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Transcription factor requirement for multiple rounds of initiation by human RNA polymerase II

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ABSTRACT **We have investigated conditions that allow multiple rounds of transcription initiation from the adenovirus major late promoter in an *in vitro* system derived from HeLa cell nuclear extracts. Templates containing guanine-free cassettes provided a direct assay for discriminating between reinitiated transcripts and transcripts generated by a first-round of transcription initiations. When reactions were reconstituted with the previously characterized class II transcription factors (TFIIA, TFIIB, TFIID, TFIIE/F), transcription by human RNA polymerase II from the adenovirus major late promoter was essentially restricted to a single round of initiations. Reinitiations at previously transcribed major late templates required an additional activity, designated reinitiation transcription factor (RTF). The RTF activity could be separated from the required transcription initiation factors. Semi-purified human RTF also promoted transcription reinitiations at minimal promoters derived from the human c-*myc*, histone H4, and heat shock 70-kDa protein genes, indicating that the same reinitiation factor may be utilized by many, if not all, genes. The possible role of RTF in regulating the transcription rate of various class II genes is discussed.**

In contrast to prokaryotic RNA polymerases, human RNA polymerase II alone cannot initiate transcription from promoters but requires the participation of several general factors (1). *In vitro* investigations of transcription from the adenovirus major late (ML) promoter have revealed that the first step in class II gene activation involves binding of class II transcription factor D (TFIID) to the TATA element (2–5). This initial interaction leads to template commitment and is sufficient to prevent promoter inactivation during nucleosome assembly (6). The committed template, the other general factors, and RNA polymerase II then assemble into a preinitiation complex (7). After transcription initiation and promoter clearance by the RNA polymerase, a postinitiation complex, containing TFIID and perhaps other components, remains at the promoter (8–10). The existence of this post-initiation complex suggests that subsequent rounds of transcription initiations (i.e., reinitiations) should proceed by a different mechanism than initial gene activation. At the least, one might expect that the step in which TFIID binds to the template would not be necessary for subsequent initiations. Since this particular step appears to be generally rate-limiting (3, 11), transcription reinitiations should be kinetically favored, unless specific mechanisms exist to control them.

Experiments with various concentrations of sarkosyl to inhibit individual reaction steps in RNA polymerase II transcription have shown that transcription reinitiations take place when the ML promoter is transcribed in crude HeLa nuclear extracts (11). Reinitiations were also invoked to explain the pattern of decreasing-length transcripts sometimes observed when templates containing guanosine-free (G-free) cassettes were transcribed in reconstituted reactions (12, 13). Here we confirm that G-free cassette templates can be used to directly distinguish first-round transcripts from reinitiated transcripts *in vitro*. In addition, we present evidence for the existence of a previously unknown transcription factor, designated reinitiation transcription factor (RTF), which is absolutely required for efficient reinitiation by human RNA polymerase II *in vitro*.

MATERIALS AND METHODS

Purification of Transcription Factors and RNA Polymerase II. HeLa transcription factors TFIIB, TFIID, TFIIE, and RNA polymerase II were prepared essentially as described (8, 14). TFIIA and TFIIC [poly(ADP-ribose) polymerase] were also purified by published procedures (15, 16).

Bacterially expressed human TFIID was purified from *Escherichia coli* cells transformed with human TFIID cloned in a pET vector (a gift from M. C. Schmidt and A. J. Berk, University of California, Los Angeles). The recombinant TFIID (3.3 units/μl, 1.5 mg/ml) was purified from the soluble *E. coli* extract by chromatography on DEAE-cellulose (DE-52; Whatman) and Bio-Rex 70 (Bio-Rad).

RTF was separated from TFIIB by chromatography on a Mono S column (Pharmacia) in a buffer containing 20 mM Hepes (pH 7.9 at 23°C), 20% (vol/vol) glycerol, 0.2 mM EDTA, and 1 mM dithiothreitol (buffer A). Prior to this, the 0.5 M KCl fraction of the nuclear extract from Whatman P-11 phosphocellulose purification was chromatographed on a DE-52 column. The breakthrough was loaded onto the Mono S column in 100 mM KCl and was eluted with a gradient of increasing KCl concentration in buffer A. RTF activity was eluted in the 230 mM KCl gradient fraction, although significant amounts of activity were also recovered in the column runthrough. TFIIB activity was reproducibly found in the Mono S 350 mM KCl gradient fraction.

Protein concentrations were determined by the method of Bradford (17).

***In Vitro* Transcription Assays.** Transcription reaction mixtures (25 μl) contained 50–70 mM KCl, 2–10 mM $(NH_4)_2SO_4$, 12 mM Tris (pH 7.3 at 25°C), 12% glycerol, 3.6% (wt/vol) polyethylene glycol 8000, 40 mM Hepes (pH 8.4), 7.5 mM $MgCl_2$, 0.12 mM EDTA, 4 mM dithiothreitol, 0.6 mM ATP, 0.6 mM UTP, 25 μM [α-^{32}P]CTP (5000–10,000 cpm/pmol), and 8 units of RNasin (recombinant ribonuclease inhibitor; Promega). Unless otherwise noted, transcription reactions contained 0.3–1 unit of each of the general transcription factors and RNA polymerase II and were incubated with 400 ng of template for 45 min at 30°C (see ref. 14 for the definition of units). Reactions with linearized templates contained in addition 0.4 μg of TFIIC to decrease possible nonspecific initiations by RNA polymerase II (16). Reactions were stopped, extracted with 1:1 (vol/vol) phenol/$CHCl_3$, precip-

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Abbreviations: TFIIA, TFIIB, etc., class II transcription factors A, B, etc.; RTF, reinitiation transcription factor; ML, major late; G-free, guanosine-free.

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itated with ethanol, and analyzed by electrophoresis through a 4.5% acrylamide gel as described (18).

The ribonucleoside triphosphates used in all transcription reactions were purified by chromatography on a Polyanion SI column (Pharmacia) to remove any contamination by GTP. However, it was necessary to add 0.1 mM 3′-*O*-methyl-GTP to reactions that included TFIIA because this fraction contained a small amount of endogenous GTP.

RESULTS

G-free Cassette Templates Provide a Direct Assay for Transcription Reinitiation. Our analysis of transcription reinitiation relied on electrophoretic separation of transcripts initiated *in vitro* at a promoter cloned upstream of a G-free cassette (14). As illustrated schematically in Fig. 1 *Upper*, a first-round of transcription initiation on such a template produces specific transcripts corresponding in length to the cassette. In the absence of GTP, the RNA polymerase pauses, without disengaging, at the end of the G-free region (8). Therefore, if transcription occurs a second time on the same template, the second RNA polymerase will be arrested by the presence of the first enzyme, producing RNA transcripts shorter by some 30 nucleotides (the length of DNA covered by an elongating RNA polymerase molecule) (12). Similarly, a third round of initiation will give rise to transcripts that will be 60 nucleotides shorter than the full-length cassette, and so on.

Our initial studies were carried out with a minimal promoter containing the TATA box and initiator region of the adenovirus ML promoter (from −50 to +10) upstream of a 390-base-pair (bp) G-free cassette [plasmid pMLC$_2$ATΔ-50 (12)]. As predicted in the above model, a regular pattern of decreasing-length transcripts was observed with this template when the basic factors required for *in vitro* transcription were provided by crude fractions from HeLa nuclear extracts or when the phosphocellulose 0.5 M KCl fraction was used as a source of TFIIB and TFIIE/F (Fig. 1 *Lower*). This pattern was present whether the DNA was circular (lane 1) or linearized (lane 3).

Several control experiments were performed to verify that the particular transcript pattern in these reactions was actually due to reinitiation events. If the shorter transcripts resulted from RNA polymerases stacking at the end of the cassette, addition of GTP for a brief period of time at the end of the reaction should allow transcript extension past the G-free region. This was the case; after a chase with GTP, most of the full-length and reinitiated transcripts disappeared (Fig. 1 *Lower*, lanes 2 and 4). With the uncut template, a heterogeneous mixture of very long transcripts appeared (Fig. 1 *Lower*, lane 2). Reinitiated transcripts generated from a template cut 106 bp past the end of the G-free region (pMLC$_2$ATΔ-50/*Pvu* II) were extended to a unique 496-nucleotide run-off product by the addition of GTP (Fig. 1 *Lower*, lane 4). This would not have happened if the shorter transcripts were produced by degradation or originated from different start sites inside the G-free region.

On the other hand, eliminating the RNA polymerase II stacking sites by cutting the template at the exact end of the G-free region (pMLC$_2$ATΔ-50/*Sma* I) should allow polymerases engaged in both first-round transcription and reinitiation to run off the end of the cassette. In accord with this prediction, a single product of 390 nucleotides was observed for the pMLC$_2$ATΔ-50/*Sma* I template, with or without a GTP chase (Fig. 1 *Lower*, lanes 5 and 6).

Note that in the chase experiments, not all of the G-free transcripts could be elongated by the addition of GTP, indicating that a small fraction of the RNA polymerase II molecules did not remain bound to the template under these assay conditions, or were in some other way inactivated and unable to resume elongation. This might reflect a deficiency in our reconstituted system of transcription elongation factors such as SII (1, 19, 20).

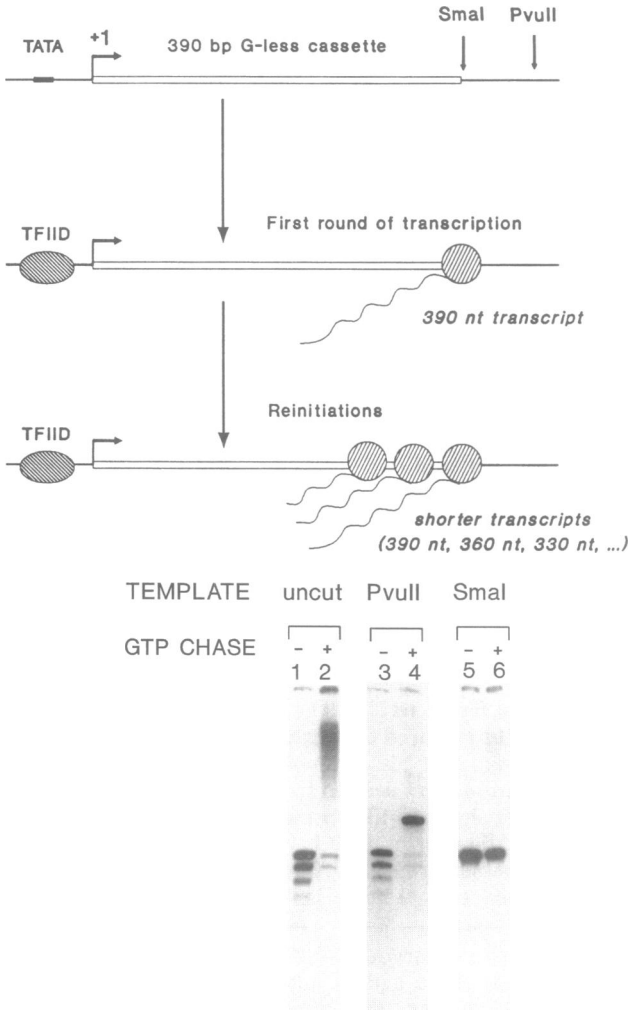

FIG. 1. Assay for transcription reinitiations using G-free cassette containing templates. (*Upper*) Schematic representation illustrating how first-round and reinitiated transcripts can be distinguished by their lengths when transcription elongation by RNA polymerase II is arrested at the end of the G-free cassette in the absence of GTP. (*Lower*) Controls for the *in vitro* reinitiation assays. Transcription reactions were carried out as described with the 0.5 M KCl step fraction from phosphocellulose chromatography as the source of TFIIB and TFIIE/F. After a 25-min incubation at 30°C in the absence of GTP, transcription elongation was allowed to proceed for an additional 5 min in the presence (lanes +) or absence (lanes −) of an additional 0.5 mM concentration of all four ribonucleoside triphosphates as indicated ("GTP chase"). The templates were either uncut pMLC$_2$ATΔ-50 (13) (lanes 1 and 2), pMLC$_2$ATΔ-50 cut with *Pvu* II 106 bp downstream of the G-free cassette (lanes 3 and 4), or pMLC$_2$ATΔ-50 cut with *Sma* I at the end of the G-free cassette (lanes 5 and 6). The resulting transcripts were resolved by polyacrylamide gel electrophoresis and detected by autoradiography.

An Additional Component Is Required for Transcription Reinitiation. It is known that the general transcription factors TFIIB, TFIID, and TFIIE/F are absolutely required for specific transcription initiation by RNA polymerase II (reviewed in ref. 1). TFIIE/F consists of more than one protein and can be resolved into at least two components, TFIIE and TFIIF (21). A requirement for other factors, TFIIA or TFIIG, is sometimes noted, depending on the source and purity of the other transcription factors used (1, 15, 22). We carried out transcription with a set of partially purified factors in which the TFIIB and TFIIE/F in the phosphocellulose 0.5 M KCl

fraction had been separated and were each further purified by two additional chromatographic steps (14) (Fig. 2). TFIIA and TFIIG were not required for transcription with this set of fractions. With this more purified reconstituted system, the very large majority of the transcripts were full-length, independent of the size of the G-free cassette (Fig. 2, lanes 1 and 3). No, or very few, reinitiated transcripts were observed, in contrast to the pattern observed with cruder fractions where a large fraction of the transcripts was due to reinitiations (Fig. 2, lanes 2 and 4). Multiple rounds of transcription initiation were not observed in transcriptions reconstituted with only TFIIA, TFIIB, TFIID, TFIIE/F, and RNA polymerase II, even if these activities were present in excess or if the reaction time were extended (not shown).

This difference in reinitiation activity of crude versus more purified reconstitutions was consistent with the existence of a factor in the phosphocellulose 0.5 M KCl fraction that is necessary for reinitiation and that is lost upon further purification of TFIIB and TFIIE/F. By adding various fractions to RNA polymerase II and the partially purified factors required for first-round transcription, we followed the chromatographic behavior of the reinitiation activity, designated RTF, starting with the phosphocellulose 0.5 M KCl fraction (Fig. 3*A*). Fig. 3*B* shows TFIIB activity at different stages of purification. RTF activity was found to comigrate with TFIIB in the first two chromatographic steps (Fig. 3*B*, lanes 2 and 3). However, chromatography on the Mono S column separated RTF and TFIIB (Fig. 3*B*, lane 4).

Reconstituted Reactions with Isolated RTF. After Mono S chromatography, the RTF activity was free of contamination by the general factors TFIIB, TFIID, TFIIE/F, and RNA polymerase II. It was thus possible to investigate the requirements for transcription initiation and reinitiation at the ML promoter in a completely reconstituted system (Fig. 4). The presence of RTF did not alleviate the absolute requirement for RNA polymerase II, TFIIB, TFIID, or TFIIE/F in the transcription reaction (Fig. 4 *Left*, lanes 5–8). However, multiple rounds of initiation were observed only when RTF was present (compare lanes 1 and 3 with lanes 2 and 4 in Fig. 4 *Left*). In the absence of RTF, a pair of transcripts of variable

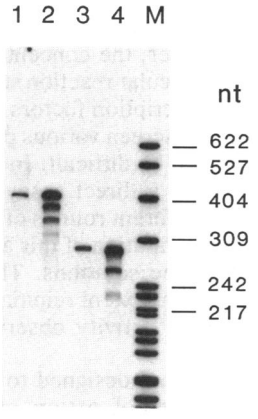

FIG. 2. Reinitiations do not take place in reactions reconstituted with purified transcription factors. Transcription reactions were carried out under standard conditions with isolated human RNA polymerase II (0.9 unit) and TFIID (0.3 unit; Bio-Rex step fraction) and either the P-11 0.5 M step fraction (5 μl, 12 μg of protein) as the source of TFIIB and TFIIE/F activities (lanes 2 and 4) or more purified TFIIB (single-stranded DNA–agarose fraction, 1 unit) and TFIIE/F (Bio-Gel A-1.5m fraction, 0.7 unit) activities (lanes 1 and 3). The ML template in these reactions was either uncut pMLC$_2$ATΔ-50 (390-bp G-free region; lanes 1 and 2) or uncut pMLC$_2$ATΔ-71 (271-bp G-free region; lanes 3 and 4). The resulting transcripts were resolved by gel electrophoresis along with an end-labeled *Hpa* II digest of pBR322 as markers (lane M). The lengths of the marker fragments [in nucleotides (nt)] are indicated at the right.

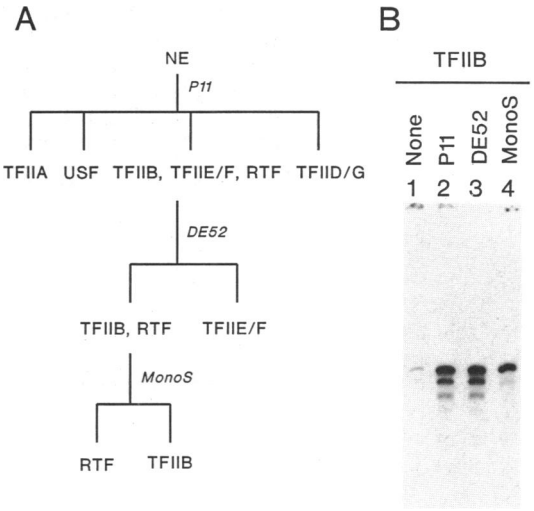

FIG. 3. Separation of RTF from the general transcription factors. (*A*) Diagram of the chromatographic steps necessary to separate the reinitiation factor (RTF) from the other transcription factors required for ML transcription. (*B*) *In vitro* transcription reconstituted with TFIIB at different stages of purification. Each reaction contained a uniform amount of pMLC$_2$ATΔ-50 template and isolated TFIID, TFIIE/F, and RNA polymerase II. TFIIB activity (see purification scheme in *A*) was added as follows: no TFIIB (lane 1), P-11 0.5 M fraction (lane 2), DE-52 fraction (lane 3), or Mono S gradient fraction (lane 4). NE, nuclear extract.

intensity was often observed near the position of second-round transcripts (Fig. 4 *Left*, lanes 2 and 4). This may simply reflect a small contamination of RTF activity in our other fractions. However, transcripts of this particular size can also be due to the existence of a weak TATA element within the G-free cassette (23). A third possibility would be that these transcripts result from a small amount of reinitiation taking place by a mechanism independent of RTF.

In these experiments, TFIIA was not required, but stimulated both first-round transcription and reinitiation (Fig. 4 *Left*, lanes 1 and 2). No RTF activity could be detected in our TFIIA fractions (Fig. 4 *Left*, lane 2). Thus, RTF is clearly distinct from all previously described class II transcription initiation factors.

Interestingly, reinitiation in the presence of RTF was supported not only by the native human TFIID isolated from HeLa cells but also by bacterially expressed cloned human TFIID (Fig. 4 *Right*). This is in sharp contrast to the gene-specific activity of upstream stimulatory factors, which cannot be mediated by the cloned TATA-box binding factor (24–28).

RTF Is Not Gene-Specific. We tested a variety of human class II promoters, each cloned upstream of a similar-size G-free cassette, for reinitiation in the presence and absence of semipurified RTF. All of these promoters were somewhat weaker in the *in vitro* reconstituted system than the adenovirus ML promoter. Otherwise, their behavior in the *in vitro* reconstituted system was identical, in that the RTF-containing fraction was always absolutely required for reinitiation to take place (Fig. 5). Obviously, since the RTF activity used in this experiment is only partially purified, this result cannot be considered definite proof that the same reinitiation factor is utilized by all of these promoters. However, it strongly suggests that many, if not all, genes require a separate reinitiation activity to be transcribed multiple times and that this activity is provided either by a single protein or by a family of related proteins that were copurified in our purification scheme.

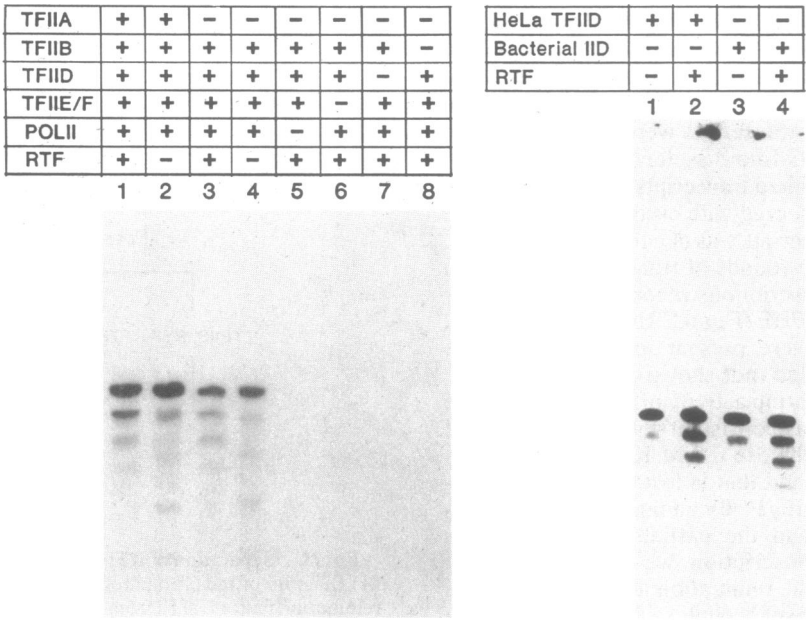

FIG. 4. Factors required for transcription reinitiations. (*Left*) Transcription of the ML gene ($pMLC_2AT\Delta$-50) in reconstituted reactions. The complete reaction (lane 3) contained human RNA polymerase II (polII) (1.2 unit), TFIIB (0.9 unit, Mono S gradient), TFIID (0.5 unit, DE-52 gradient), TFIIE/F (0.9 unit, Bio-Gel A-1.5m), and RTF (1 μl of Mono S gradient fraction). In reactions 1 and 2, TFIIA (0.25 μl; hydroxylapatite fraction) was added. In each of the other reactions, one component was omitted as indicated. (*Right*) Transcription reinitiation with cloned TFIID. Transcription reactions were reconstituted as in *A*, except that the source of TFIID activity was either 0.15 unit of native human TFIID purified from HeLa cells (lanes 1 and 2) or 0.11 unit of the cloned 38-kDa TATA factor purified from bacteria (lanes 3 and 4). RTF addition is indicated.

DISCUSSION

We have identified and separated from the required general transcription initiation factors a component of HeLa cell nuclear extract that confers on the RNA polymerase II transcription apparatus the ability to initiate transcription *in vitro* several times from a single template. The existence of such a separate activity has very important consequences for the overall control of gene activity in eukaryotes. Indeed, once a gene is activated, the simplest way its expression can be altered is by controlling the frequency at which it is transcribed. Therefore, the activity of RTF at various promoters may be a key component distinguishing between highly expressed genes and genes that are expressed at a very low level.

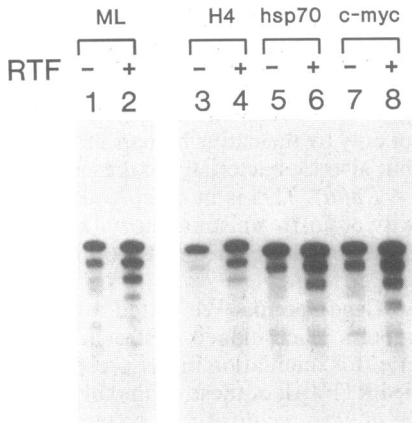

FIG. 5. RTF allows reinitiations at various class II promoters. Transcription reactions were carried out with purified general transcription factors (0.6 unit of TFIIB from mono S gradient; 0.07 unit of TFIID from DE-52 gradient; 0.6 unit of TFIIE/F from Bio-Gel A-1.5m; 0.9 unit of human RNA polymerase II) in the presence or absence of isolated RTF (1 μl of Mono S gradient fraction) as indicated. Each template contained a similar-size G-free transcription cassette inserted behind various portions of the following gene promoters: adenovirus ML (−50 to +10 region), human histone H4 (−650 to +5 region), human heat shock 70-kDa protein (hsp70) (−84 to +5 region), and human c-*myc* P_2 promoter (−44 to +4 region). The autoradiographic exposure time for lanes 3–8 was 2.5 times longer than that for lanes 1 and 2.

In studies carried out with crude or partially fractionated nuclear extracts, the detergent sarkosyl has been shown to inhibit specific steps in the RNA polymerase II transcription initiation pathway (9, 11, 29). The evidence from these investigations strongly suggests that sarkosyl can specifically block reinitiation. However, the concentrations of sarkosyl effective in inhibiting particular reaction steps varied with the purified state of the transcription factors (9), indicating that a sarkosyl-based assay to screen various protein fractions for reinitiation activity would be difficult. In following the RTF activity, we used instead a direct assay for reinitiation in which transcripts from different rounds of transcription were separated on a gel. One limitation of this assay is that it must be carried out in GTP-free solutions. Therefore, we were unable to determine to what extent reinitiation contributes to the overall transcriptional activity observed in nuclear extracts.

Our experiments were not designed to address questions relating to the mechanism of action of RTF, but some inferences that bear on this topic can still be made. First, since reinitiation does not occur without RTF, even with transcription initiation factors present in large excess, the evidence does not support a model in which RTF would restore activity to a factor released from the transcription complex after a first round of initiation. Nor does it seem that RTF stabilizes the preinitiation complex, since reinitiation is observed even though the number of first-round transcripts does not increase. On the other hand, we know from previous footprinting experiments that TFIID, and perhaps other transcription factors, remain at the promoter after a single round of transcription (8, 10). Experiments with sarkosyl in HeLa nuclear extracts also suggest that a complex remains at

the promoter after transcription initiation (9). In the absence of RTF, this postinitiation complex seems unable to function in subsequent initiations *in vitro*. Therefore, the simplest model for RTF action is that it somehow alters the postinitiation complex to permit another round of transcription. The 38-kDa TATA-binding core region of human TFIID, which was recently cloned (24, 27, 30), could be a candidate for this putative alteration by RTF, since we have found that it is as active in reinitiation as the native human TFIID. In contrast to its activity in reinitiation, the cloned human TFIID does not support stimulation by gene-specific transcription factors such as USF and Sp1 (24, 27, 28). Thus, RTF seems to control a function that is distinct from activation by upstream elements and probably provides an additional means to regulate gene expression.

It is well known from quantitation of *in vitro* class II transcription that most templates are not transcribed (9, 14). In the experiments reported here, even if all of the transcripts were from a single round of transcription, fewer than 1 in 20 templates generated a full-length transcript. Clearly, when reinitiation occurs, the ratio of second-round to first-round transcripts is greater than 1/20. Therefore, in the presence of RTF, productive initiation seems to favor previously transcribed templates. However, this may or may not be mechanistically significant, because we are unsure of the state of the untranscribed templates in our *in vitro* system. (For example, many promoters may be unavailable for TFIID binding because they are blocked by nonspecific DNA-binding proteins.)

In most of our assays with RTF, we see a pattern of transcripts indicating that three to four rounds of transcription have taken place. In previous reports, more than four rounds have been noted; however these transcriptions were carried out at the adenovirus ML wild-type promoter in the presence of the upstream factor USF (12). This may imply that USF stimulates both first-round transcription and reinitiation, as reported earlier (13). Therefore, interplay between the activities of the various upstream factors and the reinitiation factor RTF should be investigated.

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