The EMBO Journal Vol.2 No.2 pp.185–191, 1983

In vitro premature termination in SV40 late transcription

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Communicated by Y. Aloni
Received on 15 November 1982

Nuclear extracts and viral transcribing minichromosomes were prepared from SV40-infected cells and incubated *in vitro* with [α-^{32}P]UTP under conditions which allow the elongation of preinitiated RNA chains. Sucrose gradient analysis of the transcription mixtures revealed two populations of SV40-specific RNA: elongating chains that remain associated with the viral minichromosomes, and, at the top of the gradient, small free RNA detached from the template and hybridizing exclusively to the promoter-proximal region of SV40 DNA. This free RNA was shown by polyacrylamide gel electrophoresis to comprise essentially a 94 nucleotide species, which could, however, at high UTP concentration, be elongated a further few nucleotides before terminating. These results thus show that the actively transcribing minichromosomes provide a sytem in which the attenuated RNA can be released from the template. Moreover, this is the first demonstration of specific *in vitro* termination of polymerase B transcription. The conditions which lead to transcription termination are discussed.

Key words: attenuation/premature termination/SV40 minichromosome/SV40 transcription

Introduction

SV40 DNA is found in infected cells in the form of a minichromosome. It possesses a beaded structure composed of cellular histones and supercoiled viral DNA in a molecular complex which is very similar to that of cellular chromatin (Felsenfeld, 1978; Kornberg, 1977; Chambon, 1977). This has made SV40 an attractive model system in which to study the organization and expression of eucaryotic chromatin. The chromatin structure of the 5′-flanking region of several actively transcribed cellular and viral genes is known to be different from the bulk of chromatin. Such regions are generally hypersensitive to DNase I (Cremisi, 1981; Groudine *et al.*, 1981; Herbormel *et al.*, 1981; Keene *et al.*, 1981; Scott and Wigmore, 1978; Stalder *et al.*, 1980; Varshavsky *et al.*, 1979; Mathis *et al.*, 1980; Weintraub *et al.*, 1981). Consistent with this is the observation that ~25% of the SV40 minichromosomes display, precisely within the sensitive region, a stretch of DNA not contained within a typical nucleosome structure (Jakobovits *et al.*, 1980a; Saragosti *et al.*, 1980). Transcription occurs on the SV40 minichromosomes (Gariglio *et al.*, 1979; Laub *et al.*, 1980), and the late promoters map at the exposed region (Ghosh *et al.*, 1978; Horowitz *et al.*, 1978; Laub *et al.*, 1979; Canaani *et al.*, 1979). The possible role of the exposed region in directing the specificity of transcription initiation of the late genes is suggested by the observation that *Escherichia coli* RNA polymerase and the eucaryotic polymerase B initiate transcription primarily within this region *in vitro* (Jakobovits *et al.*, 1980b; Beard and Nyfler, 1982). It is assumed that the higher order structure of the actively transcribed minichromosome is involved at other levels of regulation during transcription (Mathis *et al.*, 1980; Weintraub, 1980).

Once a gene is turned on it can be advantageous for the cell to keep it in a primed state so that transcription can resume in response to physiological factors and either allow readthrough of an internal facultative termination site to produce a full length transcript, or, on the contrary, premature termination at the facultative site and generation of an abortive transcript. The latter regulation termed 'attenuation' exists in procaryotes (for review, see Adhya and Gottesman, 1978; Biro and Weissman, 1979; Rosenberg and Court, 1979; Crawford and Stauffer, 1980; Gallupi and Richardson, 1980; Gottesman *et al.*, 1980; Yanofsky, 1981). Attenuation might also play a role during transcription in eucaryotic cells and their viruses, opening a possibility of regulation at a post-initiation level (Tamm and Kikuchi, 1979; Evans *et al.*, 1979; Fraser *et al.*, 1979; Laub *et al.*, 1979; Salditt-Georgieff *et al.*, 1980; Testa *et al.*, 1980; Laub *et al.*, 1980; Gariglio *et al.*, 1981; Tweeten and Molloy, 1981). In the case of SV40 RNA polymerase molecules initiating transcription at the major initiation site of the late genes pause *in vivo* at promoter-proximal sites (see accompanying paper). Consequently, a significant fraction of potentially active transcriptional complexes accumulate in the infected cells (Hay *et al.*, 1982; Skolnik-David *et al.*, 1982). Upon *in vitro* incubation of viral transcriptional complexes (VTC) or isolated nuclei transcription resumes but stops again within a stretch of uridylic acid residues that follows a GC-rich region with dyad symmetry, yielding 94 nucleotide RNA (Laub and Aloni, 1976; Hay *et al.*, 1982; Skolnik-David *et al.*, 1982). Based on these observations we have presented a model in which a mechanism resembling attenuation in procaryotes regulates SV40 gene expression (Hay *et al.*, 1982; Aloni and Hay, 1983).

In those reports we discussed the question of whether the production of the attenuated RNA results from a pausing effect or from a termination mechanism. Here we characterize a system of actively transcribing SV40 minichromosomes and describe *in vitro* conditions for premature termination followed by the release of the attenuated RNA. This is the first demonstration of *in vitro* specific termination of polymerase B transcription.

Results

RNA synthesis directed by nuclear extracts from SV40-infected cells is a salt-sensitive reaction

At 48 h post-infection nuclei were prepared from SV40-infected cells and leached in hypotonic buffer to yield transcriptionally active viral complexes (Jakobovits and Aloni, 1980; Laub *et al.*, 1980). Figure 1 illustrates the kinetics of RNA synthesis at 30°C under various salt concentrations. Incubation of a nuclear extract for increasing lengths of time in a reaction mixture for RNA synthesis at moderate salt concentrations (30 mM ammonium sulfate or 100 mM NaCl) led

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to incorporation of labeled nucleotide with linear kinetics for only 5 min. On the other hand, incubating the nuclear extract at high salt concentrations (300 mM ammonium sulfate or 500 mM NaCl) results in continued [^{32}P]UMP incorporation. This effect is more pronounced with ammonium sulfate than with NaCl since incorporation proceeded linearly for as long as 60 min in the presence of the former salt. Addition of 0.2 μg/ml α-amanitin results in up to 80% inhibition of [^{32}P]UMP incorporation. The residual [^{32}P]RNA is not virus specific as determined by hybridization to SV40 DNA immobilized on filters (results not shown). Much of the α-amanitin-resistant NTP incorporating activity detected in the nuclear extracts is due probably to terminal nucleotidyl transferase activities (results not shown). In early experiments, they caused extensive end-labeling of contaminating tRNA and other small nuclear RNA. This difficulty could be largely alleviated by an additional wash of the nuclei before leaching. However, some non-viral small RNA species remained consistently end-labeled upon incubation with [α-^{32}P]UTP. Nevertheless when nuclear extracts were fractionated on sucrose gradients, RNA co-sedimenting with the viral minichromosomes was found to be essentially virus specific.

Promoter-proximal viral RNA species accumulate during moderate and low salt incorporation; long transcripts are synthesized at high salt incorporation

The SV40-specific RNA synthesized at moderate or high salt was characterized by hybridizing [^{32}P]RNA made in nuclear extracts to nitrocellulose filters containing five fragments of SV40 DNA in which the 'late' promoter region (fragment 'e') was designed to be the smallest of the five fragments (Figure 2A). When exposed to homogeneously labeled nick-translated SV40 DNA this fragment has the weakest signal due to its size (Figure 2B).

RNA made in nuclear extracts at moderate salt (100 mM NaCl and 30 mM ammonium sulfate) for a short period of time (5 min) hybridized almost exclusively to the viral 'late' promoter-proximal fragment 'e'. This, as previously shown, indicates the pausing *in vivo* of a large excess of RNA polymerase molecules in a promoter-proximal region and elongation along this stretch *in vitro* (Laub *et al.*, 1980; Hay *et al.*, 1982; Skolnik-David *et al.*, 1982, see accompanying paper). Increasing the time of incubation to 20 min and to 60 min led to a small accumulation of counts into the 'e' fragment with relatively few transcripts reaching the region of the adjacent 'b' fragment at the longest time. This was in agreement with the results obtained from the kinetics of synthesis at moderate salt concentration (see Figure 1).

In contrast, transcription at high salt concentration (500 mM NaCl; 300 mM ammonium sulfate) showed that while the promoter-proximal 'e' fragment was in molar excess at all times of synthesis, but primarily at the shortest time (5 min), there was a clear increase with times of incubation in the ratio of counts complementary to the adjacent 'b' and

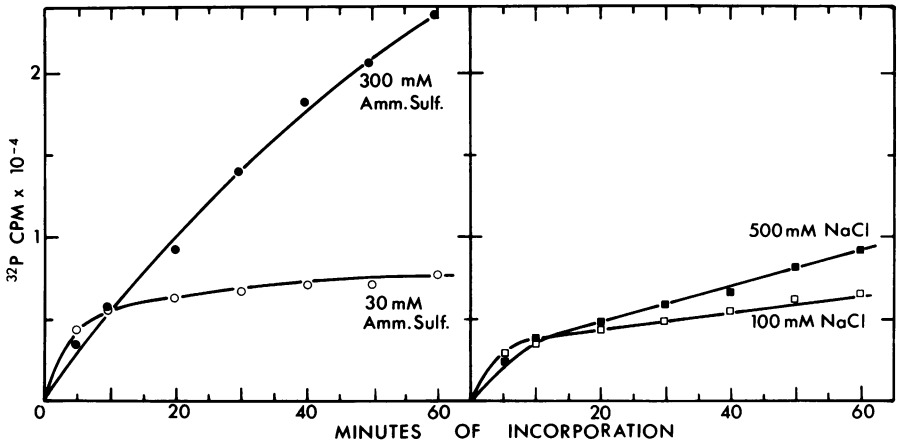

Fig. 1. Kinetics of [^{32}P]UMP incorporation directed by nuclear extracts from SV40-infected cells. Nuclei were prepared by hypotonic lysis of the cells and the nuclear extracts obtained were supplemented with 2 mM $MnCl_2$, [α-^{32}P]UTP (40 μCi/100 μl final volume) 1 mM ATP, GTP and CTP and salt as indicated. Aliquots were removed and spotted on strips of Whatman 3 MM paper and precipitated with trichloroaceteic acid (Skolnik-David *et al.*, 1982).

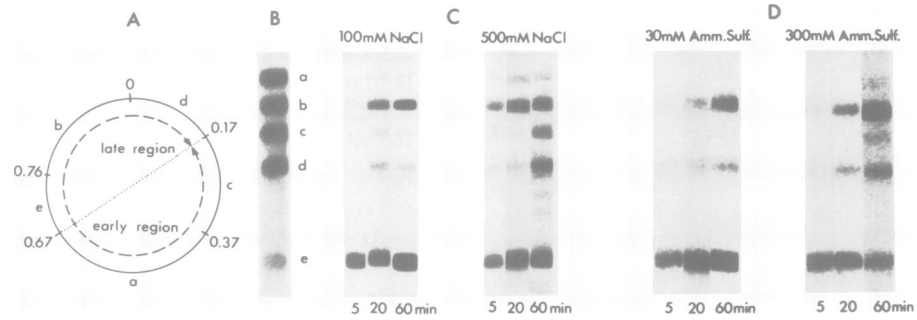

Fig. 2. Hybridization pattern of RNA synthesized by unfractionated nuclear extracts from SV40-infected cells. RNA was purified and hybridized to nitrocellulose filters bearing the five restriction fragments generated by the digestion of SV40 DNA by the restriction enzymes *Eco*RI, *Bgl*I and *Hpa*I (Hay *et al.*, 1982). **(A)** Physical map of SV40 DNA with the cleavage point and denomination of the restriction fragments. **(B)** Hybridization of SV40 DNA uniformly labeled with ^{32}P by nick-translation to the restriction fragments immobilized on nitrocellulose sheet. **(C)** Southern blot analysis of the ^{32}P-labeled RNA synthesized in the presence of moderate and high concentrations of NaCl. **(D)** The same as in **(C)** but for ammonium sulfate.

eventually also 'd' and 'c' fragments. This pattern is more pronounced with ammonium sulfate as compared with sodium chloride. Thus, RNA synthesis at high salt was partially able to continue for the full length of the 'late' region. This was also in agreement with the results obtained from the kinetics of synthesis at high salt (see Figure 1).

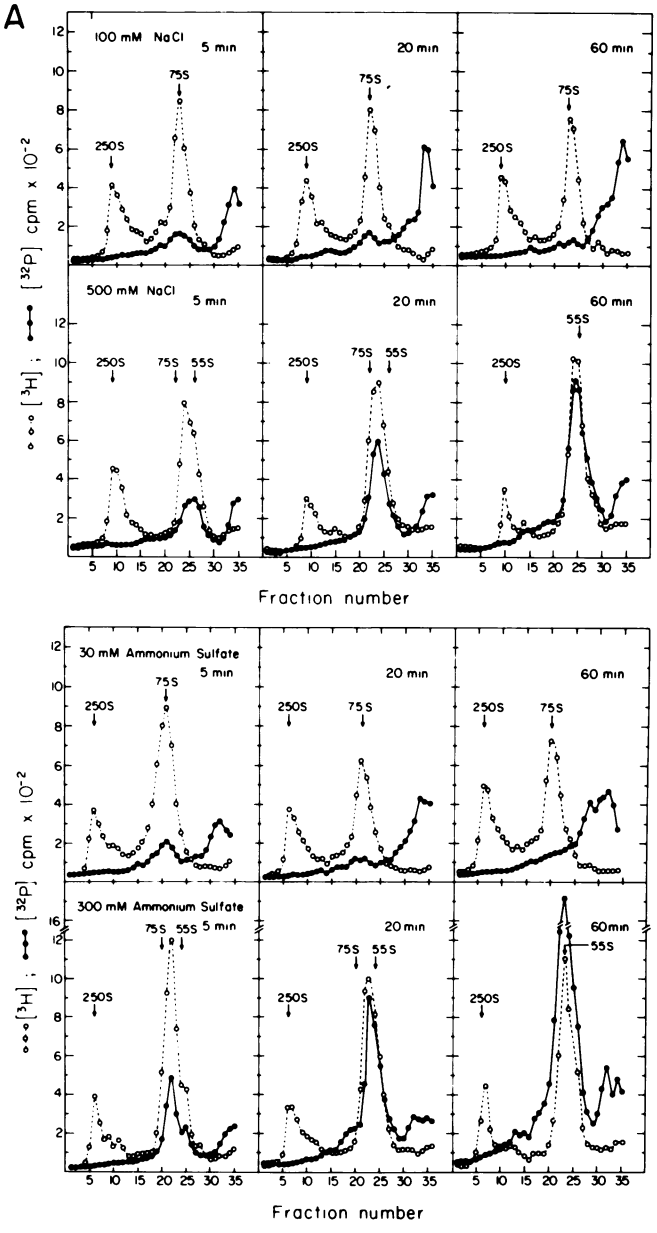

Fig. 3. Sucrose gradient analysis of transcription mixtures programmed by nuclear extracts from SV40-infected cells. Nuclei were prepared by hypotonic leaching of 21 plates of CV-1 cells at 46 h post-infection in 15 ml buffer 'H' and the nuclear extract was concentrated by dialysis against PEG 20 000 and equilibrated overnight against buffer 'H'. Transcription mixtures (300 μl) containing 240 μl nuclear extracts were adjusted to the desired salt concentration (**A**-NaCl; **B**-ammonium sulfate) and supplemented with NTPs, $MnCl_2$ and 100 μCi [α-^{32}P]UTP as described. The evolution of the acid-insoluble radioactivity upon incubation at 32°C was followed (see Figure 1) and after 5, 20 and 60 min total RNA was prepared from half the sample for hybridization analysis (see Figure 2) while the other half was chilled on ice and layered onto precooled 5–30% sucrose gradients in buffer 'H' supplemented with 100 mM NaCl. After centrifugation for 105 min in a Beckman SW41 rotor at 4°C and 32 000 r.p.m., the gradients were collected from the bottom and the acid-precipitable radioactivity was determined on 40 μl aliquots of each fraction spotted on strips of Whatman 3 MM paper. The fractions containing the minichromosome-associated and free RNA (top of the gradient) were pooled and the RNA extracted for further analysis.

Elongating viral RNA is associated with the minichromosomes while prematurely terminated RNA detaches from the template

To determine the relationship between the viral template responsible for transcription and the RNA made at the various salt concentrations, SV40-infected cells were labeled with [^{3}H]thymidine before extraction of the nuclei. The labeled nuclear extract was incubated for synthesis of ^{32}P-labeled RNA either at moderate or at high salts for varying times. The reactions were stopped by the addition of EDTA to 10 mM and the samples were loaded onto sucrose gradients for separation of the major viral nucleoprotein pools (Jakobovits and Aloni, 1980).

As shown in Figure 3, the extracts incubated at moderate salts (100 mM NaCl; 30 mM ammonium sulfate) contained two major peaks of viral nucleoprotein of which the 250S peak comprised mature and immature virions and the 75S peak, the minichromosome pool of replicating and transcribing molecules (Jakobovits and Aloni, 1980).

RNA synthesis for 5 min revealed two major populations of which one was bound to the 75S structures reflecting attachment of RNA to the minichromosome template and a second population was present at the top of the gradient in a non-bound state. Increasing the time of incubation to 20 min or 60 min led to almost total release of RNA from the 75S peak and to its accumulation at the top of the gradient as a template-free fraction. This was in contrast to the results obtained at high salt concentrations (500 mM NaCl; 300 mM ammonium sulfate) where no major fraction of unbound RNA was obtained. Rather, increasing the time of synthesis led to accumulation of RNA into the minichromosome peak as a species attached to its template. The fraction of RNA running ahead of the minichromosome peak suggests the presence of long RNA chains. As expected from the kinetics of incorporation (see Figure 1), the number of counts synthesized at high salt increased continuously with increasing time of synthesis while synthesis at low salt rapidly plateaued.

It should be noted that the minichromosome peak displayed in the sucrose gradient profile at high salt was shifted to a 55S position in the gradient. This, as previously reported, is due to the loss of histone H1 from these molecules which leads to a reduction in the compaction of the minichromosome (Fey and Hirt, 1974; Meinke *et al.*, 1975). Minichromosome-associated and unbound RNAs were purified and hybridized to Southern blots of the five restriction fragments shown in Figure 2A. Figure 4 shows that the RNA associated with the minichromosome after 60 min incubation at low salt (100 mM NaCl) hybridized primarily with fragment 'e' and to a lesser extent with fragment 'b' which lies immediately downstream, reflecting poor growth of RNA chains. Labeled RNA found at the top of the gradient, on the contrary, hybridized exclusively with fragment 'e'. The situation is even more pronounced at high salt (300 mM ammonium sulfate), where minichromosome-associated RNA hybridized to the 'late' fragments (b, d and e) as well as to the 'early' fragments (a and c). This result does not discriminate between hybridization to the 'late' or 'early' strands but indicates, on the other hand, distribution of RNA polymerase molecules over almost all the viral genome. In contrast, RNA found at the top of the gradient hybridized primarily with the

promoter-proximal 'e' fragment. This indicates that SV40-specific RNA found detached from the template both after low and high salt incubations does not originate from random degradation or accidental release of growing RNA chains, but rather points to the existence of a well defined class of RNA molecules prematurely terminated and released from their template.

The prematurely terminated RNA can be released from the template as discrete species

To establish the characteristics of the viral RNA released from the template, [^{3}H]thymidine-labeled nucleoproteins were extracted and incubated for 10 min and 60 min in low salt (30 mM ammonium sulfate). The reactions were stopped with 10 mM EDTA and centrifuged on a sucrose gradient from which the region of the 75S peaks and the top of each gradient were pooled. The labeled RNA was loaded onto a denaturing acrylamide gel. As shown in Figure 5A, the small amount of RNA associated with the 75S peak both at 10 and 60 min was heterogeneous in length with no major bands visible. The RNA derived from the released fraction, for both times of incorporation, showed a major band of 94 nucleotides in length as well as other bands. The other bands were not analysed because they were not reproducibly revealed.

The 94 nucleotide band of RNA was excised from the gel, eluted electrophoretically and hybridized to a Southern blot of SV40 DNA fragments as described in Figure 2A. The purified RNA hybridized exclusively to the viral DNA fragment spanning 0.67 – 0.76 map units (Figure 5B). To establish the viral origin of the 94 nucleotide band, the above experiment was repeated on sucrose gradient purified minichromosomes followed by the hybridization elution procedure. Figure 5C lane a shows that after 60 min incubation at low salt (100 mM NaCl) a major viral RNA band of 93 – 95 nucleotides is found at the top of the gradient. It is interesting to note the occurrence of an additional reproducible band of ~98 nucleotides present above the major band. A similar band is also found following similar analyses of Sarkosyl extracted VTC (Hay *et al.*, 1982, see accompanying paper) or isolated nuclei (Skolnik-David *et al.*, 1982).

The 94 nucleotide viral RNA band has the same fingerprint as that found for the 94 nucleotide band produced in the VTC transcribing system (see accompanying paper). The viral RNA in the major band spans, therefore, nucleotides 243 – 336 (Hay *et al.*, 1982; Skolnik-David *et al.*, 1982) (see Figure 6). Thus the actively transcribing minichromosomes provide a system in which the attenuated RNA can be released from the template. The present observation supports our previous suggestion that the eucaryotic RNA polymerase B, like the procaryotic enzyme, recognizes a hairpin structure followed by a stretch of uridylic acid residues, present at the 3′ end of the transcript, as a signal of transcription termination (Hay *et al.*, 1982; Skolnik-David *et al.*, 1982).

Transcription termination can occur at various sites along the stretch of uridylic acid residues

Careful examination of the 94 nucleotide band revealed

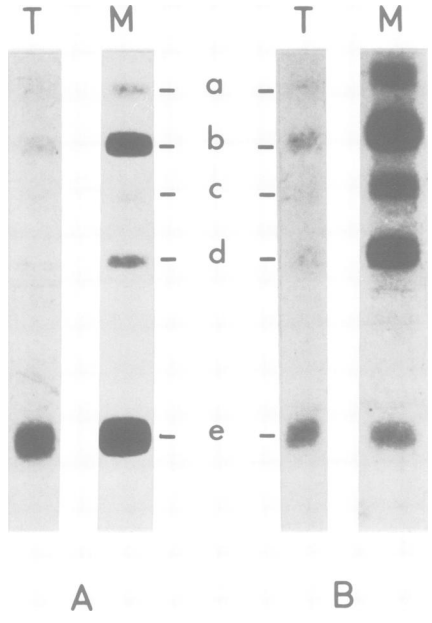

Fig. 4. Hybridization pattern of the minichromosome-associated and free RNA to Southern blots of the SV40 DNA restriction fragments depicted in Figure 2. RNA synthesized by nuclear extracts at 100 mM NaCl (**A**) or 300 mM ammonium sulfate (**B**). T: free RNA remaining at the top of the gradient; M: minichromosome-associated RNA.

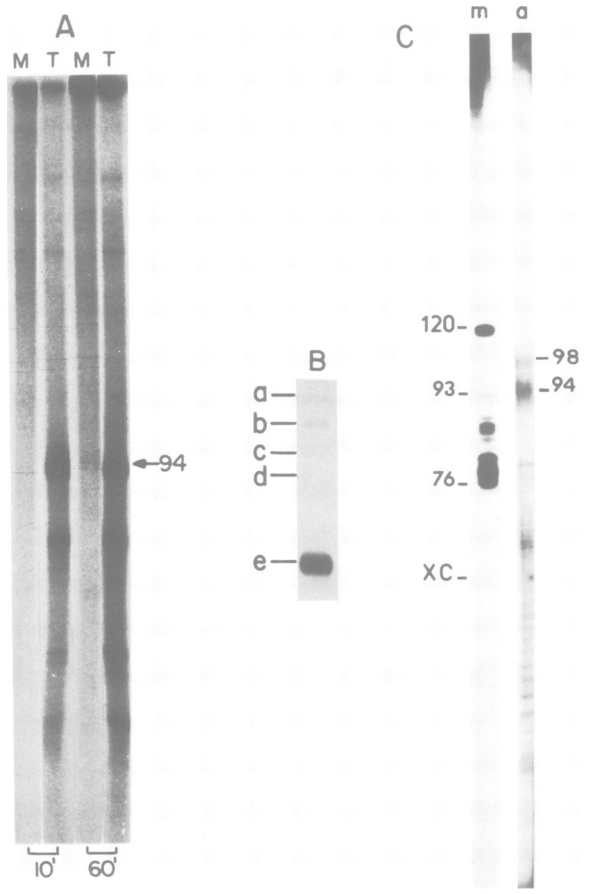

Fig. 5. Acrylamide gel electrophoresis of minichromosome bound and released RNA. Nuclear extracts were incubated for RNA synthesis in the presence of 30 mM ammonium suflate for 10 or 60 min. The reactions were stopped, the mixtures loaded onto sucrose gradients and sedimented as in Figure 3. (**A**): the regions of the 75S minichromosome peaks and the tops of the gradients were pooled, RNA purified and loaded onto the gel. M: minichromosome-bound RNA; T: released RNA. (**B**) The band of 94 nucleotides was eluted and hybridized to a Southern blot as in Figure 2. (**C**) Purified minichromosomes were incubated for RNA synthesis in the presence of 100 mM NaCl for 60 min. The mixture was loaded onto sucrose gradient and sedimented as in Figure 3. The top fractions of the gradient were pooled, RNA purified and viral RNA selected by the hybridization elution procedure and loaded onto the gel. a: released viral RNA; m: *E. coli* tRNA length markers; xc: xylene-cyanol marker.

that with increasing labeling time or with increasing UTP concentration the major band tends to shift to a position on the gel corresponding to an RNA molecule of ~100 nucleotides. Since the [α-^{32}P]UTP concentration (0.2–1 μM) used in our transcription mixture could be a limiting factor for elongation in a U-rich region, we performed pulse-chase experiments on transcribing purified minichromsomes. Figure 7 shows that the shortest RNA detectable is some 30 nucleotides long and it appears after 30 s of incubation. This observation supports Skolnik-David and Aloni's conclusion (see accompanying paper) that *in vivo* RNA polymerase molecules pause in proximity to the initiation site. The 94 nucleotide band starts to appear only after a pulse of 1 min, depending on the activity of the extracts, and reaches its full intensity after 4 min or so. A similar observation was noticed in the VTC system (see accompanying paper) indicating that the rate of RNA elongation is similar in both transcribing systems. Also, as in the VTC transcribing system, elongation to the ~100 nucleotide band requires prolonged incubation (see 60 min pulse). The ~100 nucleotide RNA species is then a terminal product, because it is stable after a 60 min chase with cold UTP following the 2, 5 and 60 min pulse (Figure 7). The viral origin of the 94 and ~100 nucleotide bands is shown in Figure 7B as they appear also after hybridization and elution from filters bearing SV40 DNA. We suggest that termination at the U-rich stretch (nucleotides 334–350; see Figure 6) is a 'wobbly' process and the exact mapping of the 3′ end of the attenuated RNA will depend on the environmental conditions.

Discussion

RNA polymerase molecules transcribing the late genes of SV40 pause *in vivo* in a promoter-proximal region (Hay *et al.*, 1982; Skolnik-David *et al.*, 1982; see accompanying paper). Upon *in vitro* incubation of VTCs, viral minichromosomes or isolated nuclei the RNA polymerase molecules resume transcription but stop again 94 nucleotides downstream from the major initiation site (Hay *et al.*, 1982; Skolnik-David *et al.*, 1982; see accompanying paper). The DNA region where transcription stops is GC-rich with dyad symmetry and the 3′ end of the transcript contains a stretch of uridylic acid residues (see Figure 6). Based on these observations it was suggested that the eucaryotic RNA polymerase B transcribing SV40 DNA responds to the same transcription termination signal as procaryotic RNA polymerase (Hay *et al.*, 1982; Skolnik-David *et al.*, 1982; see accompanying paper). However, in the above studies, no genuine *in vitro* termination of transcription was shown. Here we characterize a system of actively transcribing viral minichromosomes and we show premature termination and release of the attenuated RNA. This is the first demonstration of *in vitro* specific termination of polymerase B transcription.

High levels of premature termination occurred at low salt as compared to high salt concentration. This could be explained by the nature of nucleic acid interaction. If the mechanism of premature termination is similar to the mechanism of transcription termination in procaryotes we can predict that transcription slows down at the GC-rich regions (see Figure 6). The rU-dA duplex that is subsequently formed is exceptionally unstable at a low salt concentration. The unstable interaction could then provide a major driving force for termination and release of the transcript (Martin and Tinoco, 1980; Farnham and Platt, 1980; Bogenhagen and Brown, 1981). Increased salt concentration results, on the

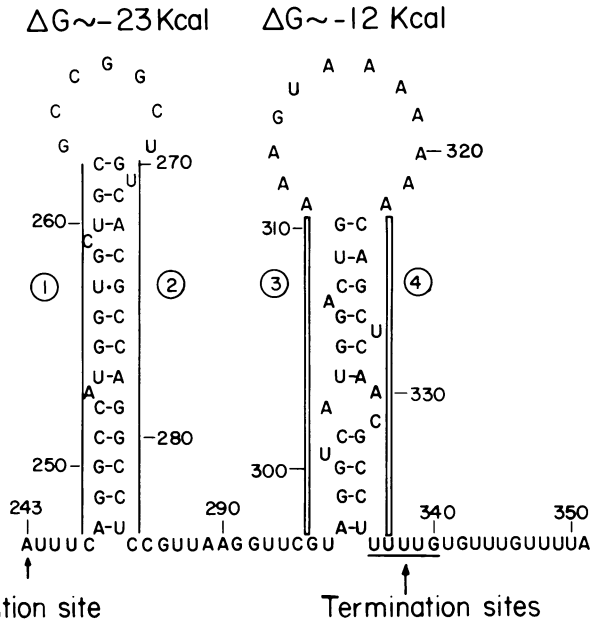

Fig. 6. The 'attenuation' conformation of the attenuated RNA. Taken from Hay *et al.* (1982). The ΔG was calculated as described (Tinoco *et al.*, 1973). Nucleotide residue number refers to the wild-type SV40 sequence of Reddy *et al.* (1978).

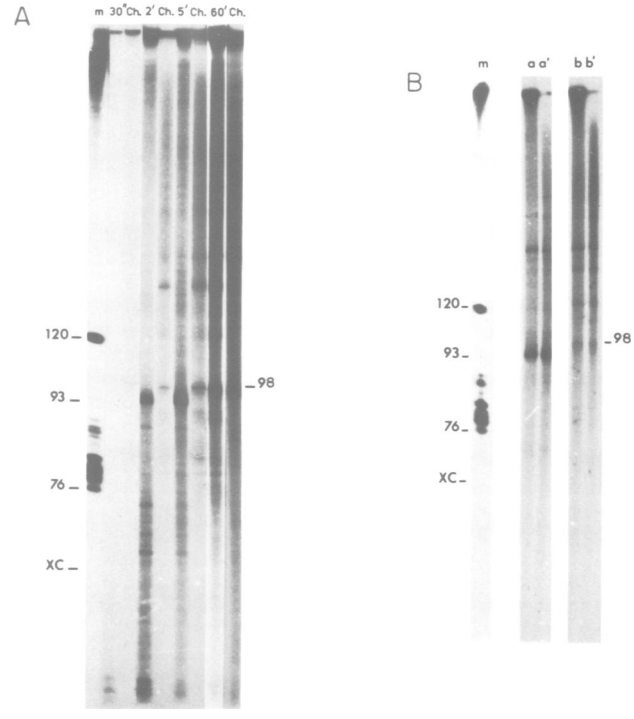

Fig. 7. (A) Production and stability of the 94 nucleotide band synthesized on purified SV40 minichromosomes. RNA was elongated *in vitro* at low UTP concentration (1.5 μM) at 30°C in 50 mM NaCl. At the indicated time points, half of the reaction mixture was withdrawn and the RNA extracted. To the other half, cold UTP was added to 1 mM final concentration, and RNA synthesis was allowed to proceed for an additional 60 min (Lanes marked 'Ch.') after which the RNA was purified and analyzed on 10% polyacrylamide gels containing 8 M urea. Lane (m): *E. coli* tRNA length markers. **(B)** Demonstration of the viral origin of the 94 and 98 nucleotide bands. RNA was extracted after synthesis and hybridized to SV40 DNA filters. **Lane (a)**: total RNA after 20 min pulse; **(a′)**: SV40-specific sequences. **Lane (b)**: total RNA after 20 min pulse followed by 60 min chase; **lane (b′)**: SV40-specific sequences.

other hand, in a partial stabilization of the rU-dA duplex and also stimulates the rate of synthesis and the enzyme then crosses this critical region at an increased rate. It is noteworthy that the UTP concentration in the transcription mixture (typically 0.2 – 1 μM) could be a condition which favours termination at the second U-residue (nucleotide 336) (see accompanying paper). This UTP concentration is not a limiting factor *per se* as testified by extensive elongation at high salt or even at low salt following proflavine treatment of infected cells (Hay *et al.*, 1982). Furthermore, a similar band was also identified when the UTP concentration was increased to 20 and 50 μM (results not shown). Indeed, it has been noticed previously that the concentration of the ribonucleotides may influence the extent of termination at any given site (Farnham and Platt, 1980; Bogenhagen and Brown, 1981). Moreover, at the end of the *trp* operon, only when the *in vitro* transcription system contains a low UTP concentration does it yield termination efficiencies approaching those seen *in vivo* (Farnham *et al.*, 1982).

Another possibility is that the production and release of the attenuated RNA is dependent upon regulatory factors that are associated with the viral minichromosomes. These factors may dissociate from the minichromosome upon high salt treatment but not upon low salt treatment.

The nature of the protein factor involved in attenuation is still unknown. A likely candidate is the agnoprotein (Dhar *et al.*, 1977). This protein binds to nucleic acids at low salt and dissociates from them at high salt (Jay *et al.*, 1981; A. Ben-Ze'ev, H. Skolnik-David and Y. Aloni, unpublished results). The agnoprotein has a short half-life typical of a regulatory protein and it was found in association with purified minichromosomes (Jackson and Chalkley, 1981; P. Pfeiffer, A. Ben-Ze'ev and Y. Aloni, unpublished results). For a possible mechanism by which the agnoprotein may regulate attenuation see Hay *et al.* (1982) and Aloni and Hay (1983).

Materials and methods

Cells and viruses

Growth of plaque-purified SV40 on CV-1 monkey cells as well as concentration and purification of the viruses from tissue-culture lysates and preparation of SV40 DNA component I have been described (Laub and Aloni, 1975). In these experiments CV-1 cells were infected with 50 – 100 p.f.u./cell of stock 777 or 776. Identical results were obtained with both viruses.

Preparation of nuclear extracts and viral minichromosomes

The hypotonic lysis procedure of Jakobovits and Aloni (1980) was used. All operations were performed in the cold. The nuclear extracts were routinely concentrated 5 to 10 times by dialysis against a solution of polyethylene glycol (PEG) 20 000 or by vacuum dialysis; both methods gave similar results, with typically 70 – 80% recovery of RNA polymerase activity for a 10-fold concentration, without loss of any of the [^{3}H]thymidine-labeled species. SV40 minichromosomes were purified by a 3 h centrifugation through 5 – 30% sucrose gradients in buffer 'H' (50 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.8) at 32 000 r.p.m. in a Beckman SW41 rotor; virions are pelleted under these conditions and minichromosomes separate well from contaminants which migrate very little into the gradient. Fractions containing the minichromosomes could be either used directly for transcription, or dialyzed to eliminate the sucrose and reconcentrated by vacuum dialysis with identical results.

In vitro transcription assays, purification and analysis of the RNA synthesized

Nuclear extracts in buffer 'H' were supplemented with 2 mM $MnCl_2$ and salt to the required concentration, preincubated for 5 min at the chosen temperature, and then the three unlabeled NTPs (final concentration 400 mM) were added together with 20 – 100 μCi per 100 μl incubation mixture of [α-^{32}P]UTP (sp. act. 400 Ci/mmol, the Radiochemical Centre, Amersham, UK).

For the preparation of total RNA, transcription mixtures were added to SDS and EDTA to 0.5% and 10 mM, respectively, and extracted with phenol-chloroform-isoamyl alcohol (Hay *et al.*, 1982). RNA was analyzed by polyacrylamide gel electrophoresis under denaturing conditions as described (see accompanying paper).

Selection of viral transcripts from transcription mixtures

SV40-specific RNA was selected from the transcription mixtures by hybridization to nitrocellulose filters containing SV40 DNA (Aloni, 1972). RNA samples resuspended in 10 mM Tris-HCl, pH 7.4, 5 mM EDTA and 0.5% SDS were first denatured for 1 min at 100°C, and hybridization was carried out for 24 – 28 h at 37°C in 70% formamide, 300 mM NaCl, 10 mM Tris-HCl pH 7.4 and 0.5% SDS. After hybridization, the filters were incubated in 35% formamide, 10 mM NaCl, 0.1% SDS, 10 mM Tris-HCl pH 7.4 and 1 mM EDTA at 37°C for 0.5 h, and then washed with 0.5 x SSC (1 x SSC is 0.15 M NaCl; 0.015 M Na_3 citrate), 0.5% SDS. The bound RNA was eluted by three successive changes of 96% formamide at 37°C for 1 h each, and precipitated with ethanol after addition of 120 μg/ml tRNA. The pelleted RNA was resuspended and treated with RNase-free DNase as previously described (Hay *et al.*, 1982).

Mapping of the viral transcripts

SV40-specific transcripts were mapped by hybridization to nitrocellulose filter-bound restriction fragments as described by Southern (1975), except that transfer was in 20 x SSC. After baking for 4 h at 80°C, the blots were directly hybridized with the ^{32}P-labeled RNA probe previously denatured by boiling for 5 min in 0.01 x SSC. Hybridization was carried out in 4 x SSC, 0.5% SDS for 24 – 28 h at 68°C. The blots were then extensively washed with 2 x SSC at 68°C, treated with RNase (20 μg/ml at 37°C in 2 x SSC), and submitted to autoradiography. Exposure time ranged from a few hours to 10 days.

Acknowledgements

We thank Ruchama Leizerowitz for technical assistance and Dr. Ann Roman for critical reading of the manuscript. P. Pfeiffer was supported by a long-term EMBO fellowship. This research was supported by USPHS research grant CA 14995 and in part by the Norman V. Wechsler Endowment Fund.

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