example, a protein termed cap-binding factor (CBF) has been found in extracts of human K562 cells and footprints on the adenovirus major late promoter between +1 and +23; methylation interference assays demonstrated that sequences from +10 to +12 are important in CBF binding (18), suggesting that its function may not be related to the major late promoter Inr element. The sequence of the CBF binding site is not strikingly similar to the ISBP binding site in MMTV, and the differing chromatographic properties of the two proteins (18) make it extremely unlikely that ISBP and CBF are the same protein.

DNA sequences corresponding to the promoter-proximal 5′ untranslated leader regions of human immunodeficiency virus types 1 and 2 have been shown to interact with a transcription factor termed leader-binding protein 1 (LBP-1) (27). In crude nuclear extracts, LBP-1 binding is required for optimal levels of basal transcription from the proviral promoters (27). However, with a more purified transcription system, this effect is less apparent and high levels of LBP-1 actually repress transcription, presumably by competing with TFIID for binding to the TATA region (30). The LBP-1 binding site has been defined as CPyAG(A/T), a sequence which is potentially related to the central core of the Inr sequence (CTCANTCT) and to MMTV sequences within the ISBP binding site from +4 to +8 (ACAGT). However, LBP-1 does not recognize the Inr element of the adenovirus major late promoter (30).

A promoter element near the transcription initiation site in the murine dihydrofolate reductase (DHFR) gene that is necessary for both efficient and accurately initiated transcription has also been defined, and a nuclear protein has been shown to footprint on the DHFR promoter between −11 and +9 (38). A similar sequence was observed near the major transcription initiation site for the SV40 late promoter, and a protein in crude nuclear extracts was shown to footprint on SV40 sequences between about −7 and +18, overlapping the region of sequence similarity with DHFR. These SV40 sequences were shown previously by mutational analysis to be required for efficient transcription initiation from the late promoter (2). The relationship between the DHFR and SV40 elements was further strengthened by the observation that a concatemerized oligonucleotide corresponding to DHFR sequences between −16 and +9 was able to inhibit in vitro transcription from the SV40 late promoter, presumably by competing for a transcription factor required to bind near the SV40 transcription initiation site. Both the DHFR and SV40 late promoters lack TATA boxes and are similar in structure to the promoters of a number of so-called housekeeping genes. Because somewhat related sequences can be identified near the transcription initiation sites for several such promoters (although their functional significance has not been tested), the protein that binds near the DHFR (and presumably SV40 late) transcription initiation sites has been termed housekeeping initiation protein 1 (38). Further studies strongly suggested that this protein is identical to the cellular transcription factor E2F (5, 6). MMTV sequences protected by ISBP are not particularly related to the E2F consensus binding site (TTTCGCGC), but the E2F site in the SV40 late promoter (TTTCAGGCC) contains a five-of-six match to MMTV sequences between +5 and +10 (CAGTCC) at exactly the same position relative to the transcription initiation site. However, our preliminary experiments suggest that an oligonucleotide containing an E2F consensus binding site does not efficiently compete with MMTV sequences for ISBP (data not shown).

Potential roles of ISBP in MMTV transcription initiation. Transcription initiation is a complex process that involves a large number of protein-DNA and protein-protein interactions. It is tempting to speculate that the position of ISBP binding near the transcription initiation site in the MMTV promoter relates to the melting of the two DNA strands at the transcription initiation site leading to the formation of an open transcription complex in which the template strand of the DNA is accessible to RNA polymerase. Alternatively, ISBP may stabilize other factors needed for transcription through protein-protein interactions. This type of function has been shown to be the case for the glial fibrillary acidic promoter in which a downstream element is required for stable binding of TFIID (43). In addition, CBF has been shown to stabilize TFIID binding to the adenovirus major late promoter and may be associated with TFIID prior to template binding (52). While there are relatively few documented cases of promoter elements 3′ to +1 having an effect on a known transcription factor, numerous proteins are known to bind 5′ to the TATA element and stabilize basal transcription factors within the transcription initiation complex (8, 9, 23, 55). Precise definition of the mechanistic role of ISBP in MMTV transcription initiation and the relationship of ISBP to proteins involved in transcription from other promoters will require further biochemical studies with the purified protein.

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