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Association of nucleosome-free regions and basal transcription factors with *in vivo*-assembled chromatin templates active *in vitro*

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ABSTRACT

Using SV40 minichromosomes assembled *in vivo*, we have studied the relationship between a nucleosome-free promoter-region and initiation of transcription by RNA polymerase II on chromatin templates *in vitro*. Our data suggest that accessibility of DNA to transcription factors, programmed into the structure of the chromatin, is crucial for initiation of transcription. First, minichromosomes competent to be transcribed *in vitro* contained nucleosome-free promoter regions. Second, tsC219 minichromosomes, most of which contain the nucleosome-free promoter region, supported transcription more efficiently both *in vivo* and *in vitro* than wild-type minichromosomes, in which only a subset contain the nucleosome-free region. We have also identified basal transcription factors associated with the *in vivo*-assembled chromatin templates. A striking correlation was observed between minichromosomes associated with *in vivo* initiated RNA polymerases and those associated with the basal transcription factors TFIID and TFIIE/F, and to a lesser extent, TFIIB. Of these associated factors, only TFIID was poised for ready assembly into preinitiation complexes and therefore for subsequent initiation of transcription. However, an active chromatin template could also be maintained in the absence of the binding of TFIID. Finally, our data are consistent with the presence of TFIIF in elongating ternary complexes on the chromatin templates.

INTRODUCTION

Gene expression within eukaryotic cells is regulated in the context of chromatin, a complex of genomic DNA, histones and non-histone proteins. One role of chromatin in the regulation of the initiation of transcription appears to be the control of DNA sequence accessibility for binding of transcription factors. Various lines of evidence suggest that nucleosomes and transcription factors may compete for binding to promoter sequences (1). Induction of transcription *in vivo* from promoters covered by precisely positioned nucleosomes (2–4) can lead to disruption of particular nucleosomes and apparent unmasking of binding sites for DNA-binding transcription factors (3,5–9). In addition, assembly of nucleosomes *in vitro* inhibits transcription from both RNA polymerase II and RNA polymerase III promoters (10–15), whereas formation of stable preinitiation complexes at the promoters prior to nucleosome assembly generates transcriptionally active templates (12,14–16).

To circumvent inhibition of transcription by chromatin assembly *in vivo*, transcriptionally active chromatin could form in several ways. Competition at promoters between the binding of transcription factors and the assembly of nucleosomes could occur during DNA replication, when regions of DNA near the replication fork are transiently free of nucleosomes (17). Alternatively, the binding of the specific DNA-binding protein itself might exclude nucleosome assembly on the surrounding DNA, thus providing promoter accessibility upon induction of gene expression at a later time (18–20). Finally, for genes transcriptionally activated in a replication-independent manner, inducible transcription factors able to bind to their recognition sites in the promoters in the presence of nucleosomes might trigger the disruption of nucleosomes, allowing the binding of additional transcription machinery and activation of the promoter.

Ultimately, the binding of basal transcription factors to the promoter is essential for initiation of transcription. The roles of the basal transcription factors have been characterized *in vitro* on naked DNA templates (21). Definition of their roles on chromatin templates will also be crucial for understanding induction of transcription from the chromatin templates *in vivo*.

To examine the roles of chromatin structural alterations and of basal transcription factors in transcription by RNA polymerase II on chromatin templates, we have studied initiation of transcription *in vitro* on *in vivo*-assembled chromatin. An abundant source of *in vivo*-assembled chromatin is SV40 minichromosomes. The SV40 genome is complexed with cellular

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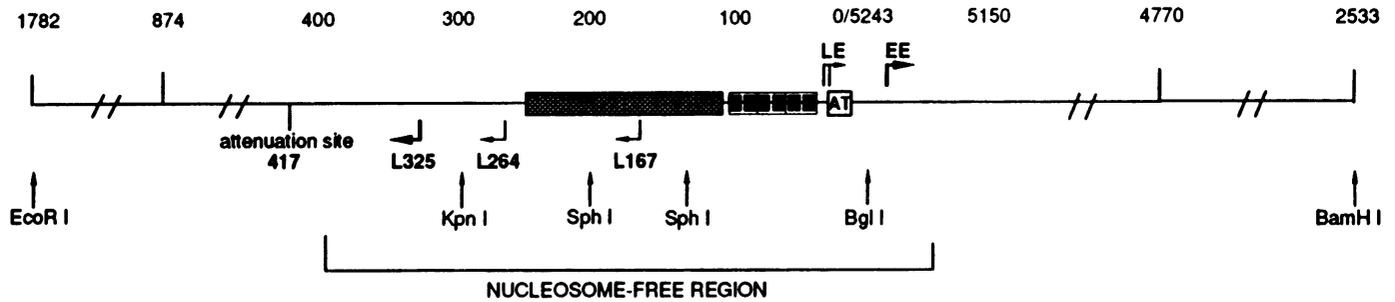

Figure 1. Scheme of the SV40 promoter region. The promoter elements of the sequence include: the enhancer, consisting in part of two 72 base pair repeats (hatched boxes); three 21 base pair repeats (solid boxes); and a 17 base pair stretch of adenines and thymidines including the early promoter TATA sequence (AT box). The promoter start sites include: the early-early (EE) and late-early (LE) initiation sites, and three late promoter initiation sites (L325, L264, and L167). Brackets mark the limits of the nucleosome-free region of the SV40 minichromosomes. Nucleotide positions 874 and 4770 define the 3′ termini of the protected late and early RNA products, respectively, following selection by hybridization with M13/SV40 recombinant DNA and digestion with T1 RNase. The late attenuation site is indicated at position 417. The cleavage sites of the following restriction enzymes are indicated: Bgl I at position 5235; Sph I at positions 128 and 200; Kpn I at position 294; EcoR I at position 1782; and BamH I at position 2533.

histones, cellular non-histone proteins, and viral proteins at all stages of the virus life cycle (22). As isolated, these chromatin complexes, called minichromosomes (MC), consist of a mixed population of complexes representing different stages of the virus life cycle. Only a portion of isolated MC contain a nucleosome-free region, mapping to the promoter/origin region of the virus (see Fig. 1; 23–29). When MC are separated by size by sucrose gradient centrifugation, the MC containing the nucleosome-free region are found in equal concentrations throughout the 90S profile of MC (29–31). In contrast, only those MC sedimenting most rapidly within the peak of 90S SV40 MC are associated with *in vivo* initiated RNA polymerases (31–33). These MC being transcribed *in vivo* comprise at most 1% of the total population at any given time (33,34); all contain the nucleosome-free region (35).

Using isolated SV40 MC, we demonstrated previously that MC sedimenting throughout the 90S sucrose gradient peak, rather than just those sedimenting most rapidly, supported efficient transcription *in vitro*. In addition, the majority of transcription from all fractions of MC resulted from initiation *de novo* (30), rather than elongation of *in vivo*-initiated transcripts. Thus, a broader population of MC are competent to initiate transcription *in vitro* than are being actively transcribed *in vivo* (30).

In this study, we have characterized further the structure and composition of the MC active for transcription *in vitro*. We demonstrate that MC templates transcribed *in vitro* contain a nucleosome-free gap in the promoter region. In addition, increasing the number of templates containing the nucleosome-free region results in concomitant increase in transcription both *in vivo* and *in vitro*. Thus, the presence of the nucleosome-free region (with associated proteins) correlates strongly with transcriptionally active templates, both *in vitro* and *in vivo*. The higher level of transcription *in vivo* accompanying an increased percentage of MC containing a nucleosome-free region correlates as well with increased levels of associated basal transcription factors, presumably reflecting higher numbers of accessible MC templates.

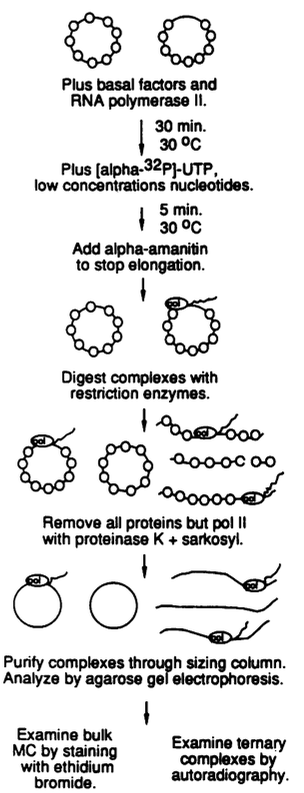

Figure 2. Scheme of protocol to examine the association of transcriptionally competent SV40 MC templates with the presence of the nucleosome-free promoter region on the MC. The beaded circles represent SV40 DNA associated with cellular nucleosomes. For the sake of simplicity, not all the nucleosomes have been drawn in, and the distances between nucleosomes is exaggerated; the nucleosomes are normally closely packed. 'pol' indicates RNA polymerase II, the smooth lines and circles indicate double-stranded SV40 DNA, and the wavy or kinky lines indicate RNA.

MATERIALS AND METHODS

Cells and virus

CV-1 cells (African green monkey kidney cell line, originally a gift from P.A. Sharp) were used for the isolation of SV40 minichromosomes (MC) and for the propagation of SV40 virus stocks. The SV40 strain 776 virus was amplified as previously described (36). The tsC219 virus stock (29) was propagated and titered at 33°C. CV-1P cells were used to titer virus stocks by plaque assays (36). Both cell lines were passaged in Dulbecco's Modified Eagle's medium containing 10% calf serum, and were maintained in Dulbecco's Modified Eagle's medium containing 2% calf serum during viral infection.

Isolation of SV40 minichromosomes

Semi-confluent monolayers of CV-1 cells (100 mm dishes) were infected with SV40 strain 776 or tsC219 virus at 5–10 PFU/cell. Infected cells to be grown at 40°C, the nonpermissive temperature for tsC219, were incubated at 37°C for the first 8 hours following infection, and then shifted to 40°C (29). The cells were labeled with 50 μCuries of [^{3}H]thymidine per dish from 24 hours to 40 hours post-infection. The SV40 MC were isolated, as previously described (30), at 40 hours post-infection for 40°C infection protocols, or at 48 hours post-infection for 37°C infection protocols. The minichromosomal DNA was quantitated as previously described (30). Various preparations of MC yielded 1 to 3 μg of wild-type SV40 DNA and 0.14 to 0.4 μg of tsC219 DNA per 100 mm dish of cells.

Viral and plasmid DNAs

To isolate SV40 viral DNA, confluent plates of CV-1 cells were infected with SV40 at 10 PFU/cell. Forty-eight hours post-infection, the viral DNA was isolated from cells lysed with 0.6% sodium dodecyl sulfate and 1 M CsC1, a modification of the Hirt extraction procedure (37). Plasmid DNA was isolated from transformed *E.coli* (strain HB101) by the alkaline lysis protocol (38). Both viral and plasmid DNAs were banded twice in a CsCl isopycnic gradient (38).

In vitro transcription assay

Transcription was promoted either by HeLa whole cell extract (39) or by a reconstituted partially purified RNA polymerase II transcription system (40–42), as previously described. Transcription reactions with whole cell extract contained either SV40 viral DNA or SV40 MC. pFLBH plasmid DNA, which contains the adenovirus type 2 major late promoter (42), was added as indicated. Finally, poly [d(I-C)]·[d(I-C)] was added to bring the total DNA concentration to 25 μg/ml. Transcription reactions performed with partially purified transcription factors and RNA polymerase II contained either SV40 DNA or SV40 MC at final concentrations of 1 to 15 μg DNA/ml.

The templates were preincubated with either 4 μl whole cell extract (at 13.2 mg of protein/ml) or a mix of the partially purified transcription factors: TFIIA, TFIIB, TFIID, TFIIE/F, and RNA polymerase II (see ref. 30 for purification and amounts of transcription factors) at 30°C for 30 minutes. Nucleotides were then added to final concentrations of 60 μM each ATP, GTP, and CTP; 1 μM UTP; 20 μCuries [α-^{32}P]UTP; and 2 mM creatine phosphate, and the reaction was incubated 10 minutes at 30°C. This was followed by the addition of 330 μM each ATP, GTP and CTP; and 1.0 mM UTP (final concentrations) and a final incubation at 30°C for 10 minutes. Some reactions also contained 0.5 μg/ml α-amanitin to inhibit MC-associated RNA polymerase II. The reaction products were analyzed as previously described (30).

Structural analysis of *in vitro* transcribed MC templates

These experiments are schematized in Fig. 2. SV40 wild-type MC were isolated at 37°C. MC from the peak 90S fraction of the sucrose gradient profile, at a final concentration of 15 μg of DNA/ml, were preincubated with basal transcription factors, followed by initiation of transcription with radioactive nucleotides for 5 minutes as described above. Transcription by RNA polymerase II was halted by the addition of 0.5 μg/ml α-amanitin. NaCl was added to generate the following final NaCl + KCl concentrations: 30 mM for cleavage with Kpn I; 60 mM for Sph I, Bgl I or Eco RI; 100 mM for Bam HI. The MC templates were then digested with 15 to 20 units of the appropriate restriction enzyme (New England Biolabs) for 30 minutes at 30°C in a final volume of 30 μl, followed by degradation of proteins with 0.2 mg/ml proteinase K in the presence of 0.8% sarkosyl for 20 minutes at 37°C, in a final volume of 40 μl (43). The remaining ternary complexes, composed of SV40 DNA, remnant RNA polymerase II, and radioactive RNA, were purified by centrifugation through a 1 ml column of Sephadex G50 (Sigma), equilibrated in 20 mM HEPES, pH 7.9; 40 mM KCl; 4 mM $MgCl_2$; 0.2 mM EDTA and 20% glycerol (38). Centrifugation was performed at 1800 g for 2.5 minutes at 20°C. Analysis of the ternary complexes proceeded by addition of bromphenol blue to the samples and electrophoresis at 4°C at 3.6 volts/cm for 14 hours through a vertical 1% agarose gel, in a buffer containing 40 mM Tris-acetate, 1 mM EDTA (38). Remaining nucleotides, migrating near the bottom of the gel, were excised prior to further manipulations. The gel was stained with ethidium bromide and photographed using ultraviolet light and Polaroid film #55. The gel was subsequently dried and exposed to preflashed XAR-5 film with an intensifying screen at −70°C.

RESULTS

Wild-type minichromosomes containing a nucleosome-free region are the templates for transcription *in vitro*

The structure of the MC templates transcriptionally active *in vitro* was examined by comparing the relative sensitivities of the templates to restriction enzymes with recognition sites either within or outside of the nucleosome-free region (schematized in Fig. 2). Those MC templates transcribed *in vitro* were radioactively tagged with short RNAs via a five minute incubation with basal transcription factors and radioactive nucleotides. Transcription was then frozen with the addition of α-amanitin, and the MC were subsequently digested with restriction enzymes. To facilitate the analysis, the MC were treated with protease and sarkosyl, as described in Materials and Methods, to obtain ternary complexes consisting of SV40 DNA, remnant RNA polymerase II (competent for elongation) and radioactive RNA (43). These complexes were partially purified by rapid chromatography through a sizing resin, and then electrophoresed through a non-denaturing agarose gel. The mobilities of the DNA and the complexes were analyzed both by staining the gel with ethidium bromide and by autoradiography.

Three major DNA bands were observed on the ethidium bromide stained gel containing the ternary complexes: Form I (supercoiled DNA), Form II (relaxed DNA) and Form III (linear DNA). As expected, only low levels of linear DNA resulted from the transcribed, but undigested MC (Fig. 3A; lane 1), but substantial levels were generated upon digestion of the MC template with restriction enzymes cleaving at a single site or at two clustered sites within the SV40 genome (Fig. 3A, lanes 2–5; see Fig. 1 for locations of cleavage sites). In particular, 50 to 60% of the MC were digested when the restriction enzymes cleaved within the nucleosome-free gap, e.g. Sph I, Bgl I, and Kpn I (Fig. 3A, lanes 2–4), whereas only 20 to 30% of the MC were digested and when the enzymes cleaved outside of the gapped region, e.g. EcoR I (Fig. 3A, lane 5) and BamH I. Viral DNA was digested to completion with all enzymes (data not shown).

Autoradiography of the same gel (Fig. 3B) revealed the presence of DNA/RNA polymerase II/RNA ternary complexes. The ternary complexes formed on undigested MC templates

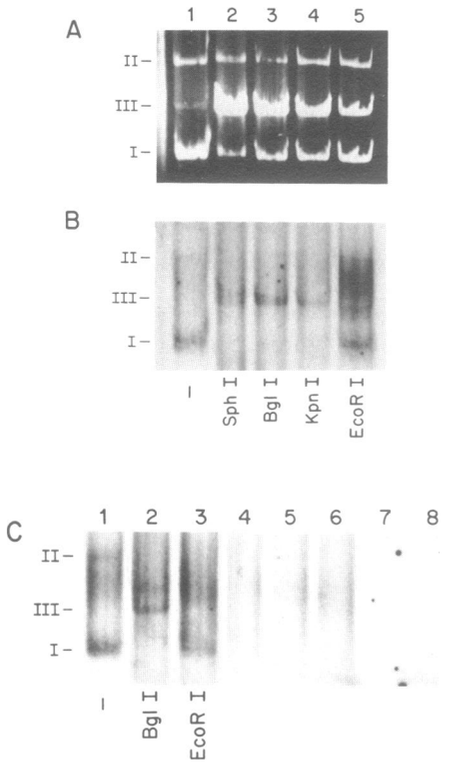

Figure 3. *In vitro* transcription is initiated on MC templates that contain a nucleosome-free gap. MC, at a concentration of 15 μg of SV40 DNA/ml, were transcribed using basal transcription factors and calf thymus RNA polymerase II, as described in Materials and Methods. The MC were derived from the 'peak' 90S fraction of a sucrose gradient profile of wild-type SV40 nucleoproteins. The resulting ternary DNA/RNA polymerase II/radiolabeled RNA complexes, frozen with the addition of α-amanitin, were prepared and analyzed as described in Materials and Methods. Panel **A**: The nucleic acid was visualized by staining the gel with ethidium bromide. Panel **B**: Autoradiogram of the gel shown in Panel A. The labels I, II, and III depict the positions of Forms I, II and III of the bulk SV40 DNA, respectively, as detected in Panel A. Lane 1: Undigested ternary complexes. Lanes 2–5: Ternary complexes digested with the indicated restriction enzymes, as described in Materials and Methods. Panel C: Autoradiogram of similar experiment with additional controls. Lane 1: Undigested ternary complexes. Lanes 2–3: Ternary complexes digested with the indicated restriction enzymes. Lanes 4–6: As lane 1, except that 0.5 μg/ml, 2.0 μg/ml, and 4.0 μg/ml of α-amanitin, respectively, were added to the transcription reactions prior to the addition of nucleotides. Lane 7: As lane 1, except that subsequent to transcription and freezing of the ternary complexes with α-amanitin, RNase A was added to a concentration of 5.0 μg/ml, and the reaction was incubated 30 minutes at 30°C. Lane 8: As lane 1, except that the sample was heated at 90°C for 5 minutes and rapidly chilled, prior to electrophoresis.

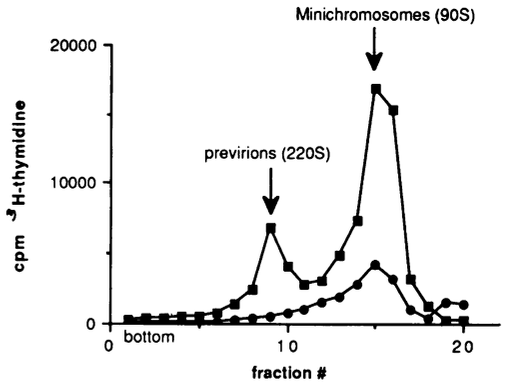

Figure 4. Sedimentation profile of wild-type and tsC219 SV40 nucleoproteins. SV40 minichromosomes labeled with [^{3}H]thymidine were isolated as described in Materials and Methods, and sedimented through a 15 to 30% sucrose gradient. Ten μl of each 500 μl fraction were counted for [^{3}H]thymidine content and plotted versus fraction number. The wild-type nucleoprotein profile is indicated by squares and the tsC219 profile is indicated by circles. The arrows at 220S and 90S mark the sedimentation positions of the SV40 previrion/virion and the minichromosomes, respectively.

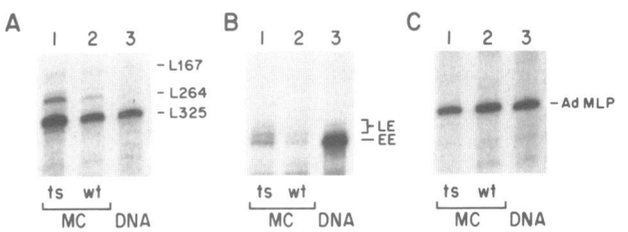

Figure 5. Comparison of transcription from wild-type and tsC219 SV40 MC in HeLa whole cell extracts. Wild-type (wt) or tsC219 (ts) SV40 minichromosomes or SV40 DNA (DNA), as indicated, were transcribed at a final DNA concentration of 1.3 μg/ml. The MC were derived from the 'peak' 90S fraction of a sucrose gradient profile of SV40 nucleoproteins (fraction 15, see Fig. 4). Transcriptions were performed with HeLa whole cell extract using the preincubation-pulse-chase protocol, as described in Materials and Methods. 1.0 μg/ml pFLBH was also included in each transcription reaction. The radiolabeled RNA was analyzed for SV40 late and early transcripts and adenovirus transcripts (Panels **A, B,** and **C**, respectively). The migration positions of transcripts from late promoter initiation sites L325 (552 bases), L264 (613 bases), and L167 (710 bases) and from the early-early (EE: 461–466 bases), the late-early (LE: 503–505 bases) initiation sites, as well as the adenovirus major late promoter (AdMLP: 197 bases) are indicated to the right of each Panel.

(Figs. 3B and 3C, lane 1) are represented in this analysis by the radioactive band comigrating with supercoiled DNA (see controls discussed below). When the MC tagged with radiolabeled RNA were digested with restriction enzymes that cut within the nucleosome-free gap (Fig. 3B, lanes 2–4 and Fig. 3C, lane 2) and the ternary complexes were analyzed, the radioactive band that comigrated with supercoiled DNA was lost and a radioactive band was generated that comigrated with linear DNA. Whereas only a subset of the bulk MC DNA was cleaved into Form III (Fig 3A, lanes 2–4), the vast majority of the radiolabeled complex was digested (Fig. 3B, lanes 2–4). In contrast, when the MC templates were digested with an enzyme that cleaves outside of the gapped region (Fig. 3B, lane 5 and Fig. 3C, lane 3), a substantial quantity of the radiolabeled ternary complexes continued to comigrate with supercoiled DNA. The numerous background bands that are particularly noticeable in Fig. 3B, lane 5, and unfortunately overlap the position at which Form III complexes migrate, apparently do not result from RNA polymerase II transcription complexes, as discussed below. These results suggest that all the MC associated with the radioactivity were susceptible to digestion within the nucleosome-free gap, but only partially susceptible to digestion outside of the gap.

To investigate the sources of the radioactivity in the various bands, a similar experiment was performed with additional controls (Fig. 3C). As in Fig. 3B, the addition of restriction enzymes to the ternary complexes resulted in either a complete or only minimal shift of radioactivity from the position of Form I (supercoiled) to Form III (linear) DNA, depending on the location of the cleavage site in the MC (Fig. 3C, lanes 2 and

3, respectively). Addition of low levels of α-amanitin to the reactions prior to the addition of nucleotides completely inhibited labeling of complexes comigrating with supercoiled and linear DNAs (Fig. 3C, lanes 4–6), although background bands migrating between Forms III and II DNAs were still detectable. This indicates that labeling of complexes comigrating with both supercoiled and linear DNAs results from transcription by RNA polymerase II, whereas the background bands result from a different source, perhaps RNA polymerase III activity. Finally, we demonstrated in two ways that the radioactivity in the complexes was due to RNA associated with the DNA. First, treatment of the ternary complex with RNase eliminated both the radioactivity comigrating with SV40 DNAs and the background bands (Fig. 3C, lane 7), without affecting the levels of SV40 DNAs (data not shown). Second, incubation of the ternary complexes at 90°C followed by rapid chilling resulted in total dissociation of the radioactivity from all complexes (Fig. 3C, lane 8), again without affecting the level or migration of supercoiled SV40 DNA. In total, these experiments suggest that MC with a nucleosome-free region are the templates competent to be transcribed by RNA polymerase II *in vitro*.

TsC219 MC, with a higher proportion of nucleosome-free regions, are transcribed *in vitro* to a greater extent than wild-type MC

If transcription can occur only on templates with nucleosome-free regions, one might predict that increasing the percentage of templates with such gaps would increase the levels of transcription. This prediction was tested by examining transcription levels both *in vivo* and *in vitro* from a temperature sensitive mutant of SV40, tsC219, which contains a point mutation in the major viral capsid protein, VP1 (44). At the non-permissive temperature, encapsidation is inhibited by the inability of the mutated VP1 protein to associate with the MC (44). We confirmed this, demonstrating that the only SV40 nucleoproteins isolated from tsC219-infected cells at the non-permissive temperature are 90S MC (Fig. 4, circles), whereas wild-type virus-infected cells at the same temperature produced both 90S and previrion (220S) MC (Fig. 4, squares). Furthermore, binding of VP1 to the MC normally results in the redistribution of nucleosomes and the concomitant loss of the nucleosome-free region (17). Thus, correlating with the lack of encapsidation, 90 to 100% of tsC219 MC isolated at the non-permissive temperature were digested by enzymes that cleave within the nucleosome-free gap (data not shown; ref. 29) in contrast to wild-type MC, where under identical isolation conditions at most 50 to 60% were cleaved (data not shown, as in Fig. 3C). However, wild-type and tsC219 MC were equally resistant to restriction enzymes that cleave outside of the gap, with 20 to 30% being cleaved (data not shown). These data indicate that tsC219, at its nonpermissive temperature, provides a unique population of MC that contain approximately three-fold more nucleosome-free gaps than the wild-type MC, making the ts virus particularly useful for the *in vitro* studies discussed below.

First, the amount of associated RNA polymerase II that had initiated transcription *in vivo* was quantitated on both the tsC219 and wild-type MC, from cells grown at 40°C. MC templates were incubated with radioactive nucleotides, allowing only elongation of transcripts initiated *in vivo*. Only late transcripts are detectable in this assay, with the majority being attenuated at nucleotide 417 (30; see Fig. 1). The tsC219 MC generated greater than six-fold more transcripts than wild-type MC (data not shown), indicating that the tsC219 MC are associated with substantially more RNA polymerase II *in vivo*.

Second, to compare the relative transcriptional potential of tsC219 and wild-type MC, equivalent concentrations of the two MC preparations were transcribed *in vitro* using HeLa whole cell extract (Fig. 5). The levels of transcription in the presence of extract were 20- to 30-fold higher on both chromatin templates than in the absence of added extract (described above), supporting previous data that most transcription observed *in vitro* from the chromatin templates derives from *de novo* initiation (30). The adenovirus major late promoter was included in each transcription reaction as an internal control (Fig. 5C). The late promoters of wild-type MC and wild-type viral DNA were transcribed with equivalent efficiency (Fig. 5A, lanes 2 and 3), as previously shown for MC isolated at 37°C (30). However, the identical SV40 late promoter within the context of tsC219 MC was transcribed four-fold more efficiently than within the context of either wild-type MC or viral DNA templates (Fig. 5A, lane 1 versus lanes 2 and 3).

In addition, the early promoter on the tsC219 MC was transcribed at higher levels (4-fold) than the promoter on wild-type MC (Fig. 5B, lanes 1 and 2). As anticipated from previous studies of transcription *in vitro* (30,45) and from *in vivo* levels of transcripts, the level of early gene expression from both the tsC219 and wild-type chromatin templates was repressed, compared to early transcription from viral DNA templates (6- and 28-fold lower, respectively; Fig. 5B, compare lanes 1 and 2 to lane 3). Thus, both early and late promoters on the tsC219 MC are transcribed more efficiently *in vitro* than the corresponding promoters on wild-type MC. This correlates with the increase in the percentage of MC with the nucleosome-free region in tsC219 MC, as compared to wild-type MC.

In vivo-transcribing wild-type MC are associated with higher levels of basal RNA polymerase II transcription factors

To investigate whether the percentage of MC with a gap correlates with levels of associated basal transcription factors, tsC219 and wild-type MC templates, isolated at 40°C, were transcribed with various subsets of the basal transcription factors (TFIIA, TFIID, TFIIB, TFIIE/F and RNA polymerase II). All these basal factors are essential for the initiation of transcription *in vitro* on the SV40 promoters (46). Assuming that all the basal transcription factors are similarly required for initiation of transcription from these promoters on chromatin templates, transcription observed *in vitro* on the MC templates in the absence of any particular basal transcription factor would indicate the presence of that factor either on the SV40 MC templates or in one of the other basal factor preparations. (The validity of the underlying assumption is addressed at the end of this section.) Thus, we analyzed the minimal cross contamination of the basal transcription factor preparations by transcribing viral DNA as a control template. Transcription from the viral DNA was either not detectable or extremely minimal when any one factor preparation was omitted (Fig. 6A, compare lane 2 to lanes 3–7 and lane 9 to lanes 10–14). On a longer exposure of the autoradiogram, late transcripts were observed in the absence of the TFIID and RNA polymerase II preparations (Fig. 6A, central panel, lanes 4 and 7, respectively), indicating that these two activities were present, albeit at low concentrations, within the other factor preparations.

Parallel experiments were performed with equivalent concentrations of tsC219 or wild-type MC templates from 90S 'peak' MC fractions. The presence of associated basal

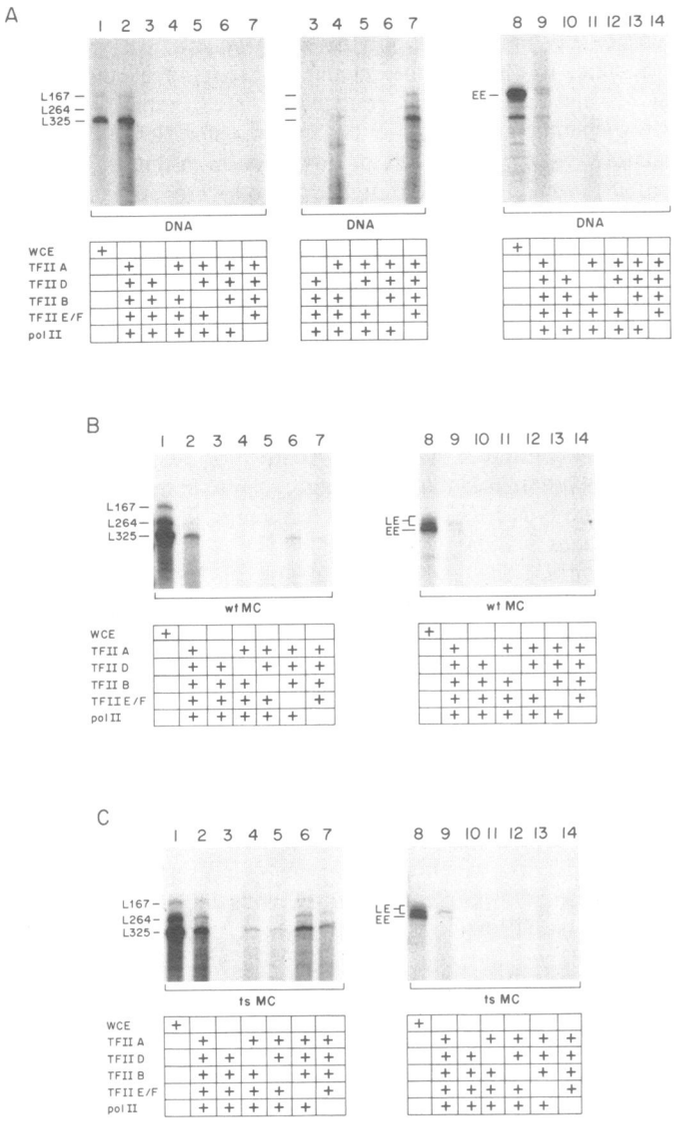

Figure 6. Differential association of RNA polymerase II basal transcription factors with tsC219 *versus* wild-type MC. SV40 DNA (Panel **A**), wild-type MC (Panel **B**), and tsC219 SV40 MC (Panel **C**) were transcribed at a concentration of 1.3 μg of SV40 DNA/ml. The MC were derived from the 'peak' 90S fraction of a sucrose gradient profile of SV40 nucleoproteins (fraction 15, see Fig. 4). The transcription reactions contained either HeLa whole cell extract (WCE) or combinations of the basal transcription factor preparations: TFIIA, TFIID, TFIIB, TFIIE/F and calf thymus RNA polymerase II (pol II), as indicated. The templates were transcribed using the preincubation-pulse-chase protocol as described in Materials and Methods. Lanes 1–7: analysis of SV40 late transcripts. Lanes 8–14: analysis of SV40 early transcripts. The migration positions of the transcripts derived from the various late and early promoter initiation sites are indicated to the left of each section. The autoradiogram shown in the central lanes (lanes 3–7) in panel A was exposed sixteen-fold longer than the autoradiograms of the remaining lanes (both late and early transcripts). The exposure lengths of the autoradiograms in panels B and C correspond to that of the central, darker lanes (lanes 3–7) in panel A. Note that the MC preparations supported transcription from the LE and L264 initiation sites at proportionately higher levels than did viral DNA. Correcting for cross-contamination seen in Panel A, the relative activities retained in the absence of TFIID, TFIIB, and TFIIE/F are 0.1, 0.07, and 0.5, respectively, for ts MC and 0.06, 0.04, and 0.3, respectively, for wt MC. Considering the 3-fold increase in total transcription on ts MC, the amounts of all three factors is at least 4-fold higher on ts versus wt MC templates.

transcription factors on the MC was indicated by the relatively small diminution in signal upon omission of certain basal transcription factors. In particular, transcription from the two MC templates was maintained in the absence of TFIIE/F (Figs. 6B and 6C, lanes 6). Levels of transcription were somewhat lower in the absence of TFIID, TFIIB or RNA polymerase II (Figs. 6B and 6C, lanes 4, 5, and 7, respectively) and undetectable in the absence of TFIIA (Figs. 6B and 6C, lanes 3). To take into account the low levels of cross-contamination of the basal factor preparations (Fig. 6A), the relative level of transcription generated in the absence of a factor on naked DNA templates was subtracted from the relative level of transcription generated in the absence of the same factor on MC templates. These corrected data indicate an associated TFIIE/F activity on both the wild-type and tsC219 MC templates, and associated TFIID and TFIIB on tsC219 MC.

The absolute requirement for the addition of the TFIIA preparation to transcription reactions containing MC templates, despite the association of TFIID activity with those MC templates, was at first puzzling. This result suggested that the TFIIA preparation played a novel role in initiation of transcription on MC templates, other than facilitating the binding of TFIID. This additional function in the TFIIA preparation must be necessary for each round of initiation on the chromatin templates. Although these TFIIA preparations are likely to be contaminated with TFIIJ (47), TFIIJ only substantially activates transcription in the presence of the TATA-binding protein (TBP) and not TFIID. Thus it is unlikely to be the source of this activity. TFIIA may be required to counteract an inhibitor of TFIID on the MC templates.

One might expect that basal transcription factors would be constituents of those MC associated with elongating RNA polymerases, upon which transcription had been initiated *in vivo*. Support for this hypothesis was obtained upon comparing levels of transcription from tsC219 and wild-type MC in these experiments. The tsC219 MC preparation, with its higher proportion of MC being transcribed *in vivo*, also generated five-times more transcripts *in vitro* in the absence of each basal factor than did wild-type MC (compare Fig. 6C and 6B, lanes 3–7).

To examine further the correlation between *in vivo* activity and association of basal factors, equivalent concentrations of 'heavy' (rapidly sedimenting) and 'light' (slowly sedimenting) wild-type MC were transcribed, omitting each basal transcription factor in turn (data not shown). In this case, both the 'heavy' and 'light' MC fractions contain similar percentages of MC with nucleosome-free gaps, but only MC that sediment on the heavy side of the 90S peak are associated with *in vivo* initiated RNA polymerase II (see 30 and references therein). The 'heavy' MC were transcribed with slightly higher efficiency than the 'light' MC when all the basal transcription factors were present (30). In the absence of particular basal factors however, transcription from the two templates was dramatically different. In particular, SV40 late transcription from the 'heavy' MC retained approximately 12-times more activity in the absence of TFIIE/F, relative to the complete reaction, and approximately 2-times more activity in the absence of TFIID than did 'light' MC. The levels of transcripts generated on 'light' MC resembled those from viral DNA, although omission of TFIID resulted in 3-times higher relative levels of transcripts from the 'light' MC than from DNA. These results indicate that there is a strong correlation between

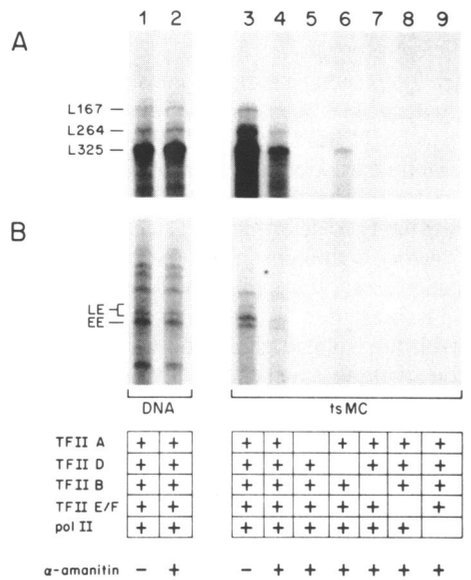

Figure 7. Ability of associated RNA polymerase II basal transcription factors to support reinitiation of transcription *in vitro* on tsC219 MC. SV40 DNA (lanes 1 and 2) and tsC219 MC (lanes 3–9) were transcribed at a concentration of 1.3 μg of SV40 DNA/ml. The MC were derived from the 'peak' 90S fraction of a sucrose gradient profile of SV40 nucleoproteins. The transcription reactions contained combinations of the basal transcription factors: TFIIA, TFIID, TFIIB, TFIIE/F and α-amanitin resistant RNA polymerase II, in the presence (+) or the absence (−) of α-amanitin, as indicated. The templates were transcribed using the preincubation-pulse-chase protocol as described in Materials and Methods. Either late or early SV40 transcripts were analyzed (panels **A** and **B**, respectively). The positions of migration of the late and early transcripts are indicated to the left of each panel. Lanes 3–9 represent seven-fold longer exposures than lanes 1 and 2.

the MC fractions transcribed *in vivo*, the 'heavy' MC, and those which contain associated basal transcription factor activity, in particular TFIIE/F and TFIID. However, some TFIID also appears to be stably associated with MC not actively being transcribed *in vivo*, the 'light' MC.

Finally, the similar requirements for basal transcription factors when comparing naked viral DNA with 'light' MC, which are not associated with RNA polymerase II *in vivo*, argues that DNA and chromatin templates require the same basal transcription factors for transcription by RNA polymerase II. This validates the assumption made in interpretation of these experiments.

Different roles of associated basal transcription factors in reinitiation of transcription *in vitro* from the chromatin templates

The ability of the associated basal transcription factors to promote reinitiation of transcription on the MC was investigated by the additions of α-amanitin, to inhibit MC-associated RNA polymerase II, and of α-amanitin resistant RNA polymerase II, to initiate transcription *de novo* (30). Due to the high levels of associated basal transcription factors, tsC219 MC were used for these experiments. Each basal transcription factor was omitted in turn. Using this protocol, α-amanitin had little effect on transcription from the viral DNA control (Fig. 7A and 7B, compare lanes 1 and 2). In the absence of the resistant RNA polymerase II, transcription was totally inhibited by α-amanitin (Fig. 7A, lane 9). As expected from previous experiments using α-amanitin resistant RNA polymerase II with MC templates actively being transcribed *in vivo* (30), α-amanitin reduced the levels of transcription from the tsC219 MC by 2- to 3-fold (Fig. 7A and 7B, compare lanes 3 and 4). This decrease with addition of α-amanitin is not observed when the chromatin templates are MC that are not being actively transcribed *in vivo* (30). The partial inhibition of transcription from the tsC219 MC is in part due to some of the transcripts labeled *in vitro* being initiated *in vivo* with polymerases that were sensitive to α-amanitin. However, this cannot account for the entire magnitude of the reduction in transcription (30). Inhibition also appears to occur by frozen polymerase/α-amanitin complexes blocking subsequent transcription of entire templates (48). The relatively high level of SV40 late transcripts generated in the absence of added TFIID was not substantially inhibited by the presence of α-amanitin (compare Fig. 6C, lane 2 versus lane 4 to Fig. 7A, lane 4 versus lane 6). In sharp contrast, the relatively high level of SV40 late transcripts generated in the absence of added TFIIE/F (Fig. 6C, lane 2 versus lane 6) was abolished when the RNA polymerase II isolated with the MC was inhibited (Fig. 7A, lane 4 versus lane 8). Thus, the chromatin-associated basal transcription factors are differentially capable of participating in reinitiation on the MC templates.

DISCUSSION

Initiation of transcription *in vitro*, as *in vivo*, occurs on minichromosome templates containing a nucleosome-free region

We have demonstrated a correlation between SV40 MC templates competent to initiate transcription *in vitro*, as *in vivo* (31, 32, 35) and those containing a nucleosome-free region. These data explain why initiation of transcription from *in vivo*-assembled chromatin templates is so efficient *in vitro* (30): transcription factor accessibility to the promoters is built into the structure of the potentially active chromatin. In sharp contrast, *in vitro* chromatin assembly of DNA containing a variety of other promoters has invariably resulted in the repression of transcription from these promoters (see Introduction). Thus, one function of the relative absence of nucleosomes at the promoters on the SV40 MC templates is to avoid chromatin-mediated inhibition. We note that the 'nucleosome-free' regions within the MC may not actually be devoid of nucleosomes, as specifically positioned nucleosomes have been mapped over SV40 promoter sequences, both in isolated SV40 MC and upon reconstitution of nucleosomes onto SV40 DNA *in vitro* (49–52).

Increasing the percentage of minichromosome templates with a nucleosome-free gap results in higher levels of transcription *in vivo* and *in vitro*

In an infected cell, both SV40 MC (approximately 50,000 copies per cell) and cellular chromatin must compete for limiting amounts of cellular transcription factors (roughly 10,000 to 50,000 molecules per cell, e.g. TFIID; see 30). This is evidenced in part by the low percentage of viral templates being utilized at any point in time. Therefore, the higher levels of transcription obtained *in vivo* from MC with a greater percentage of nucleosome-free gaps are likely to be due to a greater proportion of transcriptionally competent templates capable of competing

successfully for transcription factors, and not to increased efficiency of transcription from each template.

When transcribed *in vitro* in HeLa whole cell extract, tsC219 MC once again generate levels of late gene transcripts several times higher than wild-type MC. This is not simply a reflection of the increased level of transcription *in vivo*, because levels of transcripts generated in HeLa extracts are dramatically elevated over the levels of transcripts generated by elongation *in vitro* of RNAs initiated *in vivo* (see also 30). Instead, the elevated transcriptional potential of the tsC219 MC can be explained by a combination of promoter accessibility, activation by histone or non-histone 'structural' components, or by activation by bound specific DNA-binding proteins.

Although the nucleosome-free promoter region may be more accessible for binding of basal transcription factors in the cell extract, increased accessibility alone cannot account for the higher level of transcription from tsC219 MC, as one would expect that the even more highly accessible viral DNA templates would be more efficiently transcribed *in vitro* than MC templates. Instead, as we have shown (Fig. 5A, lanes 2 and 3), the SV40 wild-type DNA and MC templates are transcribed *in vitro* with roughly equal efficiency. Thus, greater accessibility is not the complete explanation for increased transcription.

Associated histone, non-histone chromosomal proteins, or specific DNA-binding proteins might increase transcription from the MC by recruiting additional transcription factors to the template. As precedent for such a role for structural chromosomal proteins, the N-terminus of histone H4 has been implicated in both activation and repression of specific promoters in yeast (53,54). A role for several specific DNA-binding proteins in the formation of the preinitiation transcription complex has also been shown (46,55−60), and a high proportion of isolated SV40 MC are associated with proteins bound to both enhancer and promoter elements within the 'nucleosome-free' region (61−63). In fact, these enhancer and promoter elements are required for efficient generation of the nucleosome-free region *in vivo* (64−67). All MC containing nucleosome free regions must therefore have enhancer and promoter factors bound. Finally, as our data demonstrated, TFIID activity associated with MC correlates both with transcriptional activity *in vivo* and with the percentage of MC containing gaps. Not all MC containing the gap, however, have stably associated TFIID. Any of these proteins might be responsible for accelerating transcription complex formation.

Stable association of basal transcription factors with actively transcribed or transcriptionally competent chromatin templates

Both kinetic and physical *in vitro* analyses with naked DNA templates have shown that the binding of basal transcription factors to a promoter-containing DNA template is a highly ordered process. Binding of TFIID, the only basal factor that directly binds to a specific DNA sequence (68), is followed by the binding of TFIIB, RNA polymerase II and TFIIF and finally TFIIE, TFIIH, and TFIIJ to form the complete preinitiation complex (21,69,70). Following initiation, TFIID remains bound to the TATA sequences within the promoter to participate in the formation of subsequent preinitiation complexes (55,71), whereas at least some of the other transcription factors are released, such as TFIIB (72). The only initiation factor expected from current knowledge to associate with the elongating polymerase is TFIIF, which is essential not only for the initiation of transcription, but also for efficient elongation (72−75).

By *in vivo* footprinting methodologies, TATA sequences in several promoters have been shown to be bound by an activity that appears to be TFIID (76,77). However, the association of the other basal transcription factors with promoters packaged in chromatin in the cell has not been addressed. SV40 MC offer a unique opportunity to determine the stable association of these other basal factors with a chromatin template. We have used a complementation assay, transcribing MC templates in the absence of each basal transcription factor, to determine which factors are associated with the isolated chromatin templates. The simplest interpretation of these experiments is that transcription in the absence of an added factor, as corrected for cross-contamination of the factor preparations, must be driven by basal transcription factors associated with the isolated MC. With this interpretation, our data indicate the presence of TFIID, TFIIE and/or TFIIF, and perhaps TFIIB on the chromatin templates. Two alternative interpretations of the experiments are theoretically possible, but are unlikely due to the results we have obtained. First, transcription from chromatin templates may not require all the basal factors. This is unlikely, as MC not being actively transcribed *in vivo* demonstrate a strong dependence on all the basal factors. Second, transcription may be due to the ability of the MC template with its associated specific DNA-binding proteins and chromosomal proteins to recruit low concentrations of certain basal factors more efficiently than DNA templates. Arguing against this interpretation is the accentuated ability of MC sedimenting more rapidly than the bulk of 90S MC to generate transcripts in the absence of some of the basal transcription factors. As the formation of the nucleosome-free region of SV40 MC is dependent on the binding of upstream binding proteins and as the percentage of MC containing the nucleosome-free region is invariant across the 90S peak (29−31), these specific DNA-binding proteins must also be associated with all populations of the peak. Thus, the difference in transcriptional response across the 90S peak must be due to the differential association of basal transcription factors with MC templates, potentially resulting in different sedimentation properties.

TFIID stably associated with the *in vivo*-assembled chromatin templates can support reinitiation of transcription from the chromatin promoters

We have detected functional TFIID activity stably associated with MC being transcribed *in vivo*. The high levels of transcription in the absence of exogenously added TFIID, even in the presence of α-amanitin and resistant RNA polymerase II, demonstrate that the associated TFIID activity supports reinitiation of transcription *in vitro*. Thus, TFIID remains associated with the viral late promoter after the initiation of transcription *in vivo*, which allows its participation in the formation of a new preinitiation complex with the polymerase *in vitro*. These data are consistent with the role for TFIID in reinitiation of transcription on DNA templates as defined by *in vitro* studies (71). The data also correspond with the presence of TFIID on heat shock promoters within cellular chromatin, both before and after transcriptional induction (76,77). To quantitate the amount of TFIID associated with the isolated MC, proteins extracted from the MC were analyzed by Western blotting (αTFIID antibodies were kindly provided by M.Timmers and P.A.Sharp). Less than 1% of the wild-type MC templates

were associated with TFIID (data not shown), consistent with previous determinations that 0.1 to 1% of the total MC are associated with elongating RNA polymerases. This low level of TFIID is not sufficient to account for all the potentially active MC templates. Thus, TFIID cannot be the sole determinant in both establishment and maintenance of the transcriptionally competent chromatin structure; rather other upstream DNA-binding proteins must aid in these processes. Similarly, in the *in vitro* reconstitution of chromatin, both TFIID and other DNA-binding transcriptional activators aid in preventing the inhibition of transcription by nucleosomes (78).

Finally, the point should be emphasized that in our experiments, the transcriptionally competent chromatin structure was established *in vivo*. The DNA-binding transcriptional activators present in whole cell extract were not capable of efficiently producing active chromatin over repressed SV40 promoters *de novo*. Thus, we have no evidence for an activation of preformed inactive SV40 chromatin, in contrast with the ability of Gal4-VP16 activators to activate chromatin previously reconstituted *in vitro* (79). These data support one model for how the SV40 promoters are activated *in vivo*, which is that formation of the nucleosome-free region in a lytically infected cell occurs during the process of SV40 DNA replication (17).

Basal transcription factors, stably associated with isolated MC, not poised to participate in subsequent establishment of preinitiation transcription complexes

Efficient transcription *in vitro* from MC templates actively transcribed *in vivo* (tsC219 and 'heavy' MC) was also observed in the absence of the basal transcription factors TFIIE/F. In fact, omission of TFIIE/F resulted in only a small decrease in the levels of transcripts. Our previous data, with which all these data are consistent, proved that the majority of transcripts detected *in vitro* result from initiation *in vitro* (30). Therefore, some of the transcripts produced in the absence of TFIIE/F must be generated from initiation *in vitro*. This argues that the TFIIE/F present on isolated MC is capable of being utilized for subsequent rounds of initiation. Paradoxically, transcription in the absence of exogenous TFIIE/F was completely inhibited in the stringent reinitiation assay, when both α-amanitin and the resistant RNA polymerase II were added to the reactions. The inhibition by α-amanitin must be related to the inactivation of the RNA polymerases that bound the MC *in vivo* and remained associated during the isolation procedures. Both TFIIE and TFIIF strongly interact with RNA polymerase II *in vitro*, as demonstrated by their cosedimentation with the enzyme in glycerol gradients (74,80), and the binding of the 30 and 74 kDa subunits of TFIIF to an RNA polymerase II affinity column (81–83). One can propose two mechanisms for how freezing of the RNA polymerase II with α-amanitin might lead to the apparent inactivation of TFIIE/F in subsequent rounds of initiation of transcription *in vitro*: 1) inactivation of the *in vivo* initiated polymerases might sterically block subsequent transcription of the template by the resistant enzyme (48), and if TFIIE/F could only be reutilized on the same template, due to local concentration effects, this would interfere with its reutilization, or 2) inactivation of the *in vivo* initiated polymerases might interfere with the ability of TFIIE/F to dissociate from the elongating polymerase, thus inhibiting its interaction with *de novo* preinitiation complexes. Both hypotheses presume that any TFIIF and/or TFIIE associated with the chromatin templates would be bound through RNA polymerase II, mostly in transcriptional elongation complexes. Our results are consistent with what is known about the function of TFIIF on naked DNA templates *in vitro*.

In conclusion, transcription from the MC templates seems to require the same basal transcription factors that are required for DNA templates *in vitro*. Some of these basal factors stably associate with the templates *in vivo* and remain on the chromatin templates during isolation procedures. These include TFIID, TFIIE and/or TFIIF, and possibly TFIIB. Of these associated factors, only TFIID seems to be poised to readily form preinitiation complexes with exogenously added RNA polymerase II. Thus, the steps in initiation of basal levels of transcription on chromatin templates, as assayed so far, parallel the process that has been characterized on naked DNA templates.

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