The EMBO Journal vol.12 no.12 pp.4677–4684, 1993

Synthesis of reinitiated transcripts by mammalian RNA polymerase II is controlled by elongation factor SII

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Communicated by M.Yaniv

Previous studies have revealed that the *in vitro* synthesis of reinitiated transcripts by RNA polymerase II requires an additional activity, designated reinitiation transcription factor (RTF), which is distinct from all of the general class II initiation factors. While further characterizing this activity, it was found that RTF displays properties indistinguishable from those of the RNA polymerase II elongation factor SII. In addition, Western blot analysis using SII-specific antibodies revealed that human SII is a major component in purified RTF preparations. The functional equivalence of the two proteins was established using recombinant SII, which proved fully capable of substituting for RTF in the reinitiation assay. In these reconstituted reactions, transcription complexes resulting from reinitiation events required SII to proceed through a 400 bp G-free cassette, while complexes resulting from the first round of initiations were SII-independent. Reinitiations can take place in the absence of SII; however, addition of the elongation factor is essential for full extension of the reinitiated transcripts. These results suggest that events taking place at the promoter (e.g. first-round initiations versus reinitiations) can create marked differences in the properties of RNA polymerase II elongation complexes.

Key words: RNA polymerase II/transcription elongation/transcription factor SII/transcription reinitiation

Introduction

Variations in eukaryotic gene expression during differentiation and development frequently rely on the regulation of transcription by RNA polymerase II (RNAPII). Soluble extracts and reconstituted systems have been developed to allow *in vitro* analysis of general, as well as regulatory, mechanisms in transcription initiation by RNAPII (Sawadogo and Sentenac, 1990). While much progress has been made in recent years on the purification and cloning of general factors involved in class II gene transcription (Conaway and Conaway, 1991), the mechanisms that modulate transcription initiation rates at various class II promoters remain poorly understood.

One of the features that differentiate eukaryotic transcriptional mechanisms from prokaryotic mechanisms is that post-initiation complexes can remain at the promoter after a first round of transcription initiation has occurred (Hawley and Roeder, 1987; Van Dyke *et al.*, 1988; Van Dyke and Sawadogo, 1990). Thus the mechanism leading to subsequent initiation events (reinitiations) differs fundamentally from the initial gene activation. To determine whether reinitiation is a target for regulation, the conditions that allow efficient reinitiations by RNAPII *in vitro* at the well-studied adenovirus major late (ML) promoter were investigated. First, an assay was developed based on the utilization of G-free cassette templates (Sawadogo and Roeder, 1985a), to directly distinguish first-round transcripts from reinitiated transcripts *in vitro* (Szentirmay and Sawadogo, 1991). This assay relies on the stability of RNAPII elongation complexes when artificially arrested by nucleotide deprivation. In the absence of GTP, a first round of transcription initiation on a template containing a G-free cassette yields transcripts that are identical in length to the cassette. However, when reinitiations occur, the resulting transcripts become progressively shorter because the successive polymerases stop elongating when they are blocked by the previous ones. Each round of initiation is therefore characterized by transcripts which are shorter than the previous ones by 30 nucleotides, the length of DNA covered by an elongating RNAPII (Sawadogo and Roeder, 1985b). Using this assay, significant reinitiation events (three or four rounds per template) were found in rather crude reconstituted systems. In contrast, reactions carried out with more purified initiation factors yielded only transcripts corresponding to a single round of initiations. This led to the discovery of an activity, designated reinitiation transcription factor (RTF), that was necessary for the production *in vitro* of reinitiated transcripts, but was clearly distinct from all of the initiation factors required for single-round transcription. The chromatographic fraction containing RTF allowed reinitiations at various promoters, suggesting that RTF was a general transcription factor (Szentirmay and Sawadogo, 1991).

In this study it is shown that RTF can be replaced by a known protein, the eukaryotic RNAPII stimulatory factor SII. SII and closely related proteins have been purified from a number of organisms (Sekimizu *et al.*, 1979; Sawadogo *et al.*, 1980; Reinberg and Roeder, 1987; Rappaport *et al.*, 1987; Sluder *et al.*, 1989), and clones have been isolated from human (Yoo *et al.*, 1991; Chen *et al.*, 1992), mouse (Hirashima *et al.*, 1988; Kanai *et al.*, 1991), *Drosophila* (Marshall *et al.*, 1990), yeast (Hubert *et al.*, 1983; Clark *et al.*, 1991; Nakanishi *et al.*, 1992) and vaccinia virus (Ahn *et al.*, 1990). In all cases, these proteins were originally identified because they stimulate non-specific transcription of double-stranded DNA templates by RNAPII (Seifart, 1970; Stein and Hausen, 1970; Sekimizu *et al.*, 1979; Sawadogo *et al.*, 1980). SII interacts directly with RNAPII (Sawadogo *et al.*, 1980; Rappaport *et al.*, 1988; Sluder *et al.*, 1989), allowing transcriptional readthrough at specific pause sites (Reinberg and Roeder, 1987; Reines *et al.*, 1989; Sluder *et al.*, 1989; SivaRaman *et al.*, 1990). Recently, it was found that SII allows 3′ to 5′ cleavage of the nascent RNA transcripts by RNAPII (Izban and Luse, 1992; Reines, 1992; Reines *et al.*, 1992; Reines and Mote, 1993; Wang

and Hawley, 1993; reviewed in Kassavetis and Geiduschek, 1993). It is shown here that this particular activity of SII does not interfere with the reinitiation assay. Experiments demonstrating that reinitiation can occur *in vitro* independently of SII will also be described, although efficient elongation of the reinitiated transcripts requires SII. These results suggest that the elongation complexes produced by the first round of transcription initiation differ from those created by reinitiation.

Results

RTF co-purifies with transcription factor SII

Our initial studies showed that the RTF activity, which was necessary for the production of reinitiated transcripts *in vitro*, could be chromatographically separated from all of the general class II transcription initiation factors (Szentirmay and Sawadogo, 1991). Therefore, transcription reactions, composed of semi-purified transcription factors TFIIB, TFIID, TFIIE/F/H and RNAPII, were used as a convenient complementation assay for the purification of RTF (see Figure 1B). Upon further investigation of the chromatographic behavior of RTF, it was noticed that all RTF-containing fractions stimulated non-specific transcription of native DNA templates by RNAPII, suggesting that they also contained significant concentrations of the elongation factor SII. To determine whether this was simply coincidental, or whether SII and RTF really displayed identical chromatographic behaviors, both activities were monitored during several successive chromatographic steps, as outlined in Figure 1A. This experiment revealed that non-specific transcription stimulation (SII) and transcription reinitiation (RTF) behaved identically on both cation (DEAE−cellulose) and anion exchange (phosphocellulose, Mono S) resins (Figure 1). Additional chromatography on a CM-cellulose column also failed to separate RTF and SII (not shown). Other biochemical studies provided further indications that a single protein could be responsible for the two activities. Both RTF and SII were relatively heat stable, remaining unaffected by a 7 min heat treatment at 80°C. In addition, the size of RTF estimated by gel filtration (30−35 kDa) corresponded precisely to that of human SII (34 kDa; Yoo *et al.*, 1991) (results not shown).

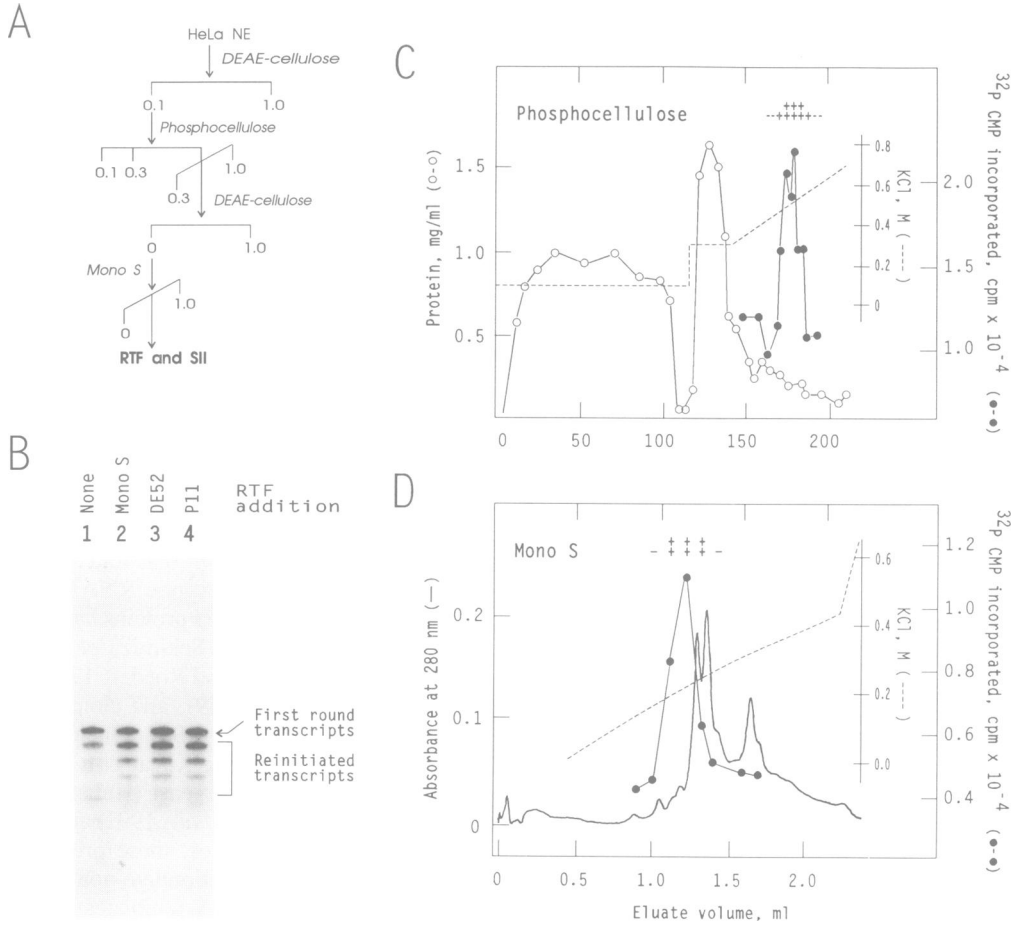

Fig. 1. Co-purification of RTF and SII. (**A**) Schematic representation of the chromatographic scheme used to purify RTF from HeLa nuclear extracts. The numbers given are KCl molarities. (**B**) The reinitiation activity of RTF was analyzed using the G-free cassette assay and $pMLC_2AT19\Delta$-50 as template (Szentirmay and Sawadogo, 1991). All reactions contained human RNAP II (2.2 U), HeLa TFIIB (Mono S fraction, 0.6 U), TFIID (DE52 fraction, 0.25 U) and TFIIE/F/H (Biogel A1.5m fraction, 0.6 U), either alone (lane 1) or in combination with RTF at various stages of purification as indicated (lanes 2−4). The migration of the full-length transcripts resulting from the first round of transcription initiation, and that of the decreasing size transcripts resulting from reinitiation, is indicated on the right. (**C**,**D**) Coelution of RTF and SII activities during chromatography on phosphocellulose and Mono S columns, respectively. Plotted are protein concentrations (○) or absorbance at 280 nm (−) and KCl concentrations (---). SII activity (●) was determined by stimulation of RNAPII in a non-specific transcription assay (see Materials and methods). Fractions which displayed RTF activity in the G-free cassette assay are indicated on the top by (+) or (++) signs; (−) indicates fractions with little or no RTF. The extent of reinitiation activity was determined as described in Materials and methods.

Identity of RTF and SII

To establish the exact relationship between RTF and SII, the mouse SII was expressed in *Escherichia coli* as a fusion protein with a stretch of six histidines at its N-terminus (Van Dyke *et al.*, 1992). This recombinant SII was purified to homogeneity using immobilized metal ion chromatography followed by an additional purification on a Mono S column. In denaturing gel electrophoresis, the purified SII–polyhistidine fusion protein migrated as a single band with an apparent molecular weight of 40 000 (Figure 2A, lane 2). This protein was used to elicit the production of SII-specific antibodies in rabbit. Western blot analysis revealed that the immune serum reacted highly specifically not only with mouse SII, but also with a 38 kDa polypeptide from HeLa cells, corresponding in all likelihood to human SII (Figure 2B, lane 1). This 38 kDa polypeptide co-purified with RTF and was the major component of the purified RTF fraction, as revealed by SDS–PAGE and Western blot analysis (Figure 2). These experiments demonstrated that by purifying RTF, human SII was isolated in a highly enriched form.

Functional assays revealed that pure recombinant SII displayed the activity of both SII and RTF. Like natural SII, it stimulated non-specific transcription of double-stranded DNA by RNAPII (Figure 3B). Like RTF, it promoted the appearance of reinitiated transcripts in a complementation assay carried out at the ML promoter with the general transcription initiation factors and human RNAPII (Figure 4). These experiments provided a final confirmation of the fact that RTF is identical to SII.

The 180 C-terminal amino acids of human SII (Agarwal *et al.*, 1991), as well as a 21 kDa C-terminal proteolytic fragment of mouse SII (Horikoshi *et al.*, 1985), have been shown to suffice for interaction with RNAPII and stimulation of non-specific transcription. Accordingly, it was found that a construct (SIIΔN), in which residues 2–125 of recombinant SII were deleted, was also fully functional in non-specific transcription stimulation (Figure 3). This truncated protein also proved active in the reinitiation assay (Figure 4), indicating that the two activities of SII might share a common mechanism.

SII-dependent transcript cleavage by RNAPII does not interfere with the reinitiation assay

Recently, an SII-dependent 3′–5′ RNA cleavage by RNAPII has been discovered (Reines, 1992). After cleavage, the transcription complex attached to the new 3′-end of the RNA remains elongation competent. This activity is most easily seen *in vitro* when nucleotides are removed from the transcription reaction mixture (Reines, 1992; Izban and Luse, 1992). Noticeably, our reinitiation assay also relies on the appearance of smaller transcripts and requires the absence of one of the normal substrates (GTP).

Having demonstrated a crucial role of SII in the production of these reinitiated transcripts, we needed to verify that SII-dependent transcript cleavage did not interfere with the reinitiation assay. Indeed, it could be argued that the absence of GTP in the reinitiation assay induces SII-dependent RNA cleavage, yielding the observed pattern of decreasing length transcripts. Note that several observations seemed to

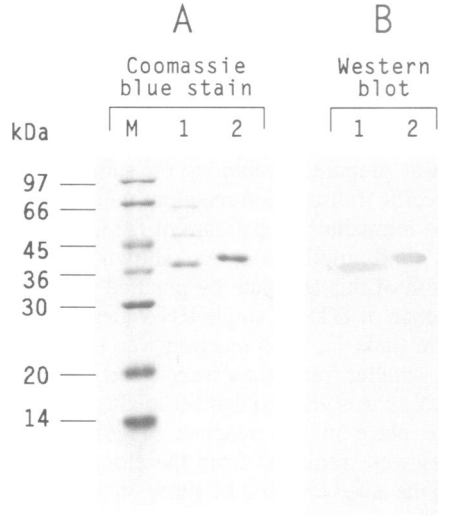

Fig. 2. Presence of human SII in the purified RTF preparation. (**A**) The polypeptide composition of purified RTF (Mono S fraction, 6 μl) was analyzed by SDS–PAGE (lane 1), along with a sample of purified recombinant mouse SII expressed as a fusion protein with a stretch of six histidines at its N-terminus (lane 2, 0.8 μg of protein) and protein molecular weight markers of the sizes shown (lane M). (**B**) Similar lanes were transferred to nitrocellulose and probed with immune serum against recombinant SII. Antibody reaction was observed with the prominent 38 kDa polypeptide present in purified RTF (lane 1), as well as with the recombinant 40 kDa SII fusion protein (lane 2).

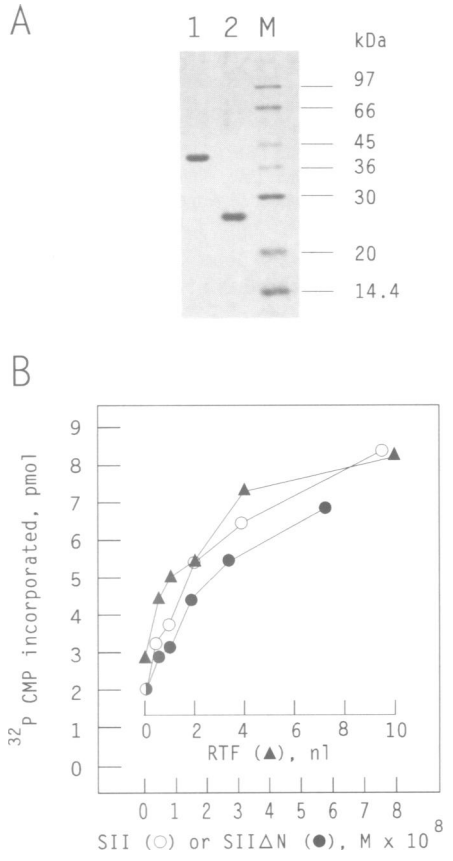

Fig. 3. Expression and activity of recombinant SII. SII clones (amino acids 1–301, 'SII', or 126–301, 'SIIΔN') were expressed in bacteria as polyhistidine fusion proteins and purified to homogeneity as described in Materials and methods. (**A**) SDS–PAGE analysis of purified recombinant SII (lane 1, 0.8 μg protein) and SIIΔN (lane 2, 1.0 μg protein). Lane M contained protein molecular weight markers of the sizes shown. (**B**) Stimulation of RNAPII non-specific transcription by RTF (Mono S fraction, ▲) and purified recombinant SII (○) or SIIΔN (●).

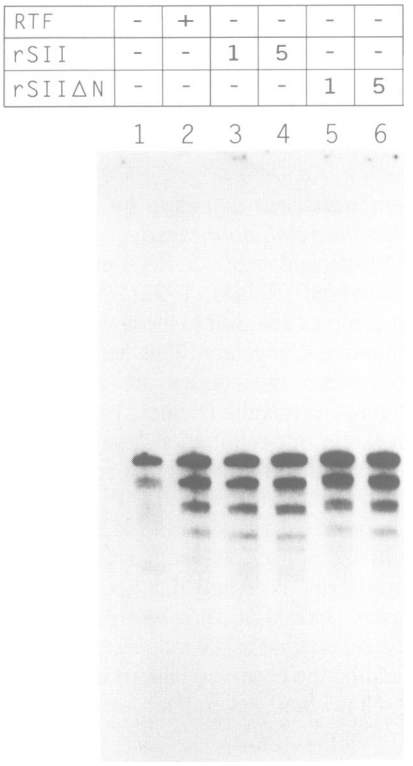

Fig. 4. Recombinant SII can substitute for RTF in the reinitiation assay. Reconstituted transcription reactions contained HeLa RNAPII (0.9 U), TFIIB (Mono S fraction, 0.5 U), TFIID (DE52 fraction, 0.25 U) and TFIIE/F/H (Biogel A-1.5 fraction, 0.5 U). RTF (1 μl, Mono S fraction), recombinant SII (rSII) or SIIΔN (rSIIΔN) were added as indicated above each lane. Numbers represent ng of purified protein added, which corresponded respectively to ~0.4 and 2 pmol of each protein.

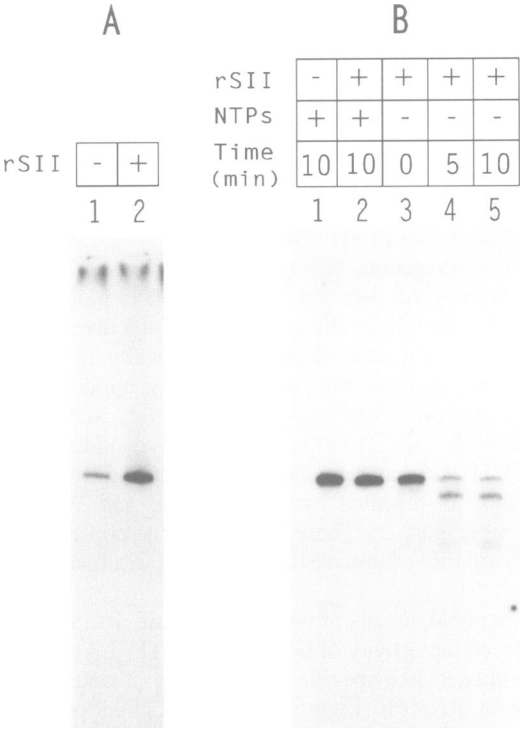

Fig. 5. Transcription of tailed G-free cassette templates by purified RNAPII. Selective initiation by RNAPII at the 5′-end of the G-free cassette was driven by addition of a polythymidine tail to the template (Kadesch and Chamberlin, 1982) as described in Materials and methods. (**A**) Effect of SII on the transcription of tailed templates by RNAPII. The reactions (25 μl) contained 384 ng of tailed template and 110 non-specific units of RNAPII alone (lane 1) or with 80 ng of recombinant SII (rSII, lane 2). (**B**) SII-dependent transcript cleavage with tailed templates. RNAPII elongation complexes present on tailed templates were separated from the nucleotides by gel filtration. These complexes were then supplemented with recombinant SII and/or nucleotides (600 μM ATP and UTP, 25 μM CTP) for the period of time indicated above each lane to allow the 3′−5′ transcript cleavage reaction to take place.

contradict such an interpretation. First, identical patterns of decreasing length transcripts, each shorter than the previous one by 30 nucleotides, had been observed at G-free cassettes of different lengths and with different 3′ DNA sequences (Szentirmay and Sawadogo, 1991). Also, as long as GTP was absent, this pattern seemed independent of the relative concentrations of the other nucleotides and remained unchanged even in the presence of 3′-*o*-methyl GTP (not shown). By contrast, transcript cleavage by RNAPII varies with the RNA sequence and the concentration of nucleotides present (Izban and Luse, 1992). More significant, a decrease in the amount of full-length transcripts was never observed upon SII addition, as compared with identical reactions lacking SII (see for instance Figures 1B and 4). Yet such a decrease would be expected if the smaller RNAs were produced by cleavage of these full-length transcripts.

Nevertheless, an experiment was designed that would directly address the possible interference of RNA cleavage in the reinitiation assay. The results of this experiment are shown in Figure 5. It is well established that (i) purified SII can assist readthrough by RNAPII in the absence of other accessory factors (SivaRaman *et al.*, 1990) and (ii) RNA cleavage is an intermediate in this reaction (Reines *et al.*, 1992). Therefore, if the shorter transcripts observed when G-free cassette templates were transcribed in the presence of SII resulted from transcript cleavage, this reaction would also be expected to take place under non-specific transcription conditions. Such a reaction was analyzed using tailed templates (Kadesch and Chamberlin, 1982). A template was prepared containing the same G-free cassette used in specific transcription reactions with a polythymidine tail located immediately upstream of the G-free region (see Materials and methods). As shown in Figure 5A, transcription of this template by purified RNAPII yielded, in the absence of GTP, a single RNA identical in length to the cassette (lane 1). This reaction was stimulated by SII. However, smaller transcripts were clearly absent (lane 2). As a control, it was verified that SII-mediated RNA cleavage could take place in this reaction (Figure 5B). For this, nucleotides were removed from the elongation complexes present on the tailed template by filtration through a desalting column. These complexes were then further incubated to allow 3′−5′ RNA cleavage. With SII present, transcript cleavage occurred readily (Figure 5B, lanes 4 and 5), but the reaction was totally inhibited when ATP, CTP and UTP were added at the same concentrations used in specific transcription reactions (lane 2). From these results, it was concluded that these nucleotide concentrations should also prevent SII-dependent cleavage of G-free transcripts by RNAPII in the reinitiation assay. Therefore, as originally postulated, the pattern and abundance of the shorter

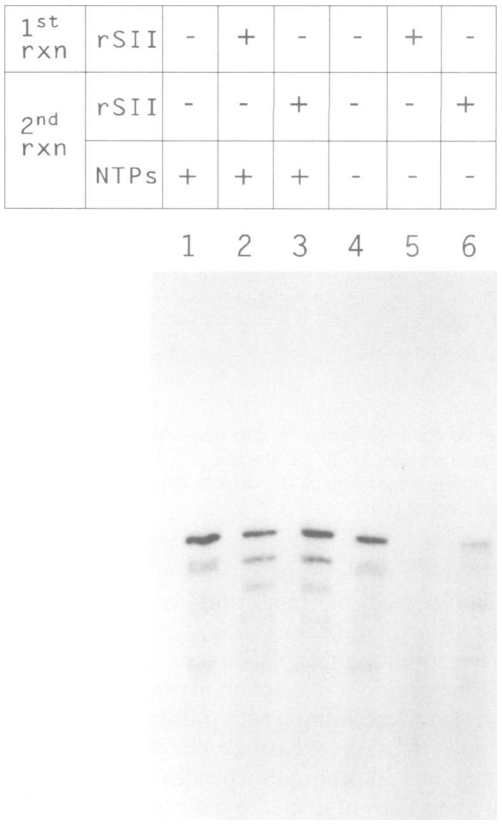

Fig. 6. SII allows extension of the reinitiated transcripts. Specific transcription reactions were carried out with human RNAPII (0.9 U), TFIIE/F/H (Biogel A-1.5 fraction; 0.5 U), recombinant TFIIB (0.15 U), recombinant TBP (0.15 U) and, when indicated, recombinant SII (15 ng). The resulting elongation complexes were precipitated, washed to remove nucleotides and resuspended in transcription buffer. Each reaction was then supplemented with recombinant SII and/or unlabeled nucleotides (600 μM ATP and UTP, 25 μM CTP), as indicated above each lane, and incubated for an additional 40 min at 30°C to allow for either extension or 3′−5′ cleavage of the transcripts.

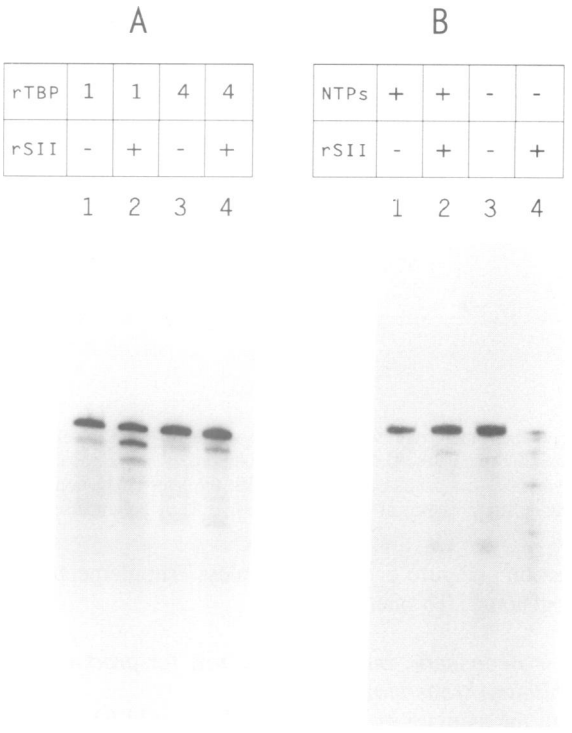

Fig. 7. Excess TBP inhibits reinitiation but not SII-dependent transcript cleavage. (**A**) High TBP concentrations inhibit reinitiation. Transcription reactions were carried out with human RNAPII (0.9 U), TFIIE/F/H (0.5 U), recombinant TFIIB (1.1 U) and either 0.06 U (lanes 1−2) or 0.25 U (lanes 3−4) of recombinant TBP. Addition of recombinant SII (15 ng) was as indicated. (**B**) High TBP concentrations do not inhibit SII-dependent transcript cleavage. Transcription reactions were carried out as in (A), with 1.5 U of TBP in the presence or absence of SII as indicated above each lane. The resulting elongation complexes were then precipitated, washed, resuspended in transcription buffer supplemented (lanes 1 and 2) or not (lanes 3 and 4) with nucleotides (600 μM ATP and UTP, 25 μM CTP) and incubated for an additional 40 min at 30°C to allow the transcript cleavage reaction to take place.

transcripts observed in specific transcription reactions must reflect the level of productive reinitiation events.

SII is required for elongation of the reinitiated transcripts

Given the differential SII requirement for production of reinitiated versus first-round transcripts (Figure 4), we next investigated whether it was the initiation or the elongation of the reinitiated transcripts that required SII. Because SII usually acts at the elongation step of transcription by RNAPII, the possibility that SII was simply allowing elongation of reinitiated transcripts that were otherwise not easily detected, because they were short and heterogeneous, was considered first. Careful examination of transcription gels carried out in the absence of SII often revealed a pair of transcripts of variable intensity near the position of the second-round transcripts, as well as several weaker, smaller transcripts. Because most of these transcripts disappeared upon addition of the elongation factor, it seemed possible that they were reinitiated transcripts that were incompletely elongated in the absence of SII.

To test this hypothesis, an initial transcription reaction was performed in the absence of SII. The resulting elongation complexes were precipitated and washed to remove all of the labeled nucleotides. These complexes were then incubated in the presence of SII and cold nucleotides to allow extension of any SII-sensitive paused complexes. As shown in Figure 6, delayed addition of SII resulted in the appearance of a pattern of reinitiated transcripts similar to that obtained when SII was present from the beginning of the experiment (compare lanes 2 and 3). This indicated that reinitiations had indeed occurred in the absence of SII and that most elongation complexes resulting from these reinitiations had entered an SII-dependent pausing mode. Furthermore, SII addition had no effect on the intensity of the first-round transcripts (compare lanes 1 and 3), yet incubation of the elongation complexes in the absence of nucleotides resulted in SII-dependent cleavage of both first-round and reinitiated transcripts (lanes 5 and 6). From these experiments, it was concluded that SII was required *in vitro* to allow elongation of the reinitiated transcripts. In contrast, addition of SII was not required for the first-round transcription complexes to proceed through the entire G-free cassette, even though these complexes were apparently devoid of endogenous SII or SII-like activity, as revealed by their inability to carry out the 3′−5′ transcript cleavage

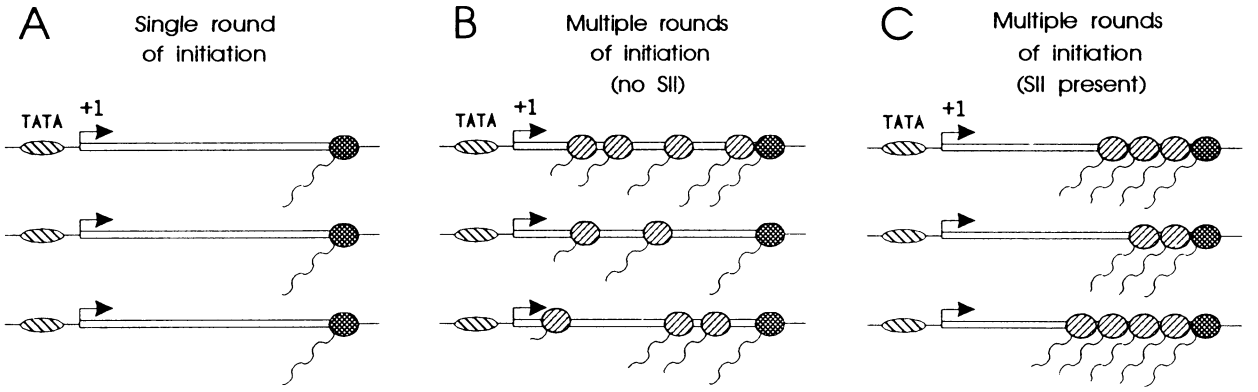

Fig. 8. Schematic representation of the variety of RNAPII elongation complexes that can be observed during *in vitro* transcription of G-free cassette-containing templates. Under certain conditions, a single round of transcription initiation is observed (**A**). In the absence of SII, multiple RNA polymerases can initiate transcription, but the later elongation complexes tend to stall at undetermined sites in the cassette (**B**). When reinitiations occur in the presence of SII, the successive RNA polymerases stack up at the end of the cassette (**C**).

reaction (Figure 6, lane 4) unless supplemented with exogenous SII (lane 6).

SII is necessary, but not sufficient, for production of reinitiated transcripts in vitro

With the availability of cloned SII, it could be ascertained easily that the concentration of this particular factor was not limiting in our reconstituted reactions. Despite this, the efficiency of reinitiation was clearly not improved by using the recombinant protein rather than the semi-purified RTF fractions (see Figure 4). This could be because SII activity was not limiting in the RTF fractions. Alternatively, factors other than SII could be rate-limiting for the reinitiation reaction. In support of this latter possibility, it was noticed that reinitiation levels seem to vary when the reactions were reconstituted with various preparations of the complementation fractions (not shown). The relative concentration of the various initiation factors also played an important role. It has been reported previously that native human TFIID and bacterially-expressed TATA binding protein (TBP) were equally capable of supporting reinitiations (Szentirmay and Sawadogo, 1991). However, it was found that the TBP concentration influenced considerably the ratio between first-round and reinitiated transcripts. Reinitiation was almost totally suppressed by high levels of TBP (Figure 7A). This suppression was not caused by an indirect effect of TBP on the activity of SII, because SII-dependent RNA cleavage, assayed by incubation of washed elongation complexes in the absence of nucleotides, was independent of the TBP concentration (Figure 7B). In contrast, high concentrations of recombinant TFIIB did not alter significantly the efficiency of reinitiations (result not shown). From these experiments, it was concluded that, while the presence of SII was necessary to ensure productive reinitiations, reinitiation levels were determined independently of SII.

Discussion

In attempting to purify an activity that controlled transcription reinitiation by RNA polymerase II *in vitro*, it was discovered that reinitiated transcripts appeared only in the presence of the transcription elongation factor SII. SII is known to possess a 'readthrough' activity that allows RNAPII arrested at certain pause sites to resume elongation (Reinberg and Roeder, 1987; Reines *et al.*, 1989, SivaRaman *et al.*, 1990). Reines and co-workers have shown that SII-dependent RNAPII cleavage of transcripts is required for this readthrough activity (Reines *et al.*, 1992).

Because no other unambiguous assay for reinitiation by RNAPII in specific transcription exists, it was essential to verify that the decreasing length transcripts observed upon transcription of G-free cassette templates resulted from reinitiation, and not from SII-dependent transcript cleavage. At least three lines of evidence indicate that this is not the case: (i) the pattern of decreasing-length transcripts was not observed under conditions of non-specific transcription of the G-free cassette; (ii) the addition of SII to transcription reactions performed in the absence of the elongation factor did not decrease the amount of full-length transcripts present (Figure 6), a clear indication that these transcripts were not cleaved by RNAPII to any significant extent at the nucleotide concentrations employed in our experiments; and (iii) excess TBP drastically decreased reinitiations but not SII-dependent RNA cleavage (Figure 7). These results, along with the previously reported control experiments (Szentirmay and Sawadogo, 1991), demonstrate that we are indeed observing reinitiation by means of the G-free cassette assay.

The absence of shorter transcripts in non-specific transcription of the G-free cassette indicates that productive reinitiations did not occur in these tailed template experiments (Figure 5). This is understandable, given that $<5\%$ of the templates were transcribed. However, this also implies that, in reactions with tailed templates, reinitiations are not favored. In contrast, reinitiations can be observed in specific transcription reactions, even though overall transcription levels are also low. In this latter case, it remains to be determined whether reinitiation levels simply vary as a function of the relative concentrations of the known initiation factors, as the experiment with high TBP might suggest (Figure 7). For example, the number of partial or complete preinitiation complexes which assemble during a given reaction could influence the ratio of initiation versus reinitiation events by competing for a limiting component. Alternatively, as yet unidentified proteins may be specifically involved in the reinitiation process and also interact with the general initiation factors. With the G-free cassette reinitiation assay firmly established, such questions can now be addressed.

During specific transcription of the minimal ML promoter, the SII requirement was clearly dependent on the origin of

the transcripts. The number of full-length transcripts resulting from the first round of initiations was unaffected by SII addition, while elongation of the subsequent transcripts required SII. These results are consistent with both the previously ascertained mode of transcription stimulation by SII at the level of elongation and the earlier observation of an SII requirement for maximum expression of the ML promoter *in vitro* in crude nuclear extracts (Sekimizu *et al.*, 1982). Unexpectedly, however, it was found that SII stimulation took place only after the initial round of transcription, and only under some circumstances. Figure 8 schematically illustrates the various patterns of transcripts that can be observed during *in vitro* transcription of G-free cassette templates. Under some specific conditions (e.g. high concentrations of TBP), a single round of initiations may be observed (Figure 8A). When reinitiation occurs in the absence of SII, the resulting elongation complexes have a greater tendency to stall than do first-round transcription complexes (Figure 8B). These stalled complexes require SII to resume elongation and stack up at the end of the G-free cassette (Figure 8C). It is unclear if certain sequences in the cassette are preferred pause sites, although several weak transcripts were often observed in the absence of SII that disappeared upon addition of the elongation factor.

Taken together, our results indicate that there is an intrinsic difference between the elongation complexes derived from the first round of initiation and those resulting from reinitiations. How does this difference arise? Clearly, the first-round elongation complexes are capable of binding SII, because SII-dependent cleavage of the corresponding transcripts could be observed after removal of the nucleotides (Figure 6). Thus, ability to bind SII does not distinguish first- and subsequent-round complexes. Furthermore, because cleavage of the first-round transcripts was not observed in the absence of exogenous SII (Figure 6, lane 4), the lack of an SII requirement for first-round transcription cannot be explained by the presence of endogenous SII, or SII-like activity, in our reconstituted system. One possibility that cannot be ruled out is that topological changes in the once-transcribed template may affect the processivity of the later transcription complexes. However, elongation complexes with different processivities were also seen originating from the HIV-1 promoter, with production of the more processive complexes being stimulated by the Tat protein (Marciniak and Sharp, 1991; Cullen, 1993). It is postulated that the first-round elongation complexes differ from the subsequent ones as a result of events occurring during initiation, perhaps via interactions between RNAPII and factors such as TFIIF or the *Drosophila* factor 5 which are known to participate both in transcription elongation and initiation (Price *et al.*, 1989). Initiation steps which produce these different complexes, as well as the varying elongation complexes themselves, are potentially points of transcriptional control, via SII or other factors.

Note that the qualitative nature of our experimental approach at this stage does not allow us to rule out that SII may also affect reinitiations directly at the initiation level, especially via a strong pause site located very close to the start point of transcription. In this case, an RNAPII stalled in the absence of SII may prevent other polymerases from binding and productively initiating more rounds of transcription. The presence of an RNAPII paused at the 5′-end of the *Drosophila hsp*70 gene is believed to prevent expression of uninduced genes while potentiating the rapid onset of high transcription levels upon heat-shock (Rougvie and Lis, 1988). Similar promoter-proximal pause sites seem to regulate the transcriptional activity of many other genes (Rougvie and Lis, 1990). Likewise, the presence on a particular gene of several engaged, but stalled, RNAPII complexes, as we have observed *in vitro*, could also facilitate rapid induction of gene transcription *in vivo*.

Materials and methods

Purification of transcription factors and RNA polymerase II

Recombinant TBP, HeLa transcription initiation factors TFIIB, TFIIE/F/H and TFIID, as well as human and mouse (MOPC21 tumors) RNAPII were prepared as previously described (Sawadogo and Roeder, 1985a; Van Dyke *et al.*, 1988). Recombinant TFIIB was produced in *E.coli* from an expression vector kindly provided by D.Reinberg (Piscataway, NJ) (Ha *et al.*, 1991) and extensively purified by chromatography on DE52 (Whatman, Hillsboro, OR) and BioRex 70 (Bio-Rad, Hercules, CA).

RTF was purified from HeLa cells following the chromatographic scheme outlined in Figure 1A. Nuclear extract (485 mg of protein) was passed through a 70 ml DEAE−cellulose (DE52; Whatman) column equilibrated with buffer A (20 mM Tris, pH 7.9, at 23°C, 20% glycerol, 0.2 mM EDTA, 0.2 mM PMSF and 10 mM 2-mercaptoethanol) containing 100 mM KCl. The unbound proteins in the DE52 flowthrough (95 mg) were loaded onto an 11 ml phosphocellulose column (P11; Whatman), which was eluted by a step to 0.3 M KCl in buffer A, followed by a gradient from 0.3 to 0.8 M salt in the same buffer. The pooled RTF-containing fractions were dialyzed against buffer A, spun to remove precipitated materials (26 000 g, 20 min, 4°C) and loaded onto a second DE52 column. An aliquot of the unbound fraction from this column was then applied to a 0.1 ml Mono S column (Pharmacia, SMART system) and the RTF activity was eluted by a gradient from 0 to 0.4 M KCl in buffer A. Protein concentrations were determined by the method of Bradford (1976).

Expression and purification of recombinant SII

A cDNA to the mouse elongation factor SII was obtained by reverse transcription of mRNA isolated from S-194 cells, amplified by PCR and inserted into the pV2 expression vector (Van Dyke *et al.*, 1992). This yielded plasmid pV2−SII, in which a stretch of six histidines was fused to the N-terminus of the full-length SII sequence (amino acids 1−301; Sekimizu *et al.*, 1979). A second expression plasmid (pV2−SIIΔN) encoding the polyhistidine region upstream of amino acids 126−301 of SII was derived by deletion of the appropriate restriction fragment from the pV2−SII construct. Extraction of bacterially produced full-length SII and SIIΔN proteins and purification by immobilized metal ion affinity chromatography were carried out essentially as described (Van Dyke *et al.*, 1992). Homogeneous preparations of both proteins were obtained by an additional chromatography on a Mono S column (Pharmacia, Uppsala, Sweden). Proteins were analyzed by SDS−PAGE according to standard protocols (Laemmli, 1970) and stained with Coomassie blue.

Polyclonal antibodies to SII

Polyclonal antibodies to SII were raised using New Zealand White rabbits. Immunization was performed by subcutaneous injection of purified recombinant mouse SII (100 μg) mixed with single-stranded calf thymus DNA (50 μg). A total of two booster injections were given at 3 week intervals. Western blots were incubated with a 100 000-fold dilution of the immune serum and reacted with alkaline phosphatase-conjugated goat antibodies to rabbit IgGs (Promega, Madison, WI) as described (Sawadogo *et al.*, 1988).

In vitro transcription assays

Specific transcription reactions were carried out in the absence of GTP as previously described (Sawadogo and Roeder, 1985a) using the G-free cassette-containing template pML(C_2AT)19Δ-50 (Sawadogo and Roeder, 1985b). Transcription gels were quantitated using a betascanner (Betascope 600, Betagen, Framingham, MA) or a densitometer (Shimadzu, Columbia, MD, C-R1B Chromatopac). From these analyses, the extent of transcription reinitiation in various reactions was estimated by comparing the amount of radioactivity incorporated into third- or fourth-round transcripts with the radioactivity incorporated into the full-length first-round transcripts.

The stimulatory activity of SII was determined in non-specific transcription reactions, which contained, in a 30 μl volume, 1 μg calf thymus DNA (Boehringer, Indianapolis, IN), 70 mM Tris (pH 7.9 at 23°C), 2 mM $MnCl_2$, 1 mM dithiothreitol, 50 mM ammonium sulfate, 400 μM each of ATP, UTP and GTP, 50 μM [α-^{32}P]CTP (1000−2000 c.p.m. per pmol),

and 2–6 non-specific units of human RNAPII (1 U being defined as the amount of enzyme that incorporates 1 pmol of CMP into RNA in 20 min at 37°C with alkali-denatured calf thymus DNA as template). After 20 min incubation at 37°C, the reaction mixtures were spotted onto DEAE paper disks, which were thoroughly washed with 0.5 M Na_2HPO_4 to remove unincorporated nucleotides. Scintillation counting was then used to determine the amount of labeled RNA synthesized.

Transcription of tailed templates

Selectively initiated G-free transcripts were generated at tailed templates (Kadesch and Chamberlin, 1982; Dedrick and Chamberlin, 1985) prepared from the pHOG19 plasmid, which contained a G-free cassette identical to that of pC_2AT19 (Sawadogo and Roeder, 1985a) with an *Eco*RV site located just 5′ of the G-free region. A polythymidine tail was added to the ends of the *Eco*RV cut pHOG19 using calf thymus terminal transferase (Promega) in a reaction mixture containing 100 μM TTP, 100 nM DNA ends, 100 mM sodium cacodylate (pH 7), 2 mM $MnCl_2$, 0.1 mM DTT, 10 μg/ml bovine serum albumin and 0.4 U/μl enzyme. After incubation for 2–5 h at 37°C, the tailed DNA template was purified by two rounds of phenol extraction and ethanol precipitation. The presence of single-stranded tails on the vast majority of the templates was confirmed by comparing the electrophoretic migration of the tailed templates with that of the original linear plasmids on agarose gels. Transcription reactions using tailed pHOG19 templates contained 20 mM Tris pH 7.5, 40 mM HEPES pH 8.4, 8% glycerol, 7.5 mM $MgCl_2$, 66 mM ammonium sulfate, 0.1% Tween 40, 600 μM each ATP and UTP, 25 μM [α-^{32}P]CTP (3000 c.p.m./pmol), 4 mM DTT, 0.32 U/μl RNase inhibitor (Promega) and 4.4 U/μl mouse RNAPII. Incubations were for 1 h at 37°C. These reactions were stopped and processed for electrophoresis as for specific transcription (Dignam *et al.*, 1983).

Isolation of transcription complexes

Specifically initiated transcription complexes were isolated by a sedimentation method similar to that described by Sollner-Webb and co-workers (Culotta *et al.*, 1985). To a 25 μl transcription reaction, 5 μl of 36% polyethylene-glycol-8000 (Sigma, St Louis, MO) was added. After centrifugation for 15 min at 4°C in a microfuge, the supernatant was removed and the pellet was resuspended by vortexing it briefly in 25 μl of transcription buffer (Sawadogo and Roeder, 1985a). After a second precipitation, performed under identical conditions, the complexes were resuspended in transcription buffer in the presence or absence of nucleotides as noted in the figure legends.

Nucleotides were removed from transcription complexes generated on tailed templates by gel filtration on a Biogel P-6DG (Bio-Rad) resin. The reaction mixture (125 μl) was applied to a 0.6 ml column equilibrated in a buffer of the same composition as the transcription reaction buffer but lacking $MgCl_2$. The excluded fractions were pooled and supplemented with $MgCl_2$ and RNase inhibitor to the same levels as in the initial reaction. This mixture was then divided into aliquots, which were incubated at 37°C after addition of nucleotides (600 μM ATP and UTP, 25 μM CTP) and/or recombinant SII as indicated.

Acknowledgements

We are grateful to Qun Lin for the construction of pV2-SIIΔN, Shona Murphy for the pHOG19 plasmid, Danny Reinberg for the TFIIB expression plasmid and Michael Van Dyke for many stimulating discussions. We also thank Sankar Maity and Walter Pagel for critical review of the manuscript. This work was supported in part by a postdoctoral fellowship from the National Cancer Institute Training Grant CA-09299 (M.N.S.) and by Grant GM-38212 from the National Institutes of Health.

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Received on June 26, 1993; revised on August 16, 1993