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RNA Polymerase II Transcription Complex Assembly in Nuclear Extracts

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In vitro transcription systems based on nuclear extracts of eukaryotic cells continue to be valuable experimental systems for assessing function of promoter sequences and defining new activities involved in transcription complex assembly and activity, but many aspects of such systems have not been experimentally examined. Here, transcription complex assembly on the promoter from the long terminal repeat of mouse mammary tumor virus was assessed in vitro with a transcription system derived from nuclear extracts of cultured HeLa cells. The extent of preinitiation complex assembly on the promoter was limited by the availability of template, even though only a small fraction of the template present in the assays participated in transcription. These results support a model for transcription complex assembly in which template DNA has two alternative fates, one leading to assembly of a functional transcription complex, and another that leads to irreversible template inactivation. The observed kinetics of assembly reflects loss of template by both pathways and is dominated by a relatively rapid rate of template inactivation. Supplementing nuclear extracts with purified TATA binding protein increased the extent as well as the apparent rate of assembly. Both effects can be explained by a TATA binding protein-dependent increase in the rate of assembly that leads to altered partitioning of template between competing pathways.

In vitro transcription Transcription complex TATA binding protein

PROMOTERS of eukaryotic genes transcribed by RNA polymerase II (pol II) are composed of discrete DNA elements to which various specific *trans*-acting proteins bind. For even the simplest pol II promoters, a complex array of proteins is required for specific transcription initiation [for review see (52)]. These proteins have been termed general transcription factors and include transcription factors (TF) IIA, IIB, IID, IIE, IIF, and IIH. TFIID, the only one of these factors that has sequence-specific DNA binding activity, is a multisubunit complex consisting of the TATA-box binding protein (TBP) and several TBP-associated factors (TAFs). TBP can substitute for TFIID in transcription from a minimal promoter, but TAFs are required for transcription to be affected by at least some of the proteins that bind to other DNA sequence elements of the promoter (10,29,46). Components of the transcription apparatus were first identified as factors required to reconstitute promoter-specific RNA synthesis in crude transcription systems in vitro, and their study has progressed through the biochemical purification of proteins to, in many cases, isolation of cDNA clones, expression of large quantities for biochemical study, and detailed determination of three-dimensional structure (8,34). Results from many labs have defined an ordered assembly of a functional pol II transcription complex from isolated components [for review see (32)]. More recently, pol II holoenzymes have been described that contain most or all of the general transcription factors necessary for initiation, suggesting that assembly of the transcription apparatus need not be nucleated around promoter DNA (25,26,35).

Understanding the biochemical mechanism(s) by which pol II transcription initiation is regulated will require detailed analysis of defined systems employ-

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ing highly purified components. Much recent progress has been made in identifying components of the transcription apparatus and in reconstituting certain aspects of regulation in purified systems (10,53). However, it is likely that many proteins involved in transcriptional regulation have not yet been identified, and the analysis of transcription in relatively crude in vitro systems will therefore remain an important tool in defining components of the transcription apparatus and characterizing novel regulatory mechanisms.

Many studies have exploited specific transcription by pol II in nuclear extracts derived from cultured cell lines, *Drosophila* embryos, or mammalian tissues to examine aspects of transcription complex assembly and transcriptional regulation of gene expression. Transcription in such systems is characterized by an inefficient use of template DNA (on the order of 1% of the added template molecules supporting productive transcription); furthermore, it is apparent that the rate-determining step in the overall synthesis of RNA is assembly of a complex that is capable of productively initiating transcription (14,18,19,22,31). In the cases in which it has been examined, assembly of functional transcription complexes proceeds with an apparent half-time ranging from about 5 to 30 min, and RNA synthesis can then initiate rapidly from the assembled complexes by addition of appropriate nucleoside triphosphates (15,18,19,31).

Activities of many transcriptional regulatory proteins can be at least partially reconstituted in crude in vitro transcription systems. In cases where it has been assessed, absence of the regulatory protein appears to have no effect on the rate of transcription complex assembly; only the number of functional complexes is altered (15,21,44,50). These results have been interpreted in a variety of ways. Most discussions have centered on differential stability of complexes dependent on the presence of a regulatory protein (21,44) or on an altered equilibrium of a reversible assembly process (15). However, both of these interpretations are inconsistent with the measured stability of assembled transcription complexes (19,22,31), which suggests that, over the normal time course of an in vitro transcription experiment, transcription complex assembly is essentially irreversible.

Here we present a kinetic analysis of transcription complex assembly on the mouse mammary tumor virus (MMTV) promoter with an in vitro transcription system based on nuclear extracts from HeLa cells. Many of our experimental results are consistent with previous experiments and exhibit many of the features discussed above. However, we offer an alternative model for describing transcription complex assembly in nuclear extracts based on multiple reaction pathways that compete for template DNA. This model can account for many observations made with nuclear extract transcription systems and provides a useful context for interpreting in vitro experiments performed with such systems.

MATERIALS AND METHODS

Plasmids

Nucleic acid restriction and modification enzymes were used according to the suppliers' (Promega or New England Biolabs) recommendations. Oligonucleotides were obtained from the Gene Technologies Laboratory, Department of Biology, Texas A&M University.

T-free cassettes were constructed by multimerization of a unit of complementary oligonucleotides of sequence 5′-GGGACAACCGACGACC-3′ and 5′-TCCCGGTCGTCGGTTG-3′. Oligonucleotides were phosphorylated with ATP and T4 polynucleotide kinase and allowed to anneal. Head-to-tail multimers were generated with T4 DNA ligase by way of the noncompatible sticky ends of the double-stranded oligonucleotide. Ends of the multimerized products were made blunt with the Klenow fragment of DNA polymerase I in the presence of dNTPs, and multimers of the desired length were purified by polyacrylamide gel electrophoresis and ligated into *Sma*I-digested pUC19 to create a series of pTfree plasmids with different length T-free cassettes. The structure of each cassette was verified by sequence analysis of double-stranded plasmid DNA (9).

MMTV promoter sequences were PCR amplified from previously described plasmids based on pLSwt (47), which contains the wild-type MMTV promoter. The 5′ primer used was pBRR1, 5′-GTATCACGAG GCCCT-3′, which hybridizes to pBR322-derived vector sequences upstream of the promoter and which lies between −419 and −405 in pLSwt (47). The 3′ primer utilized in most cases was Tfree-1, 5′-tggttcc cggGTTTGGCCTGTTGC-3′, which hybridizes between +1 and +14 on the MMTV promoter, leaving a 10-bp overhang (lower case letters) and creating a *Sma*I site. These amplified DNAs contained alterations at T residues at +8 and +11 necessary for construction of the T-free cassette. Mutation of the ISBP binding site was accomplished through use of a 3′ PCR primer of sequence 5′-TGGTTCCCGGGTTGG gtgGTTGC-3′, which hybridizes to the same region as the Tfree-1 oligonucleotide, but which incorporates several mismatches with the wild-type sequence (indicated by lower case letters) that have been shown to affect ISBP binding (38). The promoter sequences derived from PCR were cut with *Sst*I and *Sma*I and cloned into appropriate pTfree plasmids. Constructions were verified by DNA sequencing (9).

Plasmids constructed with this strategy contained MMTV promoter sequences from −105 to +14 inserted upstream of T-free cassettes. Plasmids pMBPT1 and pMBPT3 contain wild-type MMTV sequences (except for the altered Ts at +8 and +11) and generate U-free transcripts of 130 and 172 nt, respectively. Plasmids pLS(−74/−67)Tfree and pLS(−31/−24)Tfree contained clustered point mutations derived from corresponding chloramphenicol acetyltransferase expression plasmids (47) within the NF-1 binding site and TATA element of the MMTV promoter, respectively. These plasmids generate 151-nt U-free transcripts. Plasmid pLS(+6/+8)Tfree contained alterations in the ISBP site (38); correctly initiated U-free transcripts from this template are 130 nt long. pLS(−59/−38)Tfree contained mutations in MMTV octamer elements and has been described previously (23); U-free transcripts from this template are 151 nt long. pTFΔ38 was constructed by exploiting a *Bgl*II site present in the mutated region of the linker-scanner plasmid pLS(−46/−39)Tfree (23) and a unique *Eco*RI site present in the vector polylinker. A *Bgl*II/*Eco*RI digest of this plasmid cut out MMTV promoter sequences upstream of −38. The large fragment of this digest was gel isolated on 0.75% low melting point agarose and the ends made blunt with the Klenow fragment of DNA polymerase I. Ligation with T4 DNA ligase generated the deletion-containing plasmid. Transcription of pTFΔ38 yields a 151-nt U-free transcript. All plasmid sequences were verified by DNA sequencing of the final constructions (9).

Template DNAs were prepared from large-scale plasmid preparations (1L) by alkaline extraction (5), and supercoiled DNA was purified by CsCl gradient centrifugation, gel filtration on a Biogel A-5M column [equilibrated in 10 mM Tris-HCl (pH 7.9), and 1 mM EDTA, and 0.2 M NaCl], and ethanol precipitation.

Nuclear Extracts

HeLa cells were maintained at $4–8 \times 10^5$ cells/ml in JMEM supplemented with 5% horse serum (Gibco), 0.2 mM L-glutamine (Sigma), 100 U/ml penicillin G (sodium salt), 100 μg/ml streptomycin sulfate (Gibco), and buffered with 10 mM HEPES (pH 7.4) (Boehringer Mannheim). Nuclear extracts were prepared according to the method of Dignam (13), with modifications as described by Shapiro et al. (42). Protein concentration was determined by the method of Bradford (6) using bovine serum albumin as the standard.

In Vitro Transcription Assays

Nuclear extract (60 μg of protein) was incubated for 30 min on ice in a total volume of 25 μl in TM0.1 buffer [50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 12.5 mM $MgCl_2$, 20% glycerol, 100 mM KCl] containing 4 mM DTT (Sigma). We have empirically determined that this treatment with DTT allows full recovery of activity in extracts that have been stored for up to 1 year at −70°C. DNA template (1.0–2.0 μg, optimized for each extract preparation) and 40 U of RNasin (Promega) were added to reactions and diluted to a final volume of 43 μl with DEPC-treated (Sigma) distilled water. Reactions were incubated at 30°C for up to 3 h. RNA synthesis was initiated with 5 μl of a U-free NTP mix (6 mM ATP, 6 mM GTP, 1 mM 3′-dUTP, 50 μM CTP) (Boehringer Mannheim), 10 μCi [α-^{32}P]CTP (New England Nuclear) and 0.01–0.05% Sarkosyl (Sigma), and allowed to proceed for 30 min at 30°C. Reactions containing the adenovirus 2 major late (AdML) promoter template substituted 6 mM UTP and 1 mM 3′-*O*-methyl GTP for the GTP and 3′-dUTP, respectively. Reactions were terminated by addition of 350 μl of stop buffer [50 mM Tris-HCl (pH 7.5), 1% SDS, 5 mM EDTA, 25 μg/ml tRNA (Sigma)], 2 μl of 20 mg/ml proteinase K (Boehringer Mannheim), and 3000–7000 cpm of a ^{32}P-labeled recovery control RNA (described below). Protein digestion was carried out for 5 min at room temperature. Mixtures were then extracted with 400 μl of a 1:1 mixture of phenol/chloroform saturated with a solution of 100 mM Tris-HCl (pH 7.5), 10 mM sodium acetate, 0.1 M NaCl, 1 mM EDTA. RNA was precipitated with ethanol in the presence of 0.3 M sodium acetate at −20°C for at least 2 h, washed with 70% ethanol, dissolved in 53% deionized formamide (Mallinckrodt), and loaded on 6–10% denaturing polyacrylamide gels. Gels were dried and exposed 11–16 h to XAR-5 film (Eastman Kodak Co.) with an intensifying screen at −70°C. After exposure to film, selected gels were quantitated with a Betascope blot analyzer (Betagen) or by way of a Fujix BAS 2000 Phosphorimager (Fuji). Correction for background and normalization for RNA recovery between separate reactions using the added ^{32}P-labeled recovery control RNA were performed, and data from replicate experiments were normalized to allow direct comparison of derived values between experiments. All experiments were repeated at least three times using multiple plasmid and nuclear extract preparations. Because all reactions contained Sarkosyl, transcription was limited to a single round, and thus the yield of transcript was a direct measure of the number of promoters supporting an active preinitiation complex.

Synthesis of Recovery Control RNA

Recovery control RNA was synthesized using the SP6 promoter of pGEM1 (Promega) linearized at a unique *Pvu*II site at +98 relative to the start of tran-

scription by SP6 RNA polymerase. Approximately 1 μg of linearized DNA was added to a solution containing 0.5 mM ATP, GTP, UTP; 0.25 mM CTP, 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine (Boehringer Mannheim), 10 mM NaCl, 10 mM DTT, 1 U/μl RNasin, and 10 μCi [α-^{32}P]CTP. Transcription was initiated with 10 U SP6 RNA polymerase (BRL) and allowed to proceed for 30 min at 37°C. Reactions were terminated with 300 μl of stop buffer and processed as above. Precipitated RNA was resuspended in an appropriate volume of distilled water to yield 1000–5000 cpm/μl; 1–5 μl was typically added to each in vitro transcription reaction as a recovery control.

Expression and Purification of Human TATA Binding Protein

Coding sequences from the human TATA binding protein (hTBP) cDNA clone pGPP-21 (37) were subcloned into the T7 expression vector pET3C to create pET3C/hTFIID and introduced into *E. coli* strain BL21(DE3)pLYS S (43). Bacterial cultures (1 l) were grown in the presence of 50 μg/ml carbenicillin to an A_{600} of 0.5 and then induced with IPTG (1 mM) for 2 h at 28°C. Cells were pelleted at 6000 rpm for 20 min at 4°C in a Beckman model JA-10 rotor and resuspended in buffer A [25 mM Tris-HCl (pH 7.9), 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 0.1% NP-40]. The solution was sonicated with three 30-s bursts of a Branson model 450 sonicator, allowing a 30-s lag between bursts, and then subjected to two freeze–thaw cycles by freezing in liquid nitrogen and thawing at 37°C. Sonication as above was repeated until the solution was no longer viscous. Cell debris was pelleted at 11,000 rpm for 15 min at 4°C in a Beckman JA-20 rotor. Protein was precipitated from the supernatant by addition of ammonium sulfate to 33% saturation. Precipitated protein was pelleted as above and resuspended in TM0.1 buffer. The protein was then loaded on a DEAE-Sepharose column equilibrated in TM0.1 and run at 6 column volumes/h. The flow-through was collected and loaded directly on a heparin-agarose column run at 2 column volumes/h. Retained protein was eluted in two steps by raising the concentration of KCl to 0.3 and 0.5 M, respectively. The 0.5 M fraction contained the majority of rhTBP in a nearly homogeneous state. After dialysis against TM0.1 buffer, it was quick-frozen in liquid nitrogen and stored at −70°C.

RESULTS and DISCUSSION

In Vitro Transcription System

The core promoter of MMTV is located within the long terminal repeat (LTR) of the provirus and is composed of several elements that serve as binding sites for transcription factors (Fig. 1A), including a binding site for nuclear factor 1 (NF-1), two adjacent binding sites for the octamer binding protein Oct-1, a TATA box, and a binding site for a protein we have termed initiation site binding protein (ISBP) (20,23, 24,38,47). Other regulatory sequences have also been identified within the LTR and include a hormone response element (HRE) that confers inducibility mediated by several classes of steroid hormone receptors [for review see (16)], a mammary cell-specific enhancer (28,51), and several negative regulatory elements (7,36,40), including a distal negative regulatory element defined in our lab (27,33). In addition, many experiments have shown that MMTV transcription can also be affected by the specific chromatin conformation of proviral DNA (2,3,17,39,48,49).

We have developed a transcription system to assess MMTV promoter activity in vitro based on a T-free cassette transcription template. This type of template is directly analogous to the G-free cassette originally described by Sawadogo and Roeder (41). Although a G-free template has been used with the MMTV promoter (1), several G residues important for transcription are altered in such templates, including Gs at +1 and +7, the latter of which has been shown to be important for recognition by ISBP (38). The templates we employed contain no T residues on the nontemplate strand downstream of +1, and thus a transcript can be made in the absence of UTP. Background from cryptic promoters within vector sequences is reduced by a run of six naturally occurring Ts in the MMTV promoter between −16 and −11, and at −2 and −1. We have demonstrated that RNAs synthesized in this system are dependent on MMTV promoter sequences, as a T-free cassette lacking these sequences yielded no detectable transcript (data not shown). Furthermore, the transcription signal is completely inhibited by 2 μg/ml α-amanitin, indicating that the transcribed RNAs are the result of pol II activity (data not shown). Thus, a homogeneous population of RNAs of defined length can be synthesized during the transcription reaction and easily identified after electrophoresis on denaturing polyacrylamide gels.

To determine whether our in vitro system faithfully reproduced in vivo promoter activity, we assessed whether the same factors that act in vivo function in vitro. Several MMTV promoters with linker scanning mutations in defined promoter elements (23,47) were transferred into the context of the T-free cassette vectors (Fig. 1A), and the resulting templates were assayed for transcription activity in vitro. These templates included pLS(−74/−67)Tfree, containing mutations in the NF-1 site (Fig. 1B, lane 1),

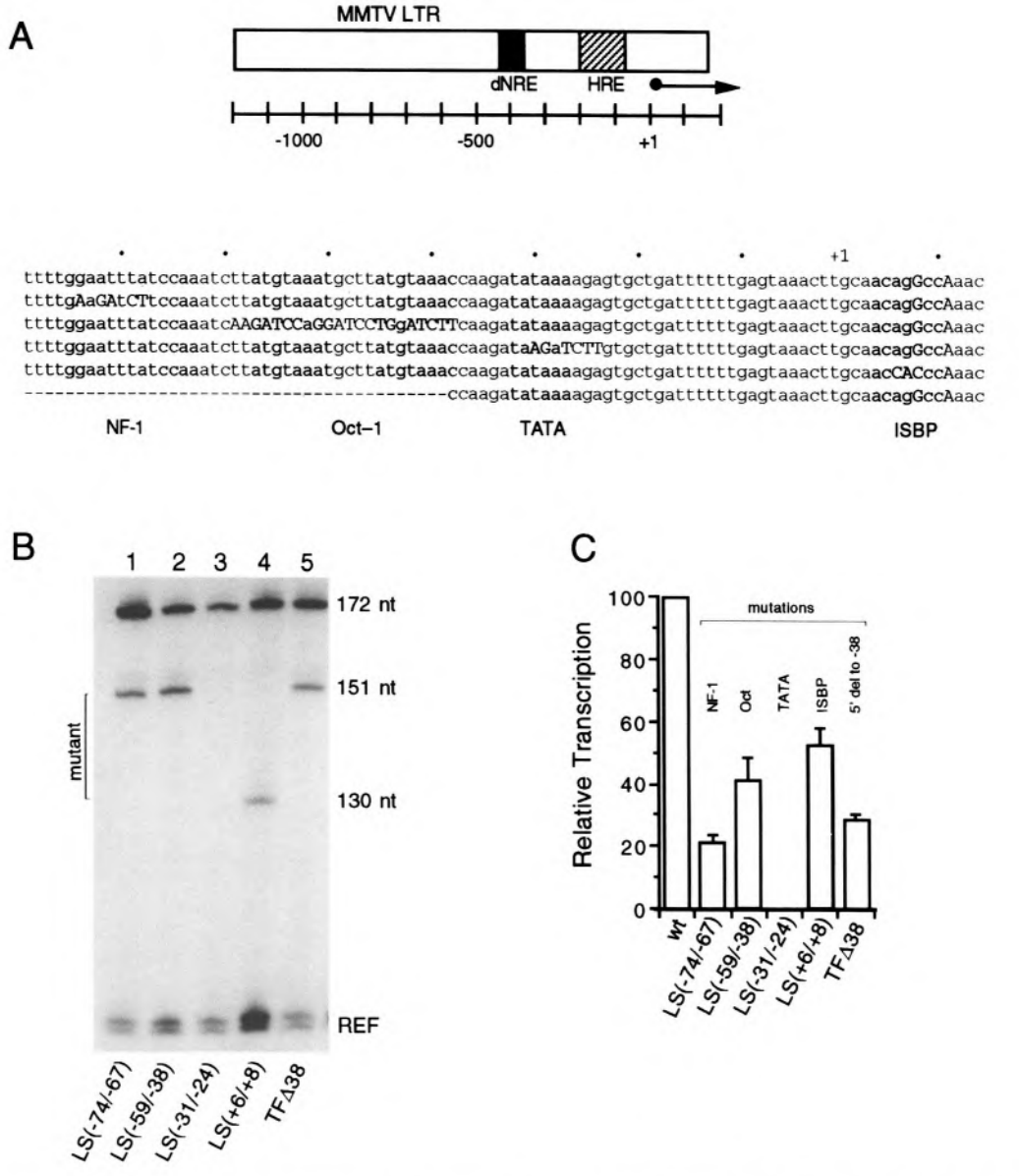

FIG. 1. Structure and activity of mutated MMTV promoters. (A) Structure of MMTV LTR and promoter. The MMTV LTR is depicted as a box with positions of the hormone response element (HRE) and distal negative regulatory element (dNRE) shown. Sequences from −79 to +14 are shown for wild-type and mutated MMTV promoters. Mutations are shown in capital letters. T residues at positions +8 and +11 are altered in all promoters to maintain the T-free cassette required in our in vitro assays; these changes do not affect ISBP function. The TATA box, along with the sequences recognized by NF-1, Oct-1, and ISBP, are shaded. Mutated promoters contain alterations in the NF-1 site [pLS(−74/−67)Tfree], Oct sequences [pLS(−59/−38)Tfree], TATA box [pLS(−31/−24)Tfree], or ISBP site [pLS(+6/+8)Tfree]. The TFΔ38 promoter contains a deletion of MMTV sequences upstream of −38. (B) In vitro transcription from mutated MMTV promoters. Each reaction contained two templates (10 μg/ml each). One template had a wild-type MMTV promoter (pMBPT3), which generated a U-free transcript of 172 nt, and the other template, which generated a U-free transcript of either 151 (lanes 1–3 and 5) or 130 nt (lane 4), contained the indicated mutations in the MMTV promoter. Transcription reactions were allowed to assemble for 60 min, NTPs (including [α-^{32}P]CTP) and Sarkosyl (0.025%) were added, and RNA synthesis was terminated after 30 min. Reaction products were subjected to denaturing polyacrylamide gel electrophoresis, and an autoradiograph of the dried gel is shown. Templates containing mutated promoters were included as follows: lane 1, pLS(−74/−67)Tfree (mutations in NF-1 site); lane 2, pLS(−59/−38)Tfree (mutations in Oct sites); lane 3, pLS(−31/−24)Tfree (mutations in TATA box); lane 4, pLS(+6/+8)Tfree (mutations in ISBP site); lane 5, pTFΔ38 (5′ deletion of promoter sequences to −38). A ^{32}P-labeled control RNA (REF) was added to all samples immediately after termination of RNA synthesis to assess recovery of sample during preparation for gel electrophoresis. (C) Quantitation of the effects of MMTV promoter mutations in vitro. Transcripts were quantitated as described in Materials and Methods. Transcription from each of the mutated promoters is shown relative to that from the wild-type MMTV promoter obtained in the same reaction, which was defined as 100. Error bars represent the SEM for at least three separate experiments.

pLS(−59/−38)Tfree, containing mutations in Oct-1 binding sites (Fig. 1B, lane 2), pLS(−31/−24)Tfree, containing mutations in the TATA box (Fig. 1B, lane 3), pLS(+6/+8)Tfree, containing mutations in the ISBP site (Fig. 1B, lane 4), and pTFΔ38, a mutation in which promoter sequences upstream of −38 (containing the NF-1 and Oct-1 sites) were deleted (Fig. 1B, lane 5). These templates supported transcription in vitro at levels 22%, 41%, 0%, 53%, and 29% of the wild-type MMTV promoter, respectively (Fig. 1C). The effects of all of these MMTV promoter mutations in vitro are consistent with their quantitative effects in vivo (47), suggesting that all of the DNA binding transcription factors required for optimal MMTV promoter activity are functional in our transcription system.

Analysis of the pLS(+6/+8)Tfree template (mutations in the ISBP site) is particularly important for our in vitro assay. In all of our T-free cassette templates, the naturally occurring T residue at +8 was changed to a G to establish the T-free cassette (Fig. 1A). This residue lies within the ISBP binding site as determined by DNase I footprinting (38). Although this single change was not previously assayed for its effect on transcription or ISBP binding, several clustered point mutations that contain this transversion have only weak effects on ISBP binding; in contrast, the mutations present in pLS(+6/+8)Tfree have been shown to dramatically decrease the ability of ISBP to recognize the MMTV promoter (J. Pierce and D. O. Peterson, unpublished observations). The decreased activity of pLS(+6/+8)Tfree confirms that transcription in vitro is dependent on ISBP, even in the context of the T-free cassette templates.

Kinetics of Transcription Preinitiation Complex Assembly

Initial experiments revealed that the time course of assembly of functional transcription complexes on the MMTV promoter in our in vitro system was relatively slow (Fig. 2). Template DNA (pMBPT1) containing MMTV promoter sequences extending to −105 was incubated with nuclear extract, and at various times NTPs were added to initiate RNA synthesis. At the same time, Sarkosyl (0.025%) was also added to block further assembly and limit transcription to a single round (18,19). We have confirmed that transcription complex assembly is inhibited by this concentration of Sarkosyl [(24) and data not shown] and that no reinitiation occurs in our assays under these conditions. The observed transcription signal is therefore due solely to a single round of initiation and is directly proportional to the number of promoters on which a functional preinitiation complex has assembled. RNA synthesis proceeded for 30 min, a time sufficient for maximum accumulation of RNA (data not shown). Reactions in which complexes were allowed to assemble for up to 3 h exhibited no significant increase in transcription over that measured at 90 min (data not shown). Transcription complex assembly under these conditions is characterized by a lag of about 5–10 min (Fig. 2B). Thus, our data cannot be fit by a simple first-order process (Fig. 2B, dotted line). A much better fit is provided

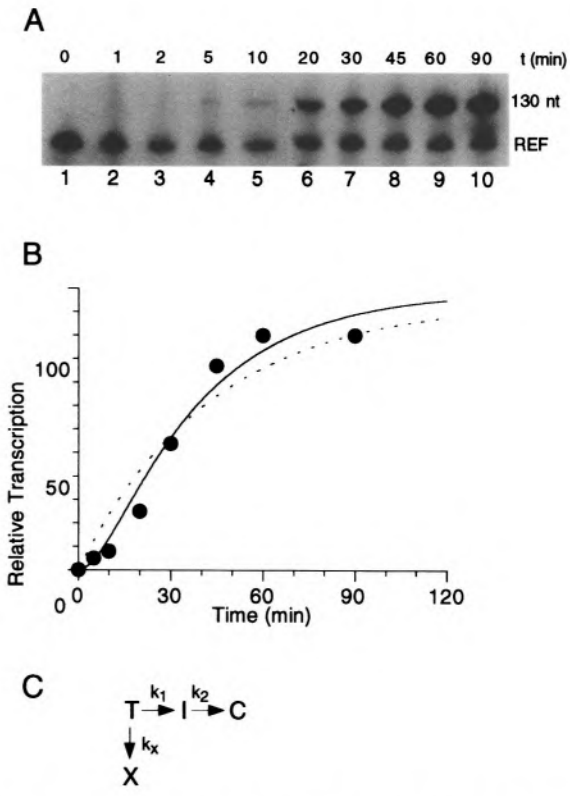

FIG. 2. Kinetics of transcription complex assembly on the MMTV promoter. (A) Transcription complex assembly. Template containing a wild-type MMTV promoter (pMBPT1) was incubated with nuclear extract for varying times. NTPs and Sarkosyl (0.025%) were then added, and RNA synthesis was allowed to proceed for 30 min. Reaction products were subjected to denaturing polyacrylamide gel electrophoresis, and an autoradiograph of the dried gel is shown. Bands corresponding to promoter-specific transcript (130 nt) and ^{32}P-labeled recovery control RNAs (REF) are indicated. Times allowed for assembly are indicated over the respective lanes. (B) Time course of transcription complex assembly. Specific transcripts were quantitated from experiments like that shown in (A). Each point represents the average of at least three independent experiments. The dotted line represents a first-order fit to the data. The solid line in (B) was generated from a kinetic simulation with the computer program HopKinSim (4) using the two-step assembly model shown in (C), in which template (T) has two alternative fates, one that leads to a productive transcription complex [C] through intermediate I, and a second that leads to inactivation with first-order rate constant k_x. For the simulation shown, the following parameters were used: T = 20,000; $k_1 = 1.8 \times 10^{-4}$; $k_2 = 0.12$; $k_x = 0.03$. With these parameters approximately 0.5% of the template forms a functional complex.

by a two-step assembly model (Fig. 2C) that can generate the observed lag (Fig. 2B, solid line). Even better empirical fits can be made with assembly mechanisms that incorporate additional steps (data not shown); however, the important point is that it appears that transcription complex assembly under these conditions is characterized by more than one kinetically important process.

The time required for assembly of a preinitiation complex on the MMTV promoter in our experiments is significantly longer than the times reported for other promoters in some studies. For example, transcription complexes on the adenovirus 2 major late (AdML) promoter in HeLa nuclear extracts (19) or the Krüppel gene promoter in *Drosophila* embryo extracts (22) assembled with apparent first-order kinetics with half-times of 9 and 3 min, respectively. To determine if the relatively long assembly time was specific to the MMTV promoter, we determined the time course of transcription complex assembly with a template containing the AdML promoter in the context of a G-free cassette. In our transcription system, the observed rate of transcription complex assembly on the AdML promoter was comparable to the rate observed with the MMTV promoter (data not shown). The activities of the two promoters are quite different, however, and the Ad2ML promoter consistently supported levels of transcription approximately fivefold higher than MMTV. The time required for complete assembly of a transcription complex in our in vitro reactions appears to be characteristic of our transcription system and thus is not a unique feature of the MMTV promoter.

As reported by others (19,22,30), only a small fraction of the template DNA present in our assays supported assembly of a functional transcription complex. We estimate that under the conditions of the experiment described in Fig. 2, approximately 0.1–0.5% of the template supports active transcription. We considered several possible explanations for this observation. One obvious possibility is that transcription complexes assemble until some required component is depleted. Similar to several previous reports (14,19), several experiments strongly implicate template DNA as this depleted component. This is most clearly demonstrated by the experiment described in Fig. 3A, in which we allowed transcription complexes to assemble for 60 min, a time that is sufficient for assembly to proceed essentially to completion (Fig. 2). Additional template was then added, and a second round of assembly occurred with kinetics similar to the first (see below), indicating that no necessary transcription factors had been depleted from the reaction during the first round of assembly. The total number of transcription complexes formed in the two rounds of assembly (Fig. 3A, closed circles) was essentially identical to that obtained in a single round from the same total amount of template (open square). A second round of assembly did not occur if the DNA added at 60 min did not contain a promoter, and, through the use of templates containing promoters linked to different length T-free cassettes, we showed that the second round of assembly occurs exclusively on the template added at the later time (data not shown). In combination, these observations suggest a model in which the extent of transcription complex assembly is limited by a competing reaction in which template DNA is irreversibly inactivated (19). In such a scenario, the apparent rate of transcription complex assembly reflects the rates of template utilization in both the assembly and inactivation pathways, whereas the extent of transcription complex assembly on a particular promoter (measured as an RNA transcript in our assays) is indicative of partitioning of the template between the two pathways (see model in Fig. 2C). Furthermore, if the rate of inactivation is rapid relative to the rate of transcription complex assembly, then the apparent rate of assembly measured in our assays largely reflects the disappearance of template by inactivation, and assembly terminates before required components of the transcription apparatus become depleted. In Figs. 2B and 3A, the solid curves are based on a kinetic simulation with the computer program HopKinSim (4) using the kinetic model in Fig. 2C. In this simulation the ratio of rate constants k_1 and k_2, which determines the extent of assembly, were chosen so that approximately 0.5% of the template present eventually forms a functional transcription complex.

The inactivation of template could result from several possible reactions. Nucleases present in the extract could degrade template; however, we have not observed extensive degradation of template during incubation with nuclear extract (data not shown). Alternatively, template could interact with nonspecific DNA binding proteins, such as histones, that could block transcription complex assembly. Nuclear extracts contain histones, and histone H1 has been shown to inhibit transcription complex assembly in vitro [(11) and data not shown]. We have no evidence that a significant fraction of template DNA in our assays is assembled into nucleosomes.

If a competing pathway by which template is inactivated is operational, inclusion of nontemplate DNA in our assays might be expected to limit inactivation and allow more template DNA to be partitioned into the transcription complex assembly pathway. Such effects have been reported by others (12), and were confirmed by the experiment shown in Fig. 3B. In the absence of added pUC19 DNA, the maximum

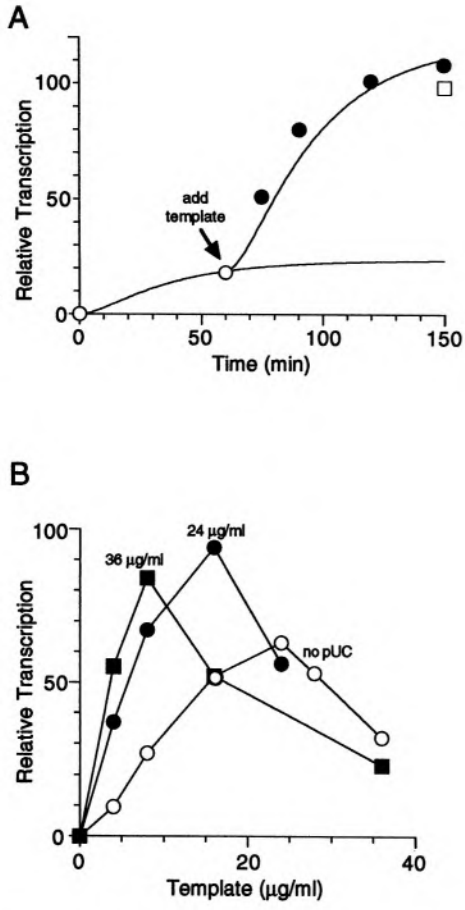

FIG. 3. Effect of DNA on transcription complex assembly. (A) Template added after one round of assembly is complete. Template (4 µg/ml) containing a wild-type MMTV promoter (pMBPT3) was incubated with nuclear extract for 60 min. The template concentration was then increased to a final concentration of 20 µg/ml and the incubation continued for an additional 90 min. NTPs and Sarkosyl (0.025%) were added at various times, and RNA synthesis was allowed to proceed for 30 min. Reaction products were subjected to denaturing polyacrylamide gel electrophoresis and quantitated. Levels of transcription complex assembly prior to (○) or after (●) increasing the template concentration are indicated. The level of transcription complex assembly observed if the template concentration is 20 µg/ml from time zero is shown by the open square. The solid curves are kinetic simulations with the computer program HopKinSim (4) as described in the legend to Fig. 2, with the exception that for the lower curve T = 4000, and for the upper curve T = 16,000 beginning at 60 min. (B) Nonspecific DNA and template dependence of in vitro transcription. Transcription complexes were assembled for 60 min on a template containing the wild-type MMTV promoter (pMBPT3). NTPs were added, and RNA synthesis was allowed to proceed for 30 min. Reaction products were subjected to denaturing polyacrylamide gel electrophoresis and specific transcripts quantitated. Template concentration was varied in the absence of any added pUC19 DNA (○) or in the presence of pUC19 such that the total DNA concentration in each reaction was 24 µg/ml (●) or 36 µg/ml (■).

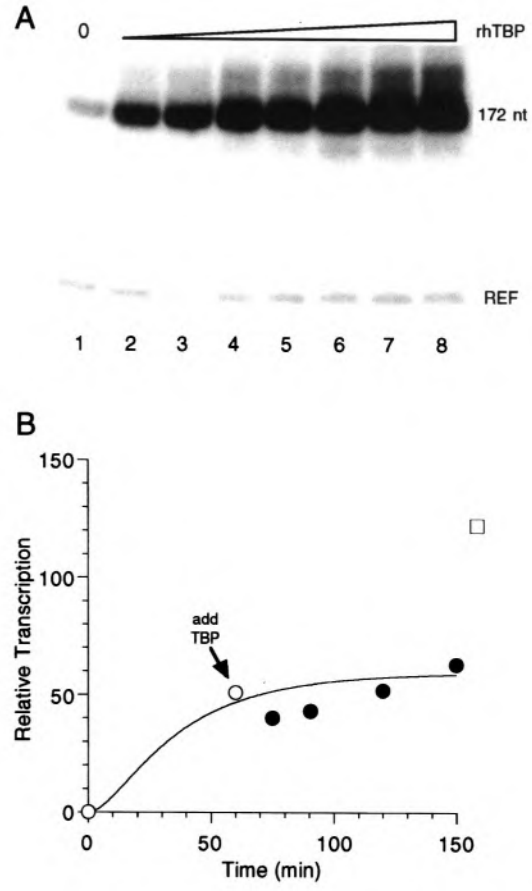

FIG. 4. Effect of rhTBP on transcription complex assembly on the MMTV promoter. (A) Effect of rhTBP on the extent of assembly. In vitro transcription reactions containing a template with the wild-type MMTV promoter (pMBPT3) were supplemented with varying concentrations of rhTBP. Transcription complexes were assembled for 60 min. NTPs and Sarkosyl (0.025%) were added, and RNA synthesis was allowed to proceed for 30 min. Reaction products were subjected to denaturing polyacrylamide gel electrophoresis, and an autoradiograph of the dried gel is shown. The 172-nt band corresponds to the MMTV promoter-specific transcript, and REF shows the position of a recovery control RNA added after transcription reactions were terminated. Reactions contained the following concentrations of rhTBP: lane 1, no rhTBP; lane 2, 100 ng/ml; lane 3, 200 ng/ml; lane 4, 300 ng/ml; lane 5, 400 ng/ml; lane 6, 600 ng/ml; lane 7, 800 ng/ml; lane 8, 1000 ng/ml. (B) Effect of rhTBP added after assembly is complete. Template (10 µg/ml) containing a wild-type MMTV promoter (pMBPT3) was incubated with nuclear extract for 60 min in the absence of added rhTBP. rhTBP was then added to a final concentration of 30 ng/ml and the incubation continued for an additional 90 min. NTPs and Sarkosyl (0.025%) were added at various times, and RNA synthesis was allowed to proceed for 30 min. Reaction products were subjected to denaturing polyacrylamide gel electrophoresis and quantitated. Levels of transcription complex assembly prior to (○) or after (●) addition of rhTBP are indicated. The level of transcription complex assembly observed if 30 ng/ml rhTBP is present from time zero is shown by the open square. The solid curve is a kinetic simulation with the computer program HopKinSim (4) as described in the legend to Fig. 2, with the exception that T = 10,000,

number of transcription complexes that could be assembled in our assays peaked at a template concentration of about 24 mg/ml. In the presence of pUC19, the maximum number of functional transcription complexes that assembled was relatively unchanged, but the concentration of template at which this maximum was obtained was significantly decreased. At the lowest template concentrations tested, pUC19 DNA stimulated transcription complex assembly by as much as sixfold. To the extent that pUC19 can be considered as a completely nonspecific DNA, the observation that it affects template partitioning suggests that template inactivation does not result from inappropriate or partial assembly of transcription complexes. If inactivation occurred only on partially assembled complexes, nonspecific DNA would not alter the fraction of template on which transcription complexes assemble. However, pUC19 contains several TATA-like sequences that are potential binding sites for TBP, and thus it may not be completely nonspecific.

Effect of Recombinant TBP on MMTV Promoter Activity

If, as suggested above, the apparent rate of transcription complex assembly is determined by the rate of a faster, competing pathway of template inactivation, then the kinetics of assembly as measured in our assays is not reflective of the actual assembly process. However, we reasoned that if the relatively slow rate of assembly could be increased to become significant with respect to the rate of template inactivation, then the apparent rate of assembly as determined in our assays would become sensitive to differences in this actual rate.

To begin to test this idea, we supplemented our transcription complex assembly reactions with purified recombinant human TBP (rhTBP) expressed in bacteria. Varying concentrations of rhTBP were added, and complexes were allowed to assemble for 60 min at 30°C. In the presence of rhTBP, transcription was increased by as much as 8–10-fold (Fig. 4A). Assay conditions limited transcription to a single round by appropriate addition of Sarkosyl (0.025%), and thus the increase in transcription signal was directly proportional to an increased number of promoters that supported assembly of a functional transcription complex.

The stimulation of transcription by rhTBP allowed us to perform an additional experiment to confirm the idea that availability of template limits the extent of transcription complex assembly (Fig. 4B). Transcription complexes were allowed to assemble for 60 min in the absence of added rhTBP; rhTBP was then added to a final concentration of 30 ng/ml, and assembly as followed for an additional 90 min (closed circles). rhTBP had no effect on assembly under these conditions, even though the same amount of rhTBP increased transcription by approximately threefold when it was present from time zero (open square). This result is consistent with the idea that template becomes limiting after 60 min of assembly, and addition of transcription factors therefore cannot promote further template utilization.

To determine whether addition of rhTBP altered the rate of transcription complex assembly, the time course of assembly in the presence of rhTBP was followed (Fig. 5). At a saturating concentration of rhTBP (600 ng/ml), the apparent rate of complex formation was dramatically increased; assembly proceeded with apparent first-order kinetics with a half-time of approximately 5 min. This is most easily seen in Fig. 5B (triangles), where the data have been normalized to allow rate comparisons to be made more easily. Consistent with the data in Fig. 4A, transcription complex assembly in the supplemented reactions also resulted in approximately an eightfold increase in the number of transcription complexes being assembled (Fig. 5A). These results suggest that in our unsupplemented nuclear extract, the binding of TFIID to the template is a rate-determining step in transcription complex assembly. Increasing the concentration of TBP by addition of recombinant protein presumably increases the rate of transcription complex assembly to a point where it becomes significant with respect to the rate of template inactivation. The result is a dramatic increase in the final level of transcription complex assembly due to increased partitioning down the assembly pathway, as well as an increase in the apparent rate of transcription complex assembly, which is now determined, at least in part, by the actual rate of assembly. The changes in both extent and apparent kinetics of assembly can be explained by the change in the rate of assembly.

It is important to note that at saturating concentrations on rhTBP, the extent of transcription complex assembly is not determined by template inactivation. Under these conditions, which support a level of transcription about eightfold higher than that obtained with unsupplemented extracts, and unlike the results obtained with unsupplemented extracts (Fig. 3A), addition of a second template after assembly has reached a plateau does not lead to formation of additional complexes (data not shown). Thus, the number of complexes assembled under these conditions reflects depletion of some required transcription factor from the nuclear extract. This component is clearly not TBP, as rhTBP was added in excess, nor is it TFIIB, because addition of recombinant human

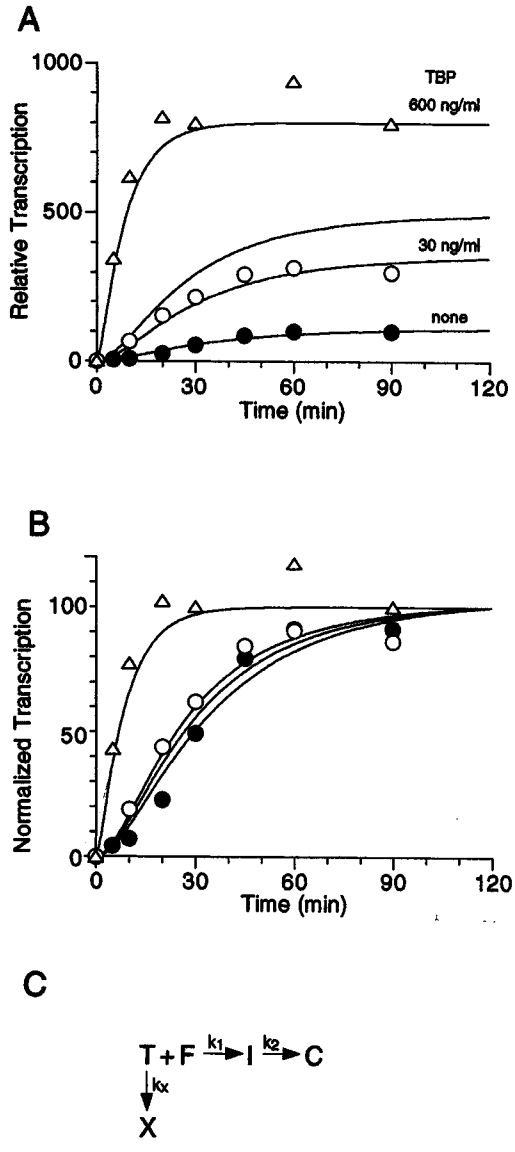

FIG. 5. MMTV transcription complex assembly in the presence of rhTBP. Template containing a wild-type MMTV promoter (pMBPT3) was incubated for varying times with nuclear extract with no supplement (●) or supplemented with rhTBP at a concentration of 30 ng/ml (○) or 600 ng/ml (△). NTPs and Sarkosyl (0.025%) were then added, and RNA synthesis was allowed to proceed for 30 min. Reaction products were subjected to denaturing polyacrylamide gel electrophoresis, and specific transcripts were quantitated. Points represent the average of at least three different experiments. (A) Effect of rhTBP on the extent of assembly. Specific transcription is shown relative to the level obtained with unsupplemented extract as shown in Fig. 2. Curves represent kinetic simulations with the computer program HopKinSim (4) using the kinetic model shown in part C. For all four of the curves shown, parameters for the simulation were: T = 20,000; F = 800; $k_2 = 0.12$; $k_x = 0.03$. The four curves shown were generated with values of k_1 of 5.0×10^{-5}, 1.5×10^{-6}, 9.0×10^{-7}, or 2.3×10^{-7} (top to bottom). (B) Effect of rhTBP on the kinetics of assembly. Specific transcription is shown normalized to the same final extent of assembly (defined as 100). Curves represent kinetic simulations identical to those in (A) but normalized to the same extent of reaction. (C) Kinetic model for transcription complex assembly. This model differs from that in Fig. 2C by the inclusion of a factor (F) that limits the extent of transcription complex assembly.

TFIIB did not result in formation of additional transcription complexes under these conditions (data not shown). The presence of this limiting factor is explicitly incorporated into our model in Fig. 5C, where factor F is present in sufficient quantity to support only about eightfold more transcription complexes than normally are made in unsupplemented reactions. Although factor F is shown to participate in the first step of the reaction where partitioning between productive and nonproductive pathways compete, an equivalent model can be made in which the limiting factor participates at a step subsequent to partitioning.

We also monitored the kinetics of transcription complex assembly in the presence of a nonsaturating concentration of rhTBP (30 ng/ml) that resulted in only about a threefold increase in the extent of assembly (Fig. 5, open circles). Under these conditions, assembly approximates apparent first-order kinetics with a half-time of approximately 20 min. This rate is reproducibly faster than that obtained in the unsupplemented reactions (compare open and closed circles in Fig. 5B). As with the reactions at saturating rhTBP, the effect on both the rate and extent of transcription complex formation can be explained by a change in the kinetics of assembly represented by the rate constant k_1 in Fig. 5C. The change in the extent of assembly (Fig. 5A) due to the presence of 30 ng/ml rhTBP is most closely approximated by an increase in k_1 of about fourfold, whereas the change in the rate of assembly (Fig. 5B) is more closely represented by an increase of about 6.5-fold.

Effect of TBP on Activity of Mutated Promoters

We have shown that supplementing in vitro transcription reactions with rhTBP can dramatically stimulate both the rate and extent of transcription complex assembly (Fig. 5). We have also assessed the effect of rhTBP on the relative activity of wild-type and mutated promoters. An example of these results with a template that contains mutations in the MMTV NF-1 binding site (pLS(–74/–67)Tfree) is shown in Fig. 6, but the results were essentially identical for all of the mutated promoters shown in Fig. 1A. As the concentration of rhTBP increased, the overall transcription signal was dramatically enhanced, but the effect of the mutation was lost; that is, the ratio of transcription complexes assembled on wild-type and mutated promoters approached 1. A simple interpretation of these results is that at relatively high concentrations of rhTBP, a transcription complex forms that lacks a component needed for NF-1-mediated stimulation but retains the ability to support a basal level of transcription. One possibility is that TAFs necessary for activator activity become limiting at

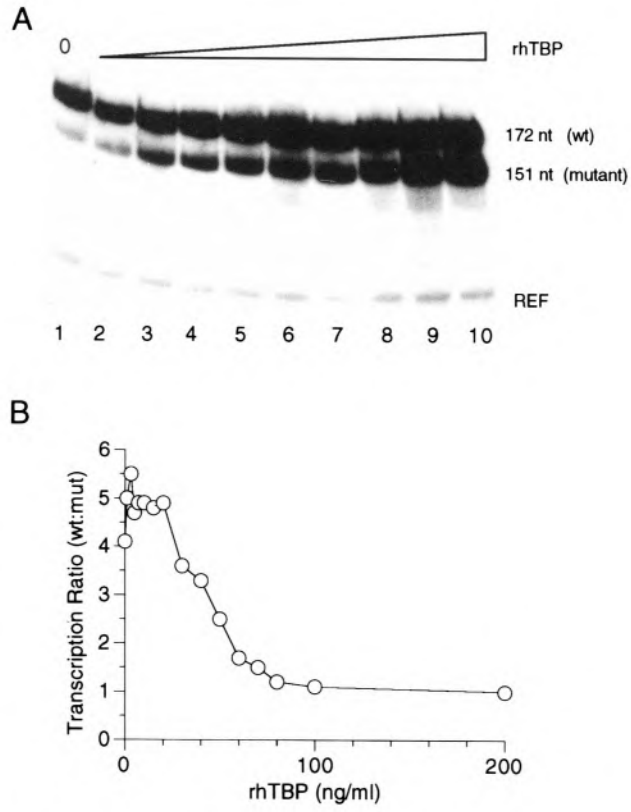

FIG. 6. Effect of rhTBP on transcription from an MMTV promoter with mutations in the NF-1 site. (A) Autoradiograph of reaction products. In vitro transcription reactions (50 μl) containing two MMTV promoter templates, one with the wild-type promoter (pMBPT3) and one with mutations in the NF-1 site [pLS(−74/−67)Tfree], were supplemented with varying amounts of rhTBP. Transcription complexes were assembled for 60 min. NTPs and Sarkosyl (0.025%) were added, and RNA synthesis was allowed to proceed for 30 min. Reaction products were subjected to denaturing polyacrylamide gel electrophoresis, and an autoradiograph of the dried gel is shown. The 172- and 151-nt products correspond to transcripts from the wild-type and mutated promoters, respectively. The position of a recovery control RNA added after transcription reactions were terminated is also shown (REF). Reactions contained the following concentrations of rhTBP: lane 1, no rhTBP; lane 2, 10 ng/ml; lane 3, 20 ng/ml; lane 4, 30 ng/ml; lane 5, 40 ng/ml; lane 6, 50 ng/ml; lane 7, 60 ng/ml; lane 8, 70 ng/ml; lane 9, 100 ng/ml; lane 10, 200 ng/ml. (B) Ratio of products from wild-type and mutated promoters. Specific transcripts from experiments like that in (A) were quantitated (including some reactions with lower concentrations of rhTBP), and the ratio of transcription from the wild-type promoter to that from the mutated promoter is shown as a function of the concentration of rhTBP.

high concentrations of rhTBP. It has been shown that TAFs are required for transcriptional enhancement from the proline-rich activation domain of NF-1 in the context of a fusion with the yeast GAL4 DNA binding domain (45). As TAFs become limiting, further increases in rhTBP lead to assembly of complexes without TAFs that are refractory to activator-mediated effects. Alternatively, other coactivators or NF-1 itself may become limiting at high concentrations of rhTBP.

Transcription Complex Assembly of Mutated Promoters in Nuclear Extract Supplemented With TBP

One significant aspect of the results in Fig. 6B is that the ratio of transcription obtained from wild-type and mutated templates was not significantly affected at very low concentrations of rhTBP. For example, addition of 30 ng/ml rhTBP had no effect on the ratio of transcription from wild-type and mutated templates (approximately 5:1), yet increased transcription from both templates by about threefold.

Because the effect of promoter mutations on the extent of transcription complex assembly was maintained at low concentrations of rhTBP, we decided to test our model for transcription complex assembly by comparing the time course of assembly for the wild-type, pLS(+6/+8)Tfree, and pTFΔ38 templates in the presence of 30 ng/ml rhTBP (Fig. 7). Consistent with the results in Fig. 6B, this low concentration had no effect on the relative levels of transcript obtained from wild-type and mutated promoters; both were increased by about threefold. Thus, any components required for NF-1, Oct-1, or ISBP to affect transcription in the presence of the increased concentration of

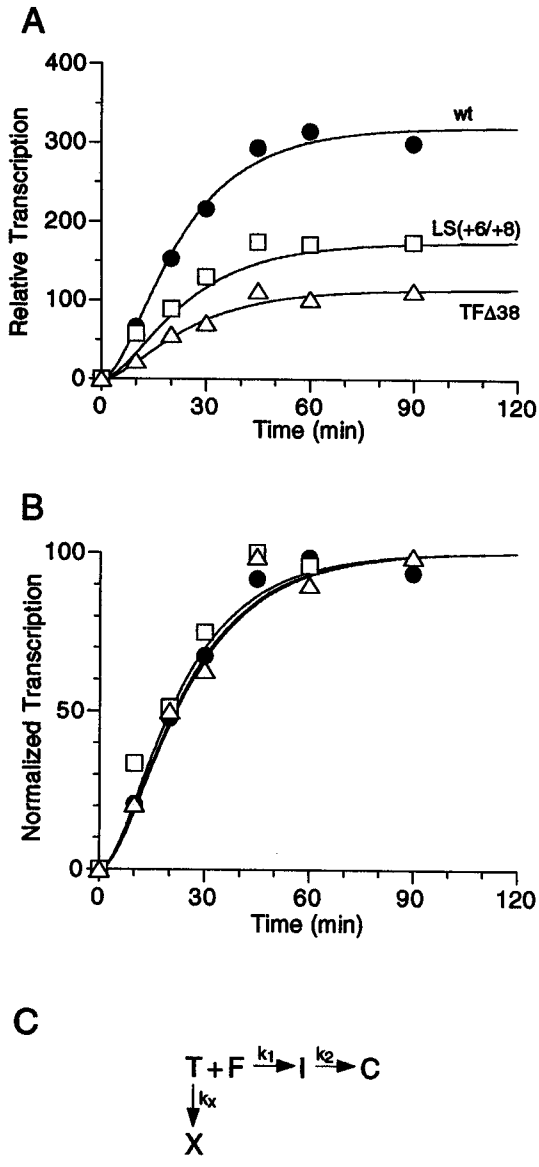

FIG. 7. Kinetics of transcription complex assembly on wild-type and mutated MMTV promoters in the presence of 30 ng/ml TBP. Template DNA was incubated with nuclear extract supplemented with 30 ng/ml of rhTBP for varying times. NTPs and Sarkosyl (0.025%) were then added, and RNA synthesis was allowed to proceed for 30 min. Reaction products were subjected to denaturing polyacrylamide gel electrophoresis, and bands corresponding to promoter-specific transcripts were quantitated. Points represent the average of at least three independent experiments. (A) Relative extent of transcription complex assembly. Assembly kinetics for the MMTV wild-type promoter (wt), as well as promoters with mutations in the ISBP site [LS(+6/+8)] or deletion of upstream sequences to −38 (TFΔ38) are shown with the extent of assembly normalized to that obtained with the wild-type promoter. Curves represent kinetic simulations with the computer program HopKinSim (4) using the kinetic model shown in (C). For all three of the curves shown, parameters for the simulation were: T = 20,000; F = 800; $k_2 = 0.12$; $k_x = 0.05$. The three curves shown were generated with values of k_1 of 1.3×10^{-6}, 6.2×10^{-7}, or 3.9×10^{-7} (top to bottom). (B) Normalized kinetics of assembly. Data and kinetic simulations from (A) were normalized to allow visual comparison of rates. (C) Kinetic model for transcription complex assembly identical to that in Fig. 5C.

TBP must be present in excess in the nuclear extract. However, similar to our results using unsupplemented extract (data not shown), changes in the rate of transcription complex assembly sufficient to account for the final levels of functional complexes (Fig. 7A) were not detectable when the apparent rates of assembly were directly compared (Fig. 7B). Thus, even though the rhTBP supplement increases the rate of assembly by a factor of about 3, the apparent rate of assembly remains dominated by the much faster inactivation pathway.

Kinetics of Transcription Complex Assembly In Vitro

Our results indicate that the apparent rate of transcription complex assembly in nuclear extract transcription systems is dependent on several factors and can, within limits, be manipulated by supplementation of nuclear extracts with rhTBP. In our hands, the apparent rate of transcription complex assembly in HeLa nuclear extracts is dominated by a relatively fast rate of template inactivation. Thus, potential template-specific differences in assembly kinetics are not likely to be observed under normal experimental conditions.

This model is consistent with a number of studies in which transcriptional regulators have been reported to affect the extent, but not the rate, of transcription complex assembly. Such studies have included transcriptional activators such as GAL4-VP16 (50), Sp1 (15), and UBX (21), as well as the negative regulator EVE (21). Some mechanistic interpretations of these results have emphasized potential effects on stability of assembled complexes (15,21). However, several studies have shown that assembled transcription complexes are quite stable to challenge by excess promoter-containing DNA (19,22) or to inhibitors of assembly such as Sarkosyl (19,22,31). We have confirmed this stability with the MMTV transcription complexes employed in our studies; assembled complexes are completely stable for at least 2 h in the presence of 0.025% Sarkosyl (data not shown). In the absence of data demonstrating that stability differences can quantitatively account for the observed differences in the extent of assembly, our kinetic partitioning model, which can roughly account for changes in both the rate and extent of assembly under a variety of assembly conditions, seems more plausible.

Until all aspects of regulated eukaryotic transcription can be reconstituted with purified components, relatively crude in vitro systems will continue to be useful. Despite the limitations of crude systems, a more complete understanding of the way such sys-

tems work can only enhance mechanistic conclusions derived from studies in which they are employed.

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