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Analysis of Premature Termination in c-*myc* during Transcription by RNA Polymerase II in a HeLa Nuclear Extract

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Transcriptional regulation of the human c-*myc* gene, an important aspect of cellular differentiation, occurs in part at the level of transcript elongation. In vivo, transcriptional arrest, due to either pausing or termination, occurs near the junction between the first exon and first intron and varies with the growth state of the cell. We have tested the transcription of c-*myc* templates in HeLa nuclear extracts. We did not observe significant arrest under standard conditions, but we found that a considerable fraction of transcription complexes stopped at the c-*myc* T_{II} site (just past the first exon-intron junction) when the KCl concentration was raised to 400 mM during elongation. Transcriptional arrest at T_{II} also was observed at KCl concentrations as low as 130 mM and when potassium acetate or potassium glutamate was substituted for KCl. Under these conditions, arrest occurred at the T_{II} site when transcription was initiated at either the c-*myc* P2 promoter or the adenovirus 2 major late promoter. Further, the T_{II} sequence itself, in forward but not reverse orientation, was sufficient to stop transcription in a HeLa nuclear extract. By separating the T_{II} RNA from active transcription complexes by using gel filtration, we found that arrest at T_{II} at 400 mM KCl resulted in transcript release and thus true transcriptional termination. The efficiency of termination at T_{II} depended on the growth state of the cells from which the extracts were made, suggesting that some factor or factors control premature termination in c-*myc*.

The control of gene expression through changes in transcriptional elongation, either by attenuation or by antitermination, is common in bacteria (23, 41, 42). Both transcriptional pausing and transcriptional termination are important components of these mechanisms. Termination occurs when the transcription complex dissociates and releases the RNA transcript, whereas pausing means that RNA polymerase stops until it resumes elongation either spontaneously or from the action of another molecule, but it does not irreversibly terminate transcription.

Changes in elongation also have been postulated to regulate transcription in eukaryotes. These events have been inferred primarily from the detection of prematurely truncated RNAs in cells or oocytes and from the decreased density of RNA polymerase II (pol II) on distal segments of certain genes, as detected with nuclear run-on assays (58, 73). However, the mechanisms by which elongation of transcription is controlled in eukaryotes are not fully understood. In particular, it has been difficult to distinguish among transcriptional pausing, termination, and posttranscriptional processing as explanations for the phenomena. The best characterized example of a eukaryotic gene regulated by changes in transcriptional elongation is the mammalian proto-oncogene c-*myc* (73).

c-*myc* encodes an unstable DNA-binding protein that is involved in the control of cellular proliferation (46). It is expressed at high levels during proliferative growth and in some transformed cells, but at much lower levels in differentiated cells (8, 20, 36, 45, 51). The c-*myc* gene is composed of three exons, the first of which is noncoding. Initiation of c-*myc* transcription occurs primarily at two promoters, P1 and P2. P2, which directs initiation 162-bp downstream from P1, is generally the stronger promoter. Regulation of c-*myc* is complex and occurs by several mechanisms (32); however, an important control of c-*myc* gene expression appears to result from changes in transcriptional elongation near the junction between the first exon and first intron. Transcriptional arrest near the exon-intron junction has been detected by nuclear run-on assays in cells undergoing terminal differentiation (8, 20, 39) and by direct analysis of RNAs made both in *Xenopus* oocytes injected with c-*myc* DNAs (9) and in vitro during transcription of c-*myc* by purified calf thymus pol II (33). Further, resting cells stimulated by growth factors or mitogens increase c-*myc* expression in part by the release of the block to elongation (19, 45, 53).

The site of transcriptional arrest in the human c-*myc* gene has been mapped to two locations near the exon-intron junction, designated sites I (T_I) and II (T_{II}) (8, 9). T_I contains a run of 7 T's in the nontranscribed strand and is located 20 bp upstream from the junction; T_{II} is located 30 bp downstream of the junction in the sequence TTTAATTTAT TTTTTTAT in the nontranscribed strand. The murine c-*myc* first exon is very similar to the corresponding human sequence and also is subject to transcriptional arrest (9, 78). Site T_I is preceded by dyad symmetries which are similar to those that specify alternative RNA secondary structures in prokaryotic attenuators and which may have a role in c-*myc* transcriptional arrest (9, 20). In the human c-*myc* gene, transcription stops at both T_I and T_{II} when c-*myc* templates are injected into *Xenopus* oocytes (9). However, purified pol II only pauses at T_I even though it terminates at T_{II} (33). Arrest at T_{II} during transcription in HeLa whole-cell extracts has been reported in one study (17), but it was not reported in other studies that utilized either HeLa whole-cell (25) or nuclear extracts (56).

The arrest of c-*myc* transcription in cells apparently occurs only when pol II initiates transcription at certain promoters (9, 52, 74). Initiation at the c-*myc* P2 promoter, but not at the P1 promoter, allows arrest near the exon-intron junction (52). In some Burkitt's lymphoma cell lines that constitutively transcribe c-*myc* in its entirety, transcrip-

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tion initiation is shifted from P2 to P1 (43, 74). Heterologous promoters from the herpes simplex virus thymidine kinase gene, from Rous sarcoma virus (9), and from the α-globin gene (78) support transcriptional arrest. Bentley and Groudine (9) reported that transcription complexes that initiate transcription at the adenovirus 2 major late promoter (Ad2MLP) in *Xenopus* oocytes do not arrest at c-*myc* T_I or T_{II} sequences located 500 bp after the cap site. However, arrest has been observed more recently in constructs where Ad2MLP and the T_I-T_{II} sequences are closer (7).

Several studies have defined conditions that block elongation during the transcription of a variety of DNA templates in either whole-cell or nuclear extracts. Normally, efficient arrest is observed only when perturbing agents such as sarkosyl or heparin are added or at elevated salt concentrations (4, 6, 26, 27, 77, and references therein). During transcription of the adenovirus major late transcription unit in vitro, sarkosyl-dependent arrest occurs at position +186 in both nuclear extracts (27, 77) and in a partially purified transcription system (26). Arrest is stimulated by low GTP (presumably the next nucleoside triphosphate [NTP] to be added), and the effects of sarkosyl apparently can be reversed by dilution or by raising the salt concentration (35). However, KCl at 200 mM or more is reported elsewhere to increase arrest at the Ad2MLP +186 site and at synthetic sites that resemble bacterial ρ-independent terminators (6). One drawback to many of these studies has been the difficulty of establishing that the truncated RNAs result from true transcriptional termination, rather than from transcriptional pausing or posttranscriptional processing.

To investigate the mechanism of transcriptional arrest, we have studied the transcription of c-*myc* DNAs in a HeLa nuclear extract. We found that, although arrest did not occur under standard in vitro transcription conditions, true transcriptional termination occurred at the c-*myc* T_{II} element at 400 mM KCl. A similar pattern of arrest was observed at KCl, potassium acetate, or potassium glutamate concentrations around 200 mM. Transcriptional arrest at this site occurred when pol II initiated transcription either at the c-*myc* P2 promoter or at the Ad2MLP, as well as at a synthetic T_{II} element that was inserted in unrelated DNA. However, the efficiency with which pol II stopped at this site depended on the growth state of the cells from which the extracts were made. In Discussion, we review what is known about the termination of transcription in eukaryotes and consider the implications of our findings for the mechanism of elongation control in genes such as c-*myc*.

MATERIALS AND METHODS

DNA manipulations and plasmid construction. A 876-bp fragment of the human c-*myc* gene containing the P2 promoter and the first exon-intron junction was recovered by digestion with *Xho*I and *Sac*I of plasmid pHSR-1 (1) obtained from the American Type Culture Collection (ATCC 41010). DNA for use as a transcription template was recovered from plasmid pLL253 (Fig. 1A), which carries the *Xho*I-*Sac*I fragment in the polylinker of pBSIIKS- (Stratagene). To form the Ad2-*myc* fusion, a 318-bp fragment carrying the Ad2MLP was excised from plasmid pMLC38 (obtained from D. Hawley, University of Oregon) and ligated upstream from the c-*myc* gene in pLL253 to create pRL507. Oligonucleotide-directed mutagenesis (38) was used to fuse the transcriptional start sites of the Ad2MLP and c-*myc* P2 to give plasmid pRL558 (Fig. 1B).

Plasmid pRL542 (Fig. 1C) was created from pRL507 by replacing the c-*myc* sequences after the Ad2MLP with two G-less cassettes of 262 nucleotides (nt) (*Eco*RI-*Hph*I) and 131 nt (*Fok*I-*Bam*HI) from pC_2AT (67), separated by nt 651 (*Sal*I site) to 526 (*Eco*O109I site) from pBR322. A synthetic oligonucleotide consisting of sequential *Bgl*II, *Nhe*I, *Stu*I, *Sac*II, and *Eco*47III restriction endonuclease recognition sequences was inserted within the *Sph*I site derived from pBR322. Oligonucleotide-directed mutations in this template at nt 575 to 577 created a unique *Bst*BI site. The synthetic oligonucleotides

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5'AGCTAATTTATTTTTTATTTACTTTAAACTT
      TTAAATAAAAAAATAAATGAAATTTGAATCGA-5'
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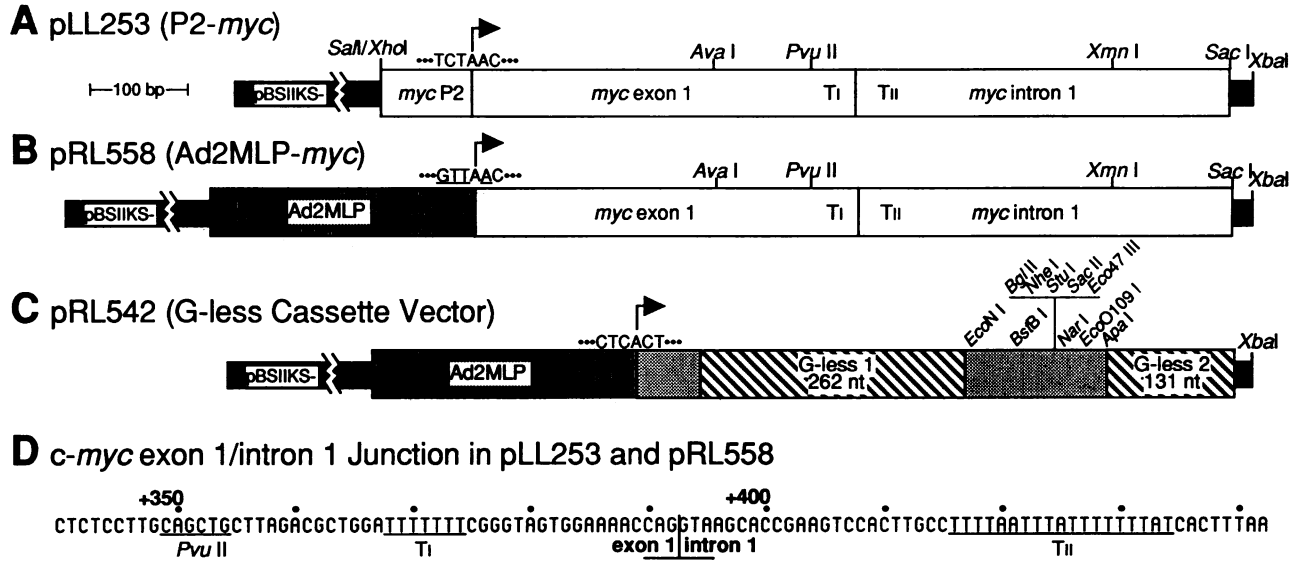
that specify the c-*myc* T_{II} site was inserted into the *Hin*dIII site of pBSIIKS+ (Stratagene) and then recovered on an *Acc*I-*Eco*RV fragment and inserted between the *Bst*BI and *Stu*I sites of pRL542 to create plasmid pRL578 (Fig. 1D). Plasmids containing the reverse orientation of the T_{II} sequence (pRL577; Fig. 1D) and two forward copies of the T_{II} sequence (pRL575; Fig. 1D) were similarly constructed from pBSIIKS+ derivatives that picked up the oligonucleotides in other orientations and combinations.

Plasmid DNA for in vitro transcription was prepared by CsCl-ethidium bromide, buoyant-density-gradient centrifugation and cleaved with *Xba*I, followed by sequential extraction with phenol, $CHCl_3$, and diethyl ether, precipitation with ethanol, and dissolution in TE buffer (10 mM Tris · HCl [pH 8.0]–1 mM Na_2EDTA).

Preparation of HeLa cell nuclear extracts. HeLa cells, maintained in RPMI 1640 (GIBCO) with 5% supplemented calf serum (Hyclone), were harvested during log-phase growth (a density of 1×10^5 to 5×10^5 cells per ml). Nuclear extracts were prepared as described by Shapiro et al. (69). Depending on the extract, the protein concentration varied from 10 to 23 mg/ml. To prepare an extract from an adherent culture, HeLa cells were grown in the same medium in 150-mm^2 T flasks without agitation, recovered by incubation for 5 min at 37°C in a solution containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, and 10 mM EDTA (pH 7.3), and then handled as described by Shapiro et al. (69).

In vitro transcription reactions. Transcription reactions, unless otherwise noted in the figure legends, were preincubated in the following solution (transcription buffer): 6 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.9), 33 mM KCl, 8 mM $MgCl_2$, 0.1 mM EDTA, 0.6 mM dithiothreitol, 5 mM creatine phosphate, 2 ng of creatine phosphokinase per ml, 16 U of RNasin (Promega) per reaction, 6% glycerol, 500 ng of template DNA, and 3 to 6 μl of a HeLa cell nuclear extract (approximately 40 μg of protein) in a final volume of 20 μl. Extract and DNA were preincubated at 30°C for 30 min in the absence of NTPs. NTPs in 5 μl of 2 mM β-mercaptoethanol (yielding final concentrations of 16 μM ATP, UTP, and CTP and 18 μM [α-^{32}P]GTP with a specific activity ~40 Ci/mmol) were added to initiate transcription. When indicated, sarkosyl (*n*-lauroylsarcosine, Na^+ salt; Sigma), KCl, potassium acetate, or potassium glutamate was added in 2.5 ml of water to the reaction 30 s after the addition of NTPs. Reactions were incubated at 30°C for the times indicated in the figure legends. When indicated, NTPs were added to 1 mM final concentration and the reactions were allowed to continue for an additional 10 min. Transcription reactions were terminated by the addition of 50 μl of proteinase K (10 μg) in 1% sodium dodecyl sulfate (SDS), 0.1 M Tris (pH 7.5), 12.5 mM EDTA, and 150 mM NaCl and allowed to incubate at room



D c-*myc* exon 1/intron 1 Junction in pLL253 and pRL558

```
          +350                                                  +400
CTCTCCTTGCAGCTGCTTAGACGCTGGATTTTTTTCGGGTAGTGGAAAACCAGGTAAGCACCGAAGTCCACTTGCCTTTTAATTTATTTTTTTATCACTTTAA
          Pvu II          TI                    exon 1|intron 1                  TII
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E DNA Sequences Inserted Between *Bst*B I and *Stu* I Sites of pRL542

For *myc* TII 1x forward (pRL578): CGTAAGCTAATTTATTTTTTTTATTTACTTTAAACTTGAT

For *myc* TII 2x forward (pRL575): CGTAAGCTAATTTATTTTTTTTATCACTTTAAAGCTTGCTTTTAATTTATTTTTTTTATCACTTTAAAGCTTGAT

For *myc* TII 1x reverse (pRL577): CGTAAGCTTTAAAGTGATAAAAAAATAAATTAAAATGGCAAGCTTGAT

FIG. 1. DNAs used as transcription templates. (A) pLL253 cleaved with *Xba*I. This plasmid contains the human c-*myc* gene, from the *Xho*I site 96 bp preceding the P2 cap site (indicated by the arrow) to the *Sac*I site 775 bp downstream from the cap site, cloned in the polylinker region of pBSIIKS-. T_I and T_{II} indicate transcriptional arrest sites I and II mapped by Bentley and Groudine (9). Restriction sites referred to in the text and the nucleotide sequence immediately around the cap site are indicated. (B) pRL558 cleaved with *Xba*I. This plasmid contains a substitution of the Ad2MLP for c-*myc* P2 in pLL253 (see Materials and Methods). The bases immediately surrounding the cap site that differ from those normally found in the Ad2MLP are underlined. (C) pRL542 cleaved with *Xba*I. This plasmid contains the Ad2MLP from pMLC38 preceding two G-less cassettes (striped regions) and sequences from pBR322 (shaded regions; see Materials and Methods). Restriction sites useful for inserting test DNAs are indicated. (D) Sequence of the human c-*myc* immediately surrounding the first exon-intron junction. (E) DNA sequences determined for plasmids pRL578, pRL575, and pRL577 which contain one c-*myc* T_{II} site in forward orientation, two c-*myc* T_{II} sites in forward orientation, and one c-*myc* T_{II} site in reverse orientation, respectively. There is some variation in the nucleotide sequences among these pRL542 derivatives that arose during the DNA manipulations used to construct them. None of these differences affects the core T_{II} sequence.

temperature for 10 min. Two hundred microliters of 0.1% SDS, 7 M urea, 20 mM EDTA, 0.1 M Tris · HCl (pH 7.5) and 10 μg of carrier tRNA were added, and the reactions were extracted once with phenol-chloroform (1:1) and precipitated with ethanol. RNA pellets were washed once with 70% ethanol, dried, and resuspended in 5 μl of loading buffer (7 M urea, 0.1% bromphenol blue, 0.1% xylene cyanol, 44 mM Tris · borate [pH 8.3], and 1.25 mM EDTA). Samples were heated for 2 min at 65°C and then electrophoresed through a 4% acrylamide-bisacrylamide (19:1) 7 M urea gel (0.4 mm by 36 cm by 48 cm) in 44 mM Tris · borate (pH 8.3)–1.25 mM EDTA (TBE buffer).

Primer-extension assay to map RNA 5′ ends. Gel-purified RNA bands were resuspended in 5 μl of 10 mM Tris · HCl (pH 8.3)–2 mM EDTA containing 0.7 M KCl and 100 fmol of 5′ ^{32}P-labeled primer (5′-CCTATTCGCTCCGGATCTCC-3′). Hybridization was conducted for 1 h at 37°C, and the reactions then were diluted by the addition of 95 μl of a buffer containing 50 mM Tris · HCl (pH 8.3), 8 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM deoxynucleoside triphosphates, and 200 U of RNasin per ml. Mouse mammary leukemia virus reverse transcriptase (200 U; GIBCO-BRL) was added, and incubation continued for 1 h an 37°C. Reactions were terminated by the addition of 10 mM EDTA, 5 μg of tRNA, NaCl to 0.3 M, and 2.5 volumes of ethanol. Electrophoresis was conducted, as described above, next to a sequencing ladder generated by using modified T7 DNA polymerase (US Biochemical) and the primer described above that had been hybridized to pLL253 DNA.

S1 nuclease-protection assay to map RNA 3′-ends. The *Ava*I-*Sac*I fragment of c-*myc* was 3′ end labeled with [α-^{32}P]dCTP with the Klenow fragment of *Escherichia coli* DNA polymerase I. Strand-separated probe was hybridized to individual gel-purified c-*myc* RNAs in 10 μl of 80% formamide–400 mM NaCl–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)](pH 6.4)–1 mM EDTA as described by Berk and Sharp (10). S1 nuclease-protection was performed in 280 mM NaCl, 30 mM sodium acetate (pH 4.6), 4.5 mM $ZnSO_4$, 200 U of S1 nuclease per ml (Sigma), and 20 μg of single-stranded DNA per ml at 20°C for 1 h. Reactions

were terminated by the addition of EDTA to 10 mM, 5 μg of tRNA, and 2.5 volumes of ethanol. Denaturing gel electrophoresis was conducted as described above. The ^{32}P-labeled DNA probe was sequenced by the method of Maxam and Gilbert (47) for use as a marker.

Gel filtration of transcription complexes. Sepharose CL-4B-200 (Sigma) was equilibrated at 4°C in transcription buffer supplemented with 20 μg of acetylated bovine serum albumin per ml, 80 μg of heparin per ml, and 30 mM KCl, and it was formed into a 9-ml-bed-volume column. Standard transcription reactions (100 μl) containing *Xba*I-cut pRL558 that had been elongated either at 30 mM KCl for 30 min, at 400 mM KCl for 4 min, or at 400 mM KCl for 30 min were chilled rapidly to 0°C and then combined. RNA size markers (230 and 690 nt) were added to mark the position of free RNAs, and the mixture was filtered through the Sepharose 4B column at 4°C. Fractions (~250 μl) containing radioactivity were collected and divided into equal aliquots. One aliquot of each pair was stored at 4°C, while the other was incubated in the presence of 1 mM NTPs at 30°C for 10 min. All samples then were phenol extracted and prepared for electrophoresis as described above.

Determination of % T. Radioactivity in the full-length and arrested RNA bands was quantitated by scanning wet gels, after autoradiography was performed, with an AMBIS radioanalytic imaging system. After correcting for the specific activity of the runoff RNAs and terminated RNAs and after making the appropriate background correction, the percentage of termination (% T) was calculated as moles terminated RNA/(moles terminated RNA + moles runoff RNA). In some cases, measurements also were made by scanning autoradiograms with a Molecular Dynamics model 300 computing densitometer. These films were prepared either by direct autoradiography or by using presensitized film and an intensifying screen, which are conditions that produce a linear film response to radioactivity. The integrated optical density in bands corresponding to full-length and terminated RNAs was converted to % T by using the corrections and formula described above.

RNase T1 digestion reactions. Both runoff and terminated RNAs generated as described above were excised from polyacrylamide gels, purified by elution into 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS, and precipitated with ethanol. RNA pellets were washed once with 70% ethanol, dried in vacuo, and resuspended in 5 μl of TE buffer. Five microliters of RNase T1 (2,000 U/ml; Sankyo) was added, and the samples were incubated at 37°C for 20 min. The reactions were terminated by the addition of 10 μl of urea-saturated loading buffer (~10 M urea, 0.1% bromphenol blue, 0.1% xylene cyanol, 88 mM Tris · borate [pH 8.3], and 2.5 mM EDTA) and electrophoresed as described above. To digest total RNAs from transcription reactions, the reactions were treated as described above, except that prior to electrophoresis the samples were resuspended in 5 μl of TE buffer that contained 1,000 U of RNase T1 per ml and incubated for 30 min at 37°C. The reactions were terminated by the addition of an equal volume of urea-saturated loading buffer and electrophoresed as described above.

RESULTS

Efficient transcriptional arrest at the c-*myc* T_{II} site occurs in the presence of 400 mM KCl, but not under standard conditions or in the presence of sarkosyl or heparin. To determine whether sequences present near the first exon-intron junction of the human c-*myc* gene cause either pausing or termination during transcription, we transcribed two different c-*myc* templates with nuclear extracts from HeLa cells. One template contained the wild-type human c-*myc* P2 promoter from −95 to the P2 initiation site at +1 and the downstream c-*myc* sequences to +780 (P2-*myc*; Fig. 1A). The other contained a precise substitution of the Ad2MLP for the c-*myc* P2 promoter (Ad2-*myc*; Fig. 1B). Thus, pol II that initiated transcription on either template produced an identical RNA transcript, and any differences in elongation behavior could be attributed to promoter-specific effects. On average, we observed 6.5-fold more 780-nt runoff transcript from the Ad2-*myc* template than from the P2-*myc* template (data not shown). Termination or pausing at the c-*myc* T_I and T_{II} sites from either template would produce RNAs of 366 and 430 nt, respectively.

As an initial test of transcriptional elongation through the first c-*myc* exon-intron region, we performed single-round transcription reactions by using the *Xba*I-cleaved Ad2-*myc* template (see Materials and Methods). In standard transcription reactions (69), we observed a full-length, amanitin-sensitive transcript of 780 nt that corresponded to transcription from +1 to the *Xba*I site by pol II (Fig. 2, lanes 1 and 2). This transcript was produced within 5 min of the addition of NTPs (data not shown). No significant amounts of prematurely arrested RNAs were detected in any of our experiments by using these standard reaction conditions (~2 mg of HeLa nuclear protein per ml, 6 mM HEPES [pH 7.9], 33 mM KCl, 8 mM $MgCl_2$, 6% glycerol). However, when either sarkosyl to 0.25% (Fig. 2, lane 3) or heparin to 2 mg/ml (Fig. 2, lane 6) was added, transcripts appeared that corresponded to arrest at numerous sites, among which T_I and T_{II} were not especially prominent. Most, if not all, of these arrested transcripts were still present after the addition of all four NTPs to a final concentration of 1 mM and incubation for 10 min (Fig. 2, lanes 4 and 7). This suggests that these RNAs did not result from transcriptional pausing but that either the RNAs were released from the transcription complex or, if still associated with the template, were in a conformation that could not be elongated.

In contrast, raising the concentration of KCl to 400 mM during elongation caused a significant accumulation of RNAs apparently ending at T_{II}, much less T_I RNA, and some, but not all, of the other prematurely arrested transcripts that were stimulated by sarkosyl and heparin (Fig. 2, lane 9). Neither the T_{II} nor the T_I transcripts induced by elevated KCl were elongated upon the addition of NTPs to 1 mM (Fig. 2, lane 10). However, a transcript (P; Fig. 2), whose 3′ end was at A-350 (40) within the *Pvu*II site (Fig. 1D) was chased by the addition of elevated concentrations of NTPs and thus appeared to be a paused transcript (Fig. 2, lane 10). That pol II pauses immediately before G-351 suggests that elongation at this position has an unusually high K_s for GTP.

Interestingly, the addition of either sarkosyl or KCl significantly reduced the elongation rate of pol II, so that production of the full-length 780-nt transcript required about 20 min (data not shown, but see Fig. 3). All of the prematurely arrested transcripts produced in the presence of sarkosyl, heparin, or elevated KCl were sensitive to 2 μg of amanitin per ml (Fig. 2, lanes 5, 8, and 11). Amanitin-resistant transcripts were observed occasionally, but only in the absence of perturbing agents (for examples, see Fig. 3A and B, lanes 2). In similar reactions, the P2-*myc* template produced qualitatively similar transcripts in the presence of sarkosyl, heparin, or elevated KCl (data not shown).

To analyze more carefully the effect of elevated KCl, we

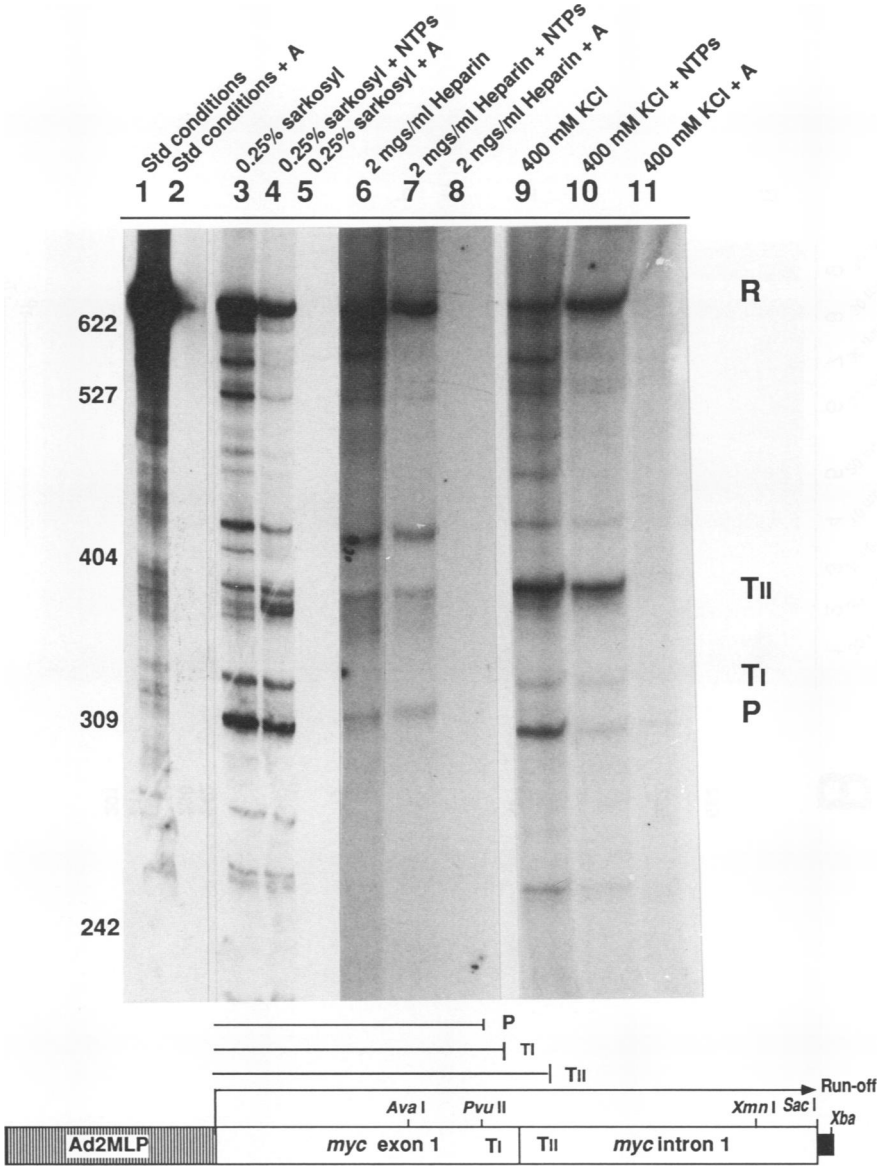

FIG. 2. Transcripts produced during transcription by the Ad2-*myc* template (pRL558) in the absence or presence of sarkosyl, heparin, or elevated KCl. Transcription reactions were performed at limiting concentrations of GTP by using extract 1 (lanes 1 to 5 and 9 to 11; see Table 1 for extract descriptions) or extract 2 (lanes 6 to 8) as described in Materials and Methods. Sarkosyl (lanes 3 to 5), heparin (lanes 6 to 8) or KCl (lanes 9 to 11) was added 30 s after the addition of NTPs. RNAs were recovered as described in Materials and Methods and electrophoresed through a 4% polyacrylamide–7 M urea–TBE buffer gel. Lanes: 1, no additions; 2, same as lane 1, but 2 μg of amanitin per ml added; 3, 0.25% sarkosyl; 4, same as lane 3, but NTPs were added to 1 mM after 30 min and the reaction continued for 10 min; 5, same as lane 3, but 2 μg of amanitin per ml added; 6, 2 mg of heparin per ml; 7, same as lane 6, but NTPs were added to 1 mM after 30 min and the reaction continued for 10 min; 8, same as lane 6, but 2 μg of amanitin per ml added; 9, 400 mM KCl; 10, same as lane 9, but NTPs were added to 1 mM after 30 min and the reaction continued for 10 min; 11, same as lane 9, but 2 μg of amanitin per ml added. R, position of the full-length runoff transcript; T_{II}, position of transcripts stopped near the c-*myc* T_{II} site; T_I, position of transcripts stopped near the c-*myc* T_I site; P, position of transcripts paused near the *Pvu*II site (see text). The positions and sizes (in nucleotides) of the pBR322 *Hpa*II fragments are indicated on the left.

performed time-course experiments using the Ad2-*myc* and P2-*myc* templates. Both the Ad2-*myc* and P2-*myc* templates produced the prominent arrested transcript of ~430 nt corresponding to stopping at c-*myc* T_{II} (Fig. 1 and 2; Fig. 3A and B, lanes 3 to 9). In addition, both the P and T_I RNAs were evident with both templates. Most of the P transcript, but neither the T_I nor the T_{II} transcripts, appeared to chase in the presence of 1 mM NTPs (Fig. 3A and B, lanes 7). Although in these particular experiments a greater fraction of transcripts appeared to halt at T_{II} when pol II initiated transcription at the c-*myc* P2 promoter (compare lanes 7 in Fig. 3A and B), we have not usually observed major differences in the elongation behavior of pol II transcribing the Ad2-*myc* and P2-*myc* templates. The strong arrest of transcription at T_{II} was not caused by the limiting GTP (18 μM) in the reaction, since there are no G's in this T-rich region of

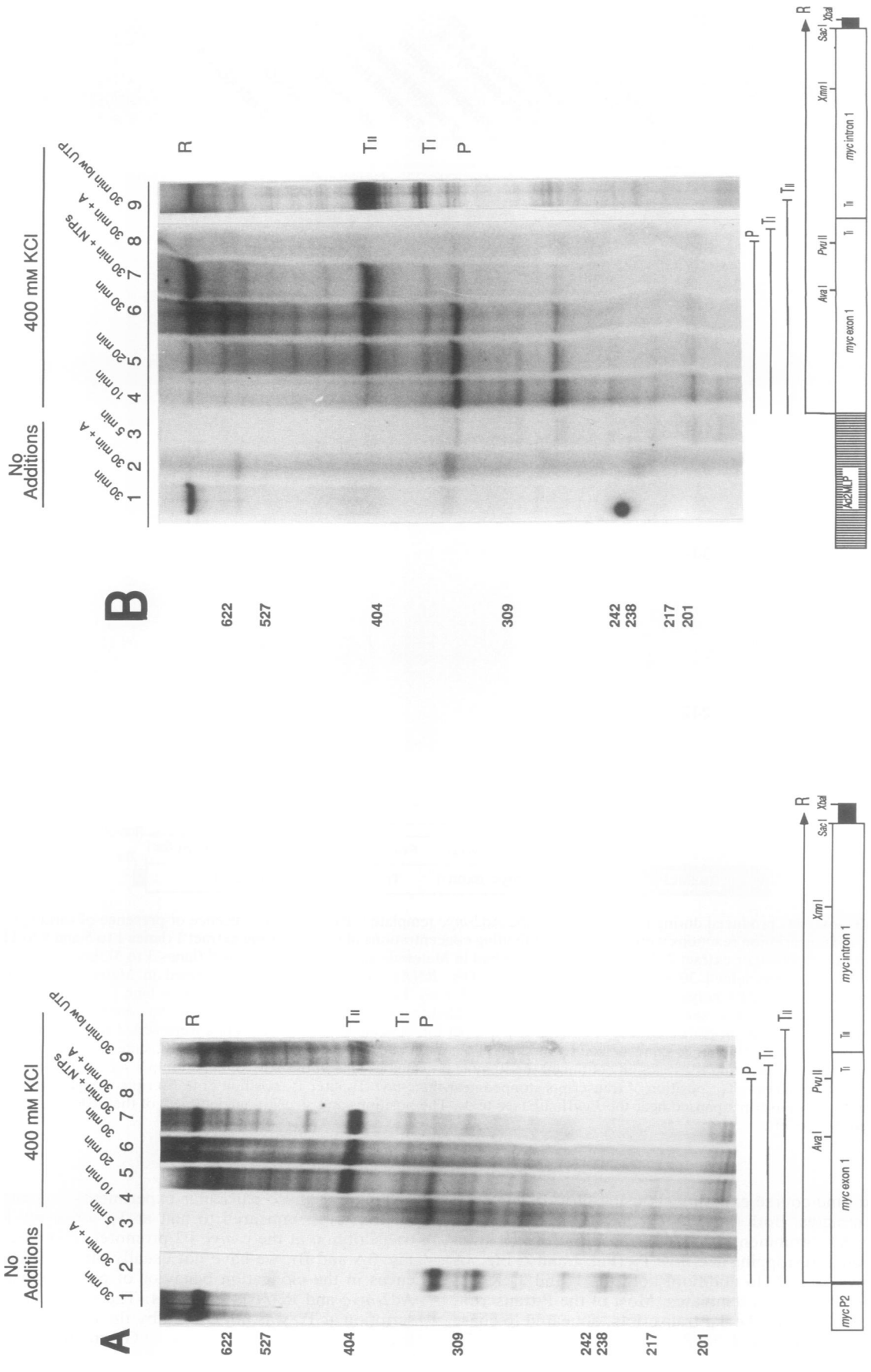
A
B
No Additions
400 mM KCl
1 2 3 4 5 6 7 8 9
30 min
30 min + A
5 min
10 min
20 min
30 min
30 min + NTPs
30 min + A
30 min low UTP
R
TII
TI
P
622
527
404
309
242
238
217
201
myc P2
myc exon 1
myc intron 1
AvaI
PvuII
XmnI
SacI
XbaI
TI
TII
A2MLP

FIG. 3. Time course of the effect of 400 mM KCl on transcriptional elongation through the first c-*myc* exon-intron region in a HeLa nuclear extract. Transcription reactions were conducted by using extract 1 (Table 1) as described in the legend to Fig. 2, except that aliquots were removed from reactions at the times indicated and electrophoresed on a 4% polyacrylamide–7 M urea–TBE buffer gel. The positions of size markers (in nucleotides) from *Hpa*II-cleaved pBR322 are indicated on the left. R, position of the full-length runoff transcript. TII, position of transcripts stopped near the c-*myc* T_{II} site; TI, position of transcripts stopped near the c-*myc* T_I site; P, position of transcripts stopped near the *Pvu*II site (see text). The background transcription that is evident in certain lanes (e.g., see Fig. 3B, lanes 3 to 6) results from nonspecific pol II transcription, presumably of DNAs that remain in the nuclear extracts. (A) P2-*myc* template (*Xba*I-cleaved pLL253; Fig. 1A). Lane 1, 30-min reaction without added sarkosyl or KCl. Lane 2, same as lane 1, but with 2 μg of amanitin per ml added before preincubation. Lanes 3 to 9, KCl added to 400 mM 30 s after the addition of NTPs. Aliquots were removed at 5 min (lane 3), 10 min (lane 4), 20 min (lane 5), and 30 min (lane 6). Lane 7, 1 mM NTPs were added to the reaction 30 min after the addition of KCl, and the reaction was continued for 10 min. Lane 8, same as lane 6, but with 2 μg of amanitin per ml added before preincubation. Lane 9, same as for lane 6, but with a limiting concentration of UTP (18 μM) and with 160 μM GTP. (B) Ad2-*myc* template (*Xba*I-cleaved pRL558; Fig. 1B). Lanes 1 and 2 are as in panel A. Lanes 3 to 9, KCl added to 400 mM 30 s after the addition of NTPs. Aliquots were removed at 5 min (lane 3), 10 min (lane 4), 20 min (lane 5), and 30 min (lane 6). Lane 7, 1 mM NTPs were added to the reaction 30 min after the addition of KCl, and the reaction was continued for 10 min. Lane 8, same as lane 6, but with 2 μg of amanitin per ml added before preincubation. Lane 9, same as lane 6, but with a limiting concentration of UTP (18 μM) and with 160 μM GTP.

c-*myc* (Fig. 1D). When we instead used limiting UTP in the presence of 400 mM KCl, a much greater fraction of transcription complexes became arrested at T_{II} (Fig. 3A and B, lanes 9), consistent with the notion that RNA polymerase was stalling in the T-rich T_{II} sequence element.

The appearance of the T_{II} transcript could be attributed either to transcriptional arrest or to processing of a longer transcript. To test for processing, we incubated gel-purified, 780-nt runoff RNA, as well as several T7 RNA polymerase transcripts that contained equivalent c-*myc* sequences, in mock transcription reactions containing 33 or 400 mM KCl. We never observed the appearance of a 430-nt RNA, nor did the added RNAs degrade substantially (data not shown). Furthermore, the appearance during transcriptional elongation of the T_{II} RNA precedes that of the full-length transcript (Fig. 3A and B).

The 430-nt T_{II} transcript begins at the P2 cap site and stops in the c-*myc* T_{II} site. To verify that the apparent Ad2-*myc* and P2-*myc* transcripts were properly initiated at the expected sites, we purified both runoff and prematurely arrested RNAs by eluting them from polyacrylamide gels and then used a primer-extension assay to determine their 5′ ends. All of the transcripts that we tested (Ad2-*myc* and P2-*myc* runoff, T_{II} from P2-*myc*, and T_{II} and P from Ad2-*myc*) gave primer extension products that corresponded to initiation at the expected sites (Fig. 4B).

To determine the 3′ ends of the most important arrested transcript, the T_{II} RNA, we performed an S1 nuclease-protection assay, using as a probe a single-stranded c-*myc* *Ava*I-*Sac*I DNA that was 3′ end labeled at the *Ava*I site (Fig. 4C). When T_{II} transcripts from GTP- and UTP-limited reactions were subjected to high-resolution gel electrophoresis, three major attenuated RNA bands were apparent (T_{IIa}, T_{IIb}, and T_{IIc}; Fig. 4A). We attempted to excise and purify these RNAs from the gel separately, with only partial success (see below), and to 3′-end map them with an S1 nuclease-protection assay. This assay was complicated by persistent cleavage in the T_I and T_{II} regions whenever RNAs extending past these sites were present (e.g., see Fig. 4C, lane 11). In control experiments, using T3 RNA polymerase transcripts that extended beyond the T_I and T_{II} region, we tested a wide variety of conditions for the S1 nuclease digestion and always observed cutting at T_I and T_{II}. Either the RNA-DNA hybrids in these regions are exceptionally unstable and thus are cut when they "breathe" or they form an unusual structure that is susceptible to cleavage by S1 nuclease. Regardless, we were able to assign approximate 3′ ends to the T_{II} RNAs because DNA cleavage products with 3′ ends in T_I or T_{II} were not observed either when RNAs spanning these regions were absent (Fig. 4C, lane 5) or when RNAs with 3′ ends which did not extend past T_I or T_{II} were present (Fig. 4C, lane 12). Although the T_{IIa}, T_{IIb}, and T_{IIc} RNAs appeared to be extensively cross-contaminated, each produced a distinct distribution of S1 cleavage sites. Typically, in S1 nuclease assays heterogeneous cleavage of the probe results from an activity that attacks RNA-DNA hybrid termini (13). Thus, when a control RNA known to end at G-351 was used, the major S1 digestion products had 3′ ends at and slightly 5′ of the corresponding end of the RNA (Fig. 4C, lane 12 and Fig. 4D). By comparing this result with those obtained with the c-*myc* T_{II} RNAs, we assigned the 3′ ends of T_{IIa}, T_{IIb}, and T_{IIc} as approximately T-428, T-423, and T-418, respectively (Fig. 4D). Interestingly, the T_{IIb} and T_{IIc} RNAs appeared to be the predominant products when limited UTP was present, whereas only T_{IIa} and T_{IIb} were evident when limited GTP was used. This suggests that arrest within the T_{II} element occurred more efficiently at low UTP concentrations, but it also confirms that significant arrest occurred even when GTP was the limiting nucleotide.

Formation of the T_{II} RNA results from transcriptional termination. To distinguish between transcriptional pausing and true termination, which necessitates the release of the transcript from the transcription complex, we tested whether T_{II} RNA formed at elevated KCl would migrate with active transcription complexes during gel filtration. We have found that paused transcription complexes can be separated from free RNA species formed in a HeLa nuclear extract by filtration through Sepharose CL-4B (preliminary data not shown, but see Fig. 5). Transcripts which elute in the void volume are bound to RNA polymerase (and DNA template) and can be elongated when NTPs are added back, whereas free RNAs are retarded by the gel filtration matrix. When a mixture of transcription complexes with short Ad2-*myc* transcripts and complexes that had formed T_{II} and runoff transcripts were combined with RNA size markers and filtered (see Materials and Methods), the paused transcription complexes eluted in the void volume and could elongate their transcripts upon the addition of NTPs to a concentration of 1 mM (Fig. 5). Interestingly, although the paused complexes could be elongated after gel filtration, they tended to stop at several positions, including the c-*myc* T_I site (Fig. 5, lanes 5 and 7), presumably because elongation factors such as TFIIS were removed during gel filtration (5). However, the majority of the T_{II} and runoff RNA species were retained on the column and eluted with the 230- and 690-nt RNA size markers (Fig. 5). Further, neither the T_{II} nor

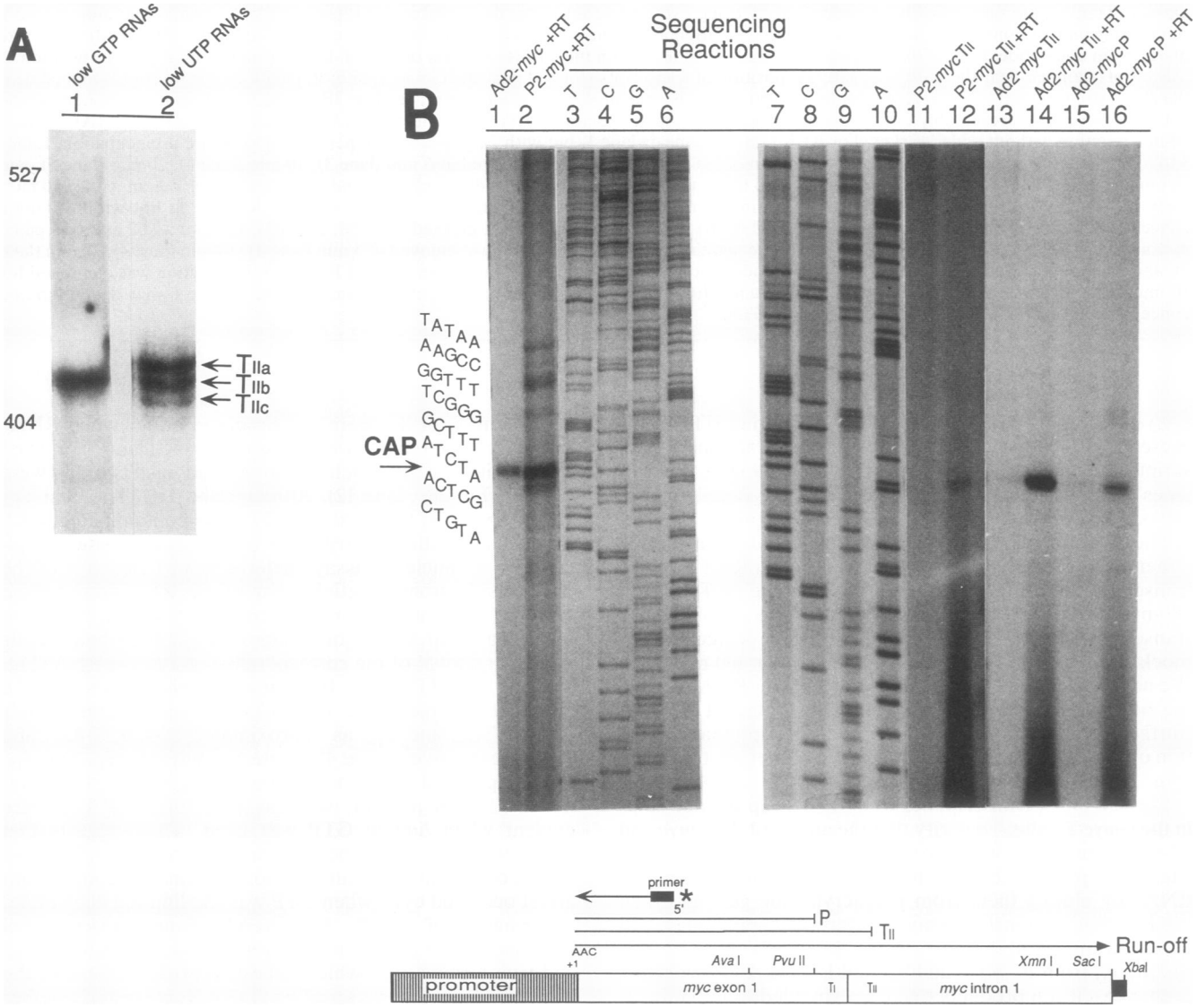

FIG. 4. Definition of the 5′ and 3′ ends of important c-*myc* transcripts. (A) High-resolution separation of the T_{II} RNAs transcribed from *Xba*I-cleaved pRL558 in the presence of 400 mM KCl by using extract 2 (Table 1) (see legend to Fig. 2). The T_{II} RNAs were electrophoresed about 44 cm through a 4% polyacrylamide–7 M urea–TBE buffer gel (0.4 mm by 58 cm). The positions at which pBR322 *Hpa*II size markers (in nucleotides) migrated are indicated on the left. On the right, TIIa, TIIb, and TIIc mark the positions of discrete bands evident within the T_{II} band. Lane 1, T_{II} RNAs made in the presence of 18 μM GTP; lane 2, T_{II} RNAs made in the presence of 18 μM UTP. (B) Primer-extension mapping of the 5′ ends of c-*myc* transcripts. Lanes 3 to 6 and 7 to 10, DNA sequencing reactions derived by dideoxy-nucleotide chain termination sequencing of pLL253 with the primer used for reverse transcriptase reactions (see Materials and Methods). The 123-nt primer extension product corresponding to the normal P2 cap site is denoted by an arrow. Reverse transcriptase reactions were performed on RNAs (Fig. 3A and B) corresponding to the Ad2-*myc* runoff transcript (lane 1), the P2-*myc* runoff (lane 2), the P2-*myc* T_{II} transcript (lane 12), the Ad2-*myc* T_{II} transcript (lane 14), and the Ad2-*myc* P transcript (lane 16). The products of control reactions containing the RNAs present in lanes to the right but lacking reverse transcriptase were electrophoresed in lanes 11, 13, and 15. Transcripts were prepared by using either extract 2 (lanes 1 and 2) or extract 1 (lanes 11 to 16) (see Table 1). (C) S1 nuclease-protection mapping of the 3′ ends of the c-*myc* T_{II} RNAs. Assays were performed as described in Materials and Methods, using RNAs prepared with extract 2 (Table 1). Lanes 1 to 4, chemical sequencing ladders generated from the probe DNA. The following RNAs, in addition to 0.5 μg of carrier tRNA, were added to the S1 assay. Lane 5, no added RNA; lane 6, T_{IIa} from Fig. 4A, lane 1; lane 7, T_{IIb} from Fig. 4A, lane 1; lane 8, T_{IIa} from Fig. 4A, lane 2; lane 9, T_{IIb} from Fig. 4A, lane 2; lane 10, T_{IIc} from Fig. 4A, lane 2; lane 11, a T3 RNA polymerase transcript with the 3′ end at the *Xmn*I site (Fig. 1A); lane 12, a T3 RNA polymerase transcript with the 3′ end at the *Pvu*II site (Fig. 1A). X, position of the protected band from the *Xmn*I RNA in lane 11. TIIa, TIIb, and TIIc mark the positions of protected species that result from the T_{IIa}, T_{IIb}, and T_{IIc} RNAs in lanes 6 to 10. TI marks the position of cleavage of the probe at the T_I site in lanes containing RNAs that extend past this site. P marks the distribution of protected species that result from the *Pvu*II RNA in lane 12. Note that the curvature of the electrophoretic paths of samples is responsible for the apparent superposition of lanes 11 and 12. (D) Sequence of the first c-*myc* exon-intron junction with the positions of S1 cleavage indicated. The different-sized arrows indicate the relative integrated optical densities of bands resulting from the different cleavages as determined with a Molecular Dynamics computing densitometer. Large arrows, strong cleavage; small arrows, weak cleavage.

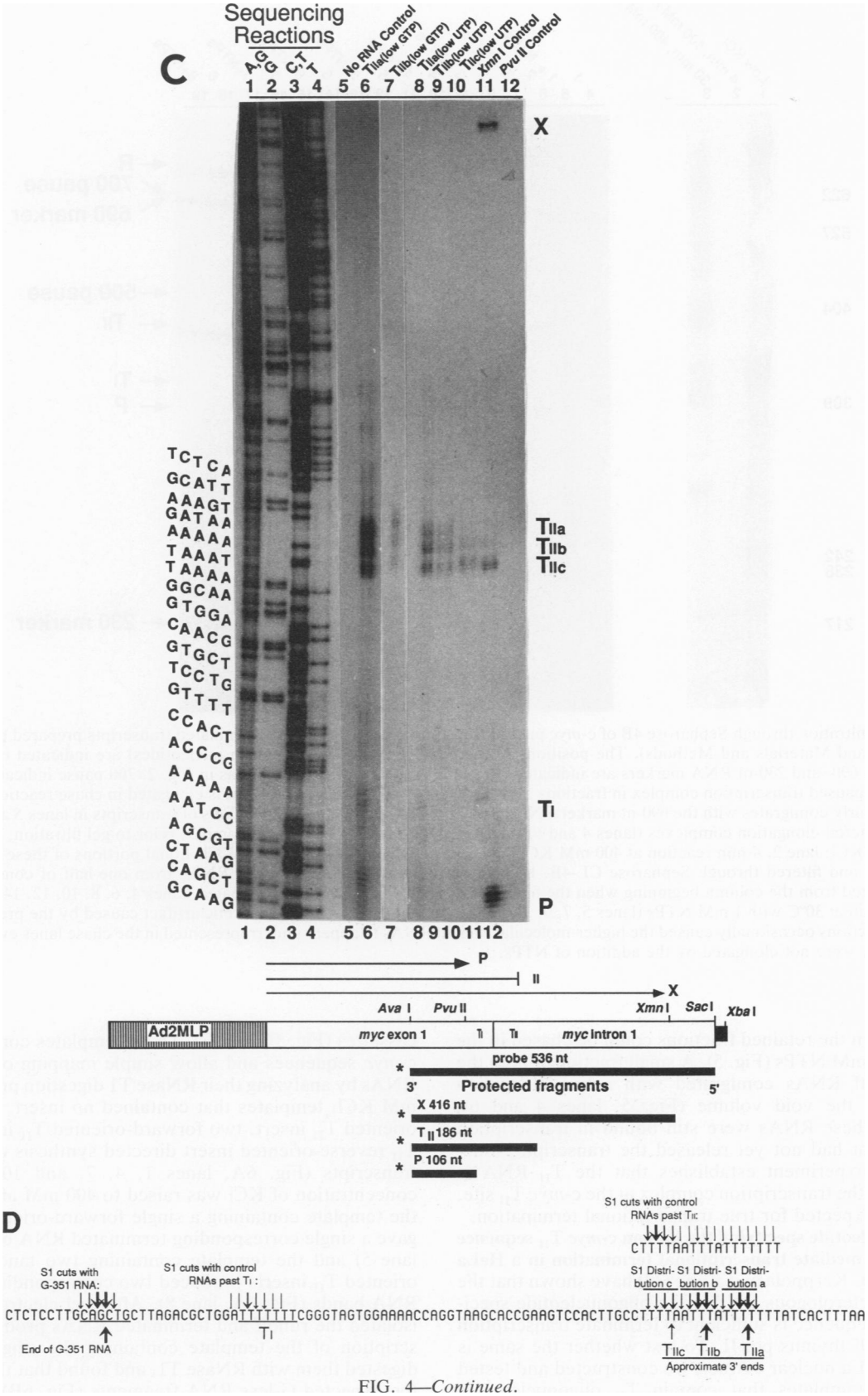

FIG. 4—*Continued.*

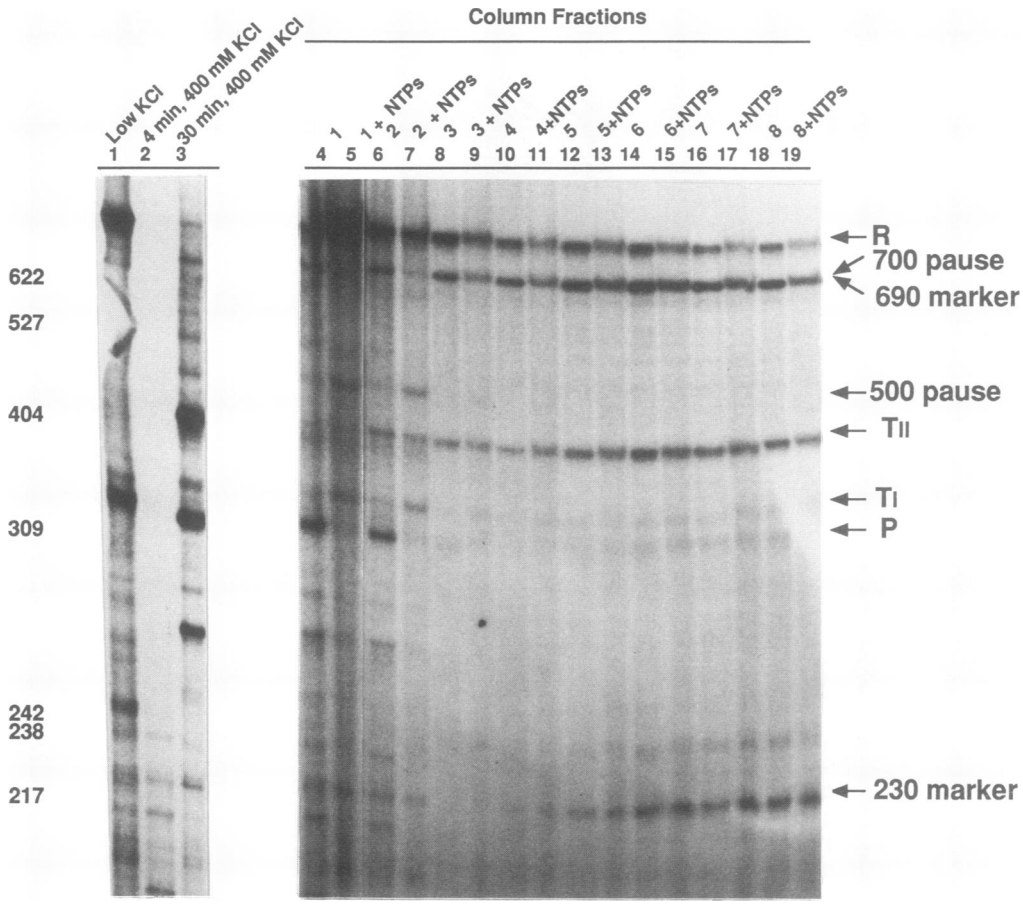

FIG. 5. Gel filtration through Sepharose 4B of c-*myc* paused transcription complexes and terminated transcripts prepared by using extract 1 (see Table 1 and Materials and Methods). The positions of the pBR322 *Hpa*II fragments (in nucleotides) are indicated on the left. The positions of the 690- and 230-nt RNA markers are indicated on the right. R, TII, TI, and P are as in Fig. 2. 700 pause indicates the position of RNA from a paused transcription complex in fractions 1 and 2 (lanes 4 and 6) whose transcript is elongated in chase reactions (lanes 5 and 7) and which nearly comigrates with the 690-nt marker RNA. 500 pause and T_I indicate the positions of transcripts in lanes 5 and 7 that result when the gel-filtered elongation complexes (lanes 4 and 6) are chased with NTPs. Left panel, samples prior to gel filtration. Lane 1, 30-min reaction at low KCl; lane 2, 4-min reaction at 400 mM KCl; lane 3, 30-min reaction at 400 mM KCl. Equal portions of these three reactions were combined and filtered through Sepharose CL-4B. Right panel, alternating lanes contain RNAs from one-half of consecutive 250-μl fractions collected from the column beginning when the first radioactivity emerged, either untreated (lanes 4, 6, 8, 10, 12, 14, 16, and 18) or chased for 10 min at 30°C with 1 mM NTPs (lanes 5, 7, 9, 11, 13, 15, 17, and 19). An electrophoretic artifact caused by the presence of NTPs in the chase reactions occasionally caused the higher-molecular-weight RNAs to appear underrepresented in the chase lanes even though they almost certainly were not elongated by the addition of NTPs.

runoff RNAs in the retained fractions could be chased in the presence of 1 mM NTPs (Fig. 5). A small fraction of both the T_{II} and runoff RNAs comigrated with active elongation complexes in the void volume (Fig. 5, lanes 4 and 6); presumably, these RNAs were still bound in transcription complexes that had not yet released the transcript. Nonetheless, this experiment establishes that the T_{II} RNA is released from the transcription complex at the c-*myc* T_{II} site, as would be expected for true transcriptional termination.

An oligonucleotide specifying the human c-*myc* T_{II} sequence is sufficient to mediate transcriptional termination in a HeLa nuclear extract. Kerppola and Kane (34) have shown that the presence in heterologous DNA of an oligonucleotide specifying the T_{II} sequence is sufficient to terminate transcription by purified calf thymus pol II. To test whether the same is true in the HeLa nuclear extract, we constructed and tested transcription templates that contain T_{II} oligonucleotides between 262-bp (upstream) and 131-bp (downstream) G-less cassettes (Fig. 1C and E). These templates contain no other c-*myc* sequences and allow simple mapping of the product RNAs by analyzing their RNase T1 digestion products. At 33 mM KCl, templates that contained no insert, one forward-oriented T_{II} insert, two forward-oriented T_{II} inserts, or one T_{II} reverse-oriented insert directed synthesis of only runoff transcripts (Fig. 6A, lanes 1, 4, 7, and 10). When the concentration of KCl was raised to 400 mM after initiation, the template containing a single forward-oriented T_{II} insert gave a single corresponding terminated RNA band (Fig. 6A, lane 5) and the template containing two tandem forward-oriented T_{II} inserts produced two corresponding terminated RNA bands (Fig. 6A, lane 8). After gel electrophoresis, we isolated the runoff and terminated RNAs produced by transcription of the template containing the single T_{II} insert, digested them with RNase T1, and found that they produced the expected G-less RNA fragments (Fig. 6B). Neither the template lacking a T_{II} insert nor the one containing the T_{II}

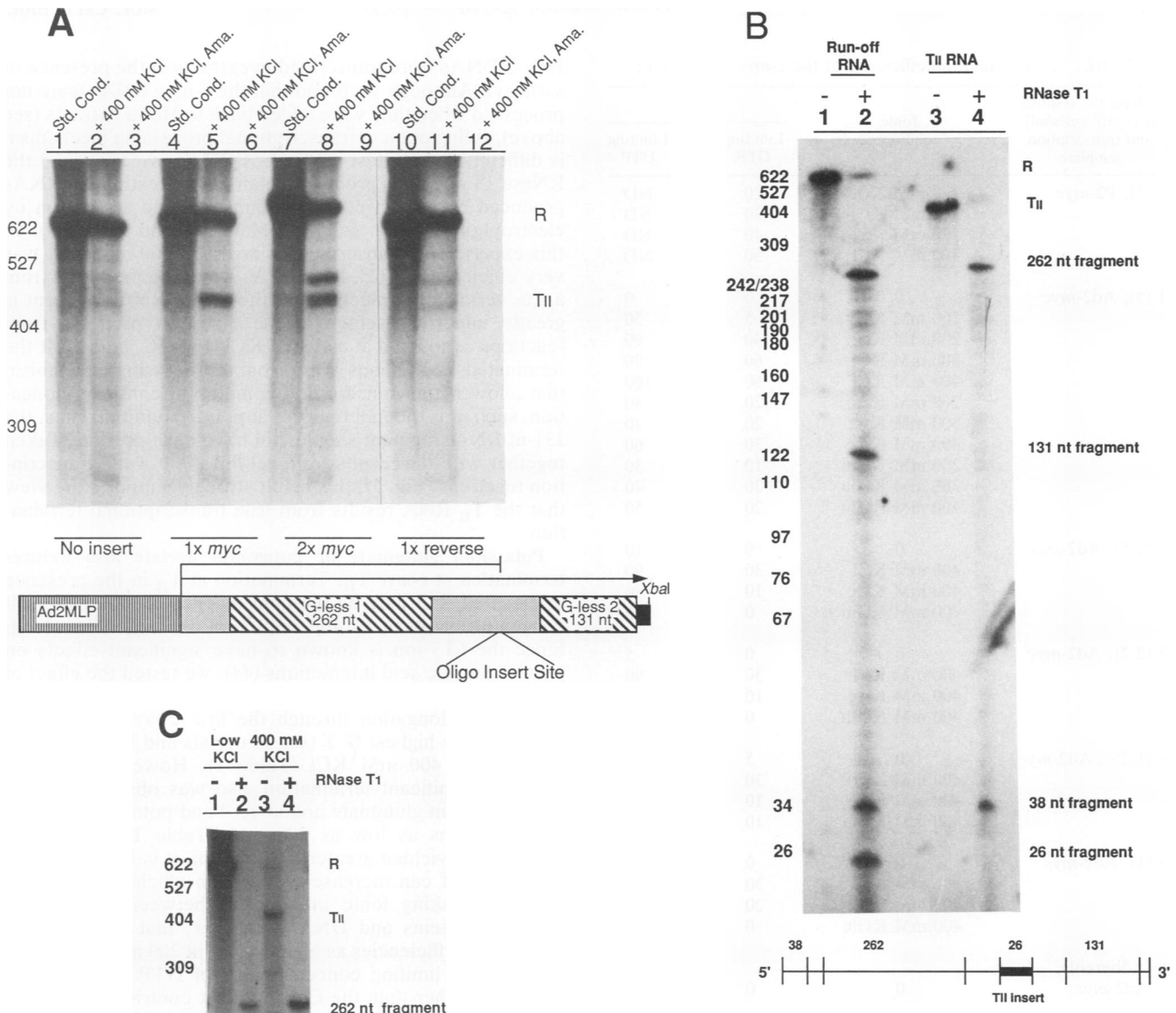

FIG. 6. Autoradiograms obtained from gels that were used to separate the transcription products of G-less cassette plasmids containing synthetic T_{II} oligonucleotides. Reactions were performed under standard conditions or with the addition of 400 mM KCl, and electrophoresis was conducted as described in Materials and Methods. Reactions were performed at limiting concentrations of UTP for 30 min, rather than at limiting concentrations of GTP, in order to label the G-less RNA fragments. (A) Transcripts prepared by using extract 2 (Table 1) from *Xba*I-cleaved pRL542, pRL578, pRL575, and pRL577 (Fig. 1D and E). The positions of pBR322 *Hpa*II size markers (in nucleotides) are shown on the left. The positions of the runoff (R) and T_{II} (TII) RNAs are indicated on the right. Lanes 1 to 3, pRL542 (no insert); lanes 4 to 6, pRL578 (one c-*myc* T_{II} insert in the forward orientation); lanes 7 to 9, pRL575 (two c-*myc* T_{II} inserts in the forward orientation); lanes 10 to 12, pRL577 (one c-*myc* T_{II} insert in the reverse orientation); lanes 1, 4, 7, and 10, 33 mM KCl added before preincubation; lanes 2, 5, 8, and 11, 400 mM KCl added as described for lanes 1, 4, 7, and 10, but after preincubation; lanes 3, 6, 9, and 10, 400 mM KCl with 2 μg of amanitin per ml added before preincubation. All reactions were incubated with 1 mM NTPs for 10 min after the standard 30 min incubation. (B) Ribonuclease T1 RNA fragments generated by cleavage of isolated runoff and T_{II} RNAs. The positions of pBR322 *Hpa*II size markers (in nucleotides) are shown on the left. The positions of the expected RNase T1 fragments are shown on the right and in the diagram at the bottom. Lane 1, runoff RNA without RNase T1; lane 2, runoff RNA with RNase T1; lane 3, T_{II} RNA without RNase T1; lane 4, T_{II} RNA with RNase T1. (C) Ribonuclease T1 RNA fragments generated by cleavage of the transcription products from *Xba*I-cleaved pRL558 (Ad2-*myc*; Fig. 1B) prepared by using extract 1. The positions of pBR322 *Hpa*II size markers (in nucleotides) are shown on the left. The positions of the 262- and 131-nt T1 RNA fragments are indicated on the right. Lane 1, transcripts produced at low KCl and not treated with RNase T1; lane 2, transcripts produced at low KCl and then treated with RNase T1; lane 3, transcripts produced at 400 mM KCl and not treated with RNase T1; lane 4, transcripts produced at 400 mM KCl and then treated with RNase T1.

TABLE 1. Termination efficiency at the c-*myc* T_{II} element

Extract no. (culture titer [10^5 cells/ml] and transcription template)	Ionic conditions[a]	% T[b]	
		Limiting GTP	Limiting UTP
1 (5), P2-*myc*	100 mM KCl	20	ND[c]
	200 mM KCl	30	ND
	300 mM KCl	40	ND
	400 mM KCl	50	ND
1 (5), Ad2-*myc*	0	0	0
	100 mM KCl	5	50
	200 mM KCl	40	90
	300 mM KCl	60	90
	400 mM KCl	60	100
	200 mM KAc	20	30
	300 mM KAc	20	40
	400 mM KAc	30	60
	200 mM KGlu	10	30
	300 mM KGlu	20	40
	400 mM KGlu	20	50
2 (2.5), Ad2-*myc*	0	0	10
	400 mM KCl	30	90
	400 mM KAc	10	50
	400 mM KGlu	0	30
3 (2.2), Ad2-*myc*	0	0	5
	400 mM KCl	30	90
	400 mM KAc	10	30
	400 mM KGlu	0	10
4 (1.25), Ad2-*myc*	0	5	10
	400 mM KCl	30	60
	400 mM KAc	10	20
	400 mM KGlu	10	5
5 (1), Ad2-*myc*	0	0	5
	400 mM KCl	30	70
	400 mM KAc	20	30
	400 mM KGlu	0	10
6 (Adherent), Ad2-*myc*	0	0	ND
	400 mM KCl	20	ND
	400 mM KAc	10	ND
	400 mM KGlu	0	ND

[a] In addition to the 33 mM KCl normally present in the transcription reaction. KAc, potassium acetate; KGlu, potassium glutamate.

[b] % T, percentage of termination. Transcription reactions were conducted with 160 μM NTPs, as described in Materials and Methods, except that either 18 μM GTP (limiting GTP) or 18 μM UTP (limiting UTP) was used. Data are rounded to a single significant figure.

[c] ND, not determined.

insert in reverse orientation produced a prematurely terminated RNA (Fig. 6A, lanes 1, 2, 10, and 11). Thus, as with purified pol II, the T_{II} sequence itself is sufficient to program transcriptional termination in a HeLa nuclear extract at elevated KCl. Further, transcription complexes that fail to terminate at a T_{II} sequence remain capable of termination, since roughly the same efficiency of termination was observed at a second T_{II} sequence when it was present downstream (Fig. 6A, lane 8).

One important question regarding termination at the c-*myc* T_{II} site is whether it results from cotranscriptional processing. Such a phenomenon was postulated by Toohey and Jones (76) to explain truncated RNAs that were produced when human immunodeficiency virus type 1 (HIV-1) and HIV-2 DNAs were transcribed in extracts in the presence of sarkosyl. Although we found that the c-*myc* RNAs were not processed when they were added back to HeLa extracts (see above), ruling out a cotranscriptional processing mechanism is difficult. As one test of this possibility, we examined the RNase T1 digestion products obtained by treating the RNAs produced by transcription reactions prior to separation by electrophoresis (Fig. 6C) (see Materials and Methods). In this experiment, transcriptional arrest at 400 mM KCl was very efficient (Fig. 6C, lane 3). As would be predicted from a true termination mechanism, the 131-nt RNA fragment is greatly underrepresented in the digestion products from reactions conducted at elevated KCl (Fig. 6C, lane 4). If the terminated RNA bands arose from a processing mechanism that allowed the transcription complex to continue elongation, such as is thought to occur at poly(A) addition sites, the 131-nt RNA fragment should still have been present. Taken together with the results from gel filtration of the transcription reactions (Fig. 5), this result strongly supports the view that the T_{II} RNA results from true transcriptional termination.

Potassium glutamate or potassium acetate also induces termination at c-*myc* T_{II}. Termination at T_{II} in the presence of elevated KCl may be the consequence of either the salt concentration itself or the nature of the anion involved. Since the Cl^- ion is known to have significant effects on protein-nucleic acid interactions (44), we tested the effect of similar concentrations of potassium glutamate or potassium acetate on elongation through the first c-*myc* exon-intron junction. The highest % T (see Materials and Methods) was observed at 400 mM KCl (Table 1). However, in some extracts, significant termination also was observed at 400 mM potassium glutamate and at KCl and potassium acetate concentrations as low as 200 mM (Table 1). Since KCl consistently yielded greater termination, it is likely that the Cl^- ion itself can increase termination efficiency, presumably by masking ionic interactions between proteins or between proteins and DNA. However, that we observed termination efficiencies as high as 20% in 200 mM potassium acetate with limiting concentrations of GTP suggests that something other than the Cl^- ion must contribute to termination at c-*myc* T_{II}.

Termination at T_{II} was more efficient in extracts from suspension cultures at high cell densities. If transcriptional arrest in c-*myc* is due, in part, to differences in some *trans*-acting factor between proliferating and differentiated cells, as might be predicted from previous work (8, 20, 36, 45, 51), then we might expect that different levels of termination would occur in vitro when different sources of cells were used to prepare the nuclear extracts. To test this possibility, we examined termination at T_{II} in five different extracts prepared from HeLa cells grown in suspension at densities ranging from 1×10^5 to 5×10^5 per ml and in an extract prepared from adherent HeLa cells grown on a solid support (plastic tissue culture dishes). All six extracts supported transcription in vitro. However, a wide range of termination efficiencies in different extracts were observed (Table 1; Fig. 7). In general, the extracts prepared from cultures with higher densities yielded the greater termination; the lowest % T was observed by using the extract prepared from adherent HeLa cells (Table 1). This suggests that some variable factor in the extracts was responsible for the efficiency of termination at T_{II}.

To test whether the variable factor was present in limiting concentrations, we performed in vitro transcription reactions by using mixtures of extracts 1 and 6 at various ratios.

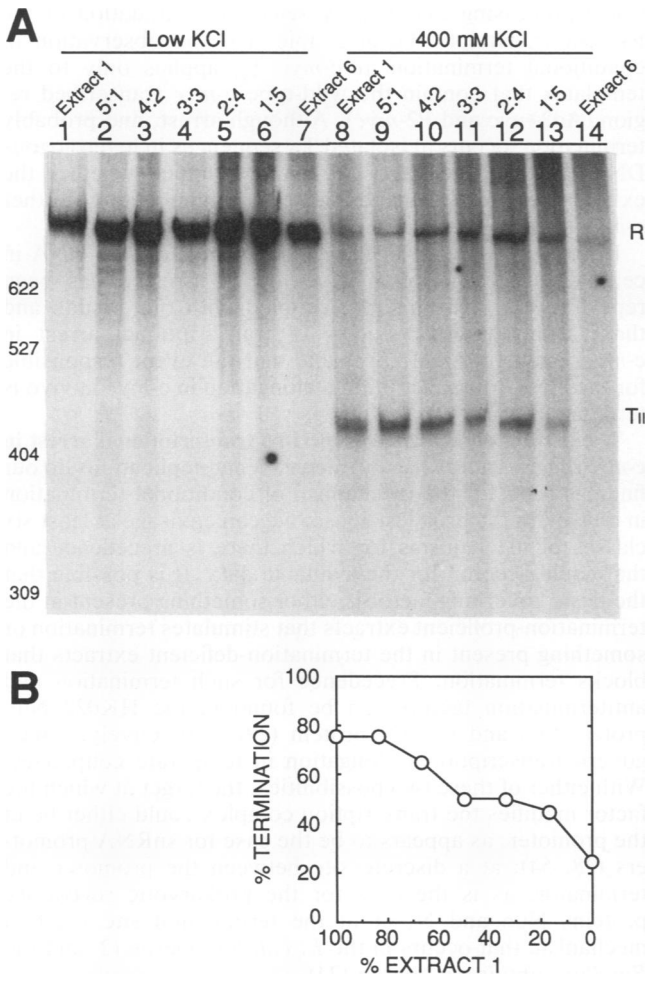

FIG. 7. Comparison of the efficiency of termination at c-*myc* T_{II} during transcription in different HeLa cell nuclear extracts. (A) Autoradiogram obtained from gel that was used to separate the transcription products from *Xba*I-cleaved pRL558 (Ad2-*myc*; Fig. 1B) by using either extract 1 or extract 6 or a mixture of the two (ratios of extract 1 to extract 6 are shown above the lanes; see Table 1), under standard conditions (lane 1 to 7) or in the presence of 400 mM KCl (lane 8 to 14). Reactions in lanes 8 to 14 have been chased with 1 mM NTPs for an additional 10 min after the 30-min incubation. The positions of pBR322 *Hpa*II size markers (in nucleotides) are shown on the left. The positions of the c-*myc* runoff (R) and T_{II} (TII) RNAs are indicated on the right. (B) Plot of percent termination versus percentage of extract 1 in the reactions. The data points (circles) were calculated from the corresponding gel lanes in panel A, as described in Materials and Methods.

At low KCl, each reaction yielded approximately the same amount of runoff transcript and no terminated RNAs (Fig. 7A, lanes 1 to 7). When the KCl concentration was raised to 400 mM after initiation in reactions containing identical ratios of extracts 1 and 6, the % T decreased from ~75 to ~25% in rough proportion to the amount of extract 6 (Fig. 7A, lanes 8 to 14). Thus, there must be at least one factor in these extracts that determines % T at c-*myc* T_{II}. If only one factor is responsible, it either must be unable to act in *trans* or it must be present in limiting concentrations in these extracts.

DISCUSSION

Conditional termination by pol II is poorly understood. Evidence is mounting that control of transcriptional elongation plays an important role in the regulation of gene expression in higher eukaryotes. In addition to the well-studied effects in c-*myc*, which formed the starting point for our work, similar mechanisms appear to control transcription of c-*myb* (3), c-*mos* (48), c-*fos* (18, 50), L-myc (36), and the adenosine deaminase gene (14). Premature arrest also has been detected during the transcription of a variety of viral genomes, including those of simian virus 40, adenovirus, minute virus of mice, polyoma virus (reviewed in reference 73), and, of great recent interest, HIV, where the TAT RNA-binding protein apparently can override premature arrest caused by the TAR element (31, 59, 68). Where they have been mapped, most of these premature termination events occur in T-rich sequences on the nontranscribed strand. In all cases, the mechanism that causes premature arrest of transcription and the means by which it is regulated remain obscure.

Termination by pol II also has been analyzed downstream from the polyadenylation sites in many genes (reviewed in reference 55), at DNA sequences downstream from the processing site in histone genes (12, 15), and in small nuclear RNA (snRNA) genes (28, 54). However, no consensus sequence for a pol II termination site has emerged from these studies. Of these sites, only the termination sequence downstream from the polyadenylation site in the gastrin gene (66) has any similarity to the T-rich c-*myc* T_{II} site. T-rich sequences also are important determinants of termination by bacterial RNA polymerase (79) and RNA polymerase III (11). In the former case, formation of secondary structure in the nascent transcript immediately upstream from the T-rich sequence is required, whereas four or more T's alone can halt RNA polymerase III. Formation of RNA secondary structure appears to facilitate some cases of termination by pol II (4, 62). In contrast, purified pol II stops at c-*myc* T_{II} even when the nascent transcript remains paired with the template strand (33), a condition that presumably precludes the formation of RNA structures. However, it is clear from studies of bacterial transcription that the same T-rich tract can cause termination either in concert with an upstream RNA hairpin or when persistent RNA-DNA hybridization blocks RNA secondary structure formation (75). On the basis of a correlation between transcriptional termination and gel mobility of a set of DNA fragments containing T-rich cassettes, including a c-*myc* T_{II} sequence, Kerppola and Kane (34) suggested that DNA bending in sequences such as T_{II} may be one determinant of termination by pol II.

There are several types of *trans*-acting factors and sites that may participate in the regulation of premature termination in c-*myc* and similar genes. Some types of factors may act during initiation of transcription and modify the potential of the transcription complex to recognize termination sites. Both termination of snRNA synthesis by pol II, which requires an snRNA promoter (28, 54), and premature termination downstream from promoters like the HIV long terminal repeat (59) may be cases in which such processes occur. Spencer and Groudine (73) have suggested that these effects could be mediated by differential phosphorylation of the heptapeptide repeat at the C terminus of the largest subunit of pol II. In the case of c-*myc*, events such as these may account for the promoter dependence of premature arrest.

The protein factors TFIIF and TFIIS are candidates for

factors that may interact with the transcription complex during elongation and change its susceptibility to termination. These factors reduce either pausing or termination by pol II (5, 22, 64, 70). The *Drosophila* DMS-II (71) and factor 5 (57) proteins exhibit effects on elongation similar to those found for TFIIS and TFIIF, respectively. A third class of factors could act directly at termination sites to stop transcription. Such termination factors have been found for RNA polymerase I (37, 49) and for mitochondrial RNA polymerase (16).

Conditional termination occurs during transcription of c-*myc* in HeLa nuclear extracts under appropriate conditions. To improve our understanding of conditional pol II termination, we have investigated transcriptional arrest in the c-*myc* gene by using nuclear extracts prepared from HeLa cells and DNAs containing various c-*myc* sequences. From these studies, we have arrived at the following conclusions.

First, significant termination at T_{II}, with the release of the nascent transcript, occurs when the concentration of neutral salts is raised to 200 mM or above, from the traditionally used 30 to 60 mM (Fig. 2 to 4 and Table 1). Further, the T_{II} sequence itself, in forward but not reverse orientation, is sufficient to stop transcription at T_{II} in a HeLa nuclear extract (Fig. 5). Termination is stimulated more by KCl than by potassium acetate or potassium glutamate, suggesting that the Cl^- ion may mask some ionic interaction that promotes read-through. However, termination does not result simply from the electrostatic effects of Cl^- ions, since we observed significant termination at 200 to 400 mM potassium acetate in some extracts (Table 1). Although these concentrations of neutral salts may seem artifactual, in fact, the total concentration of monovalent and divalent cations in tissue culture cells is about 200 mM (30). The traditionally used 30 to 60 mM KCl is required for efficient initiation in vitro (65) but differs as much from the conditions found in vivo as those in which we have observed significant termination at T_{II}.

A second important conclusion from our work is that termination occurs at the T_{II} element in a HeLa nuclear extract regardless of whether transcription initiates at c-*myc* P2 or Ad2MLP (Fig. 3A and B). The two templates we used produced exactly the same RNA transcript, so that any effect of RNA structure or DNA sequence downstream from the promoter on termination would be identical on these templates. We observed no gross differences in elongation on these two templates under any of the conditions that we tested, but we occasionally observed some small differences in the efficiency of arrest at various sites (for example, see Fig. 3); further analysis will be required to determine the significance of these minor differences.

Finally, the efficiency of termination at T_{II} varies in extracts prepared from HeLa cells grown in different conditions (Table 1). The fact that termination was approximately proportional to the amount of termination-proficient extract present when different extracts were combined (Fig. 7) suggests that a limiting concentration of some *trans*-acting factor or factors present in the extracts determine termination efficiency at c-*myc* T_{II}.

It is prudent to recognize that our conclusions regarding termination at T_{II} conceivably apply only to transcription of Ad2-*myc* in 400 mM KCl (Fig. 5), leaving open the possibility that arrest at T_{II} on other templates or with other salt concentrations that we employed may have resulted from pausing without the release of the transcript. Further, we cannot exclude the possibility that the apparent lack of transcription beyond T_{II} (Fig. 6C) resulted from cotranscriptional processing followed by selective degradation of the nascent transcript. We also note that the observation of conditional termination at c-*myc* T_{II} applies only to the templates that contain the wild-type c-*myc* transcribed region (Ad2-*myc* and P2-*myc*). Although arrest, and probably termination, occurs at isolated T_{II} sequences in heterologous DNA (Fig. 6), we have not yet determined whether the extract-dependent changes in termination require other c-*myc* sequences.

Recently, detection of an ~400-nt c-*myc* exon 1 RNA in cells where transcription of c-*myc* is arrested has been reported (60). This finding, coupled with our results and those from previous studies of transcriptional arrest in c-*myc*, makes it highly probable that the event responsible for the block to transcriptional elongation in c-*myc* in vivo is termination.

The extract dependence of in vitro transcriptional arrest in c-*myc* suggests a *trans*-acting factor. What implications do our findings have for the mechanism of conditional termination in c-*myc*? In the broadest sense, we can envisage at least six classes of mechanisms for which there is precedence and that could account for the available data. It is possible that the basic governing factor is either something present in the termination-proficient extracts that stimulates termination or something present in the termination-deficient extracts that blocks termination. Precedence for such termination and antitermination factors can be found in the HK022 Nun protein (63) and the λN protein (29), respectively, which govern transcriptional elongation in temperate coliphages. With either of these two possibilities, the target at which the factor modifies the transcription complex could either be at the promoter, as appears to be the case for snRNA promoters (28, 54); at a discrete site between the promoter and terminator, as is the case for the prokaryotic regulatory proteins Nun and N; or at the termination site itself, a mechanism that occurs in the *E. coli bgl* operon (2) and the *Bacillus subtilis trp* operon (24).

The dependence of termination efficiency on the growth conditions of cells used for the extract preparation is consistent with the idea that the variable factor in these extracts is the same as that responsible for controlling transcription arrest of c-*myc* in vivo. Thus, HeLa cells actively growing on a solid support or at low density in a suspension culture may mimic the state of proliferating cells where arrest is suppressed. HeLa cells growing in suspension at high density may contain either more of a termination factor or less of an antitermination factor and may mimic the state of differentiated cells. Such a view is consistent with findings of E. Falck-Pedersen, who has observed a greater efficiency of transcriptional arrest in quiescent HeLa cells than in actively growing HeLa cells when the cells were infected with a recombinant adenovirus containing a DNA fragment of the first c-*myc* exon-intron region (21).

If termination is governed by a *trans*-acting factor, why are elevated concentrations of neutral salts required to observe it in the extract transcription reactions? We favor the view that, in standard in vitro transcription reactions, elongation factors such as TFIIS or TFIIF may be present in great excess over regulatory factors that control termination at c-*myc* T_{II}. In these reactions, general transcription factors must be present in much greater numbers per active transcription complex than are found in vivo. Sarkosyl and elevated concentrations of neutral salts may partially or completely block the interactions of TFIIS or TFIIF, allowing the effects of termination control factors to be observed. This view is consistent with our observation that sarkosyl

and elevated KCl dramatically reduce the rate of transcription in vitro. However, only elevated neutral salts and not sarkosyl caused efficient termination at T_{II}. Since sarkosyl above 0.2% has been shown to block the actions of both TFIIS (61) and TFIIF (72), it may be that higher ionic strength tips the competition between elongation factor(s) and termination factor(s) in favor of the latter, whereas sarkosyl may block the action of both. Cl^- ions may further stimulate termination by altering some interaction between RNA polymerase and DNA or RNA so that the termination reaction becomes more efficient.

Although our results suggest that a *trans*-acting factor governs termination at c-*myc* T_{II}, they are not at odds with the view that events occurring at the promoter affect premature termination efficiency. Changes in RNA polymerase phosphorylation (73) could be governed by the factor(s) we have detected. Alternatively, this factor(s) may act in *trans* on transcription complexes that have already left the promoter. It will be critical to test for the controlling factor(s) by fractionating extracts from cells grown in various conditions and then adding the fractions to termination-proficient and termination-deficient reactions either before or after initiation.

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