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Polyadenylylation of an mRNA precursor occurs independently of transcription by RNA polymerase II *in vivo*

(RNA processing/gene expression/transcription termination)

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ABSTRACT **Most eukaryotic messenger RNAs are transcribed as precursor molecules that must be processed by capping, splicing, 3′ cleavage, and polyadenylylation to yield mature mRNAs. An important, unresolved issue is whether any of these reactions are linked either to transcription by RNA polymerase II or to each other. To address one aspect of this question, we constructed a chimeric gene containing an RNA polymerase III promoter (the adenovirus VA_I promoter) fused to the body and 3′-flanking sequences of a protein-coding gene (the herpesvirus *tk* gene). Here we show that this hybrid gene was transcribed from the RNA polymerase III promoter following transfection of human 293 cells and that the transcripts produced were stable and efficiently transported to the cytoplasm. Although a significant proportion of the transcripts were prematurely terminated at specific sites within the gene, a high percentage of the full-length RNA was accurately cleaved and polyadenylylated. These results demonstrate that cleavage and polyadenylylation of mRNA precursors are not obligatorily coupled to transcription by RNA polymerase II *in vivo*.**

The production of mature messenger RNA from a primary transcript requires several posttranscriptional modifications (1). An important question is whether, within the cell nucleus, these reactions are linked in any way. One possibility is that all or some of these reactions occur in a concerted manner with transcription by RNA polymerase II. Such a mechanism could function to limit incorrect processing and/or provide important avenues for regulating gene expression. An elegant prokaryotic precedent for this general type of regulation is the interaction of bacteriophage λ N protein with RNA polymerase. An N protein-induced modification of RNA polymerase, at a specific promoter-proximal site, modifies the polymerase so that it is no longer affected by normal transcription termination signals (2).

The formation of the mRNA 5′-cap structure (3) is an example of an RNA processing event coupled to transcription, specifically to transcription initiation. Although it is possible to uncouple these two events under certain conditions *in vitro* (4, 5), it appears that they are normally tightly linked, both *in vitro* (6–8) and *in vivo* (9, 10). Such a linkage provides a mechanism to ensure that only RNA polymerase II transcripts become capped.

Evidence exists consistent with the idea that other RNA processing reactions, if not linked, may be dependent on transcriptional events. In a HeLa whole-cell extract, splicing of an adenovirus (Ad) pre-mRNA only occurs if the transcript is capped at its 5′ end (11). However, capping appears not to be crucial for splicing in nuclear extracts of human β-globin (12) or simian virus 40 early (13) pre-mRNAs (although see ref. 14). Also, while proper cleavage of an Ad late pre-RNA at the mRNA 3′-end site requires that the RNA be transcribed *in situ* in a whole-cell lysate (15), this requirement does not exist for the same pre-mRNA (16) or a simian virus 40 early pre-mRNA (17) in HeLa nuclear extracts.

A number of studies have shown that mRNA splicing, both *in vitro* (12, 18) and *in vivo*, in microinjected *Xenopus laevis* oocytes (19, 20), need not be coupled to transcription. As mentioned above, polyadenylylation is also not obligatorily coupled to transcription *in vitro*. However, these experiments do not rule out the possibility that such a coupling might exist when RNA is transcribed from a DNA template *in vivo*. Indeed, some evidence, consistent with this notion with respect to polyadenylylation, has been presented (21, 22). Here, by analyzing RNAs made in transient expression experiments from a chimeric gene containing an RNA polymerase III promoter (the Ad VA_I promoter) fused to the herpesvirus thymidine kinase (tk) mRNA-encoding sequences, we show that transcription by RNA polymerase II is not a requirement for mRNA cleavage and polyadenylylation *in vivo*.

MATERIALS AND METHODS

Plasmid Constructions. The starting material for construction of pVAtk2 was the plasmid S2 (from S. Silverstein), a 5′-deletion mutant of the herpesvirus HSV-1 *tk* gene, which lacks the *tk* promoter and all sequences on the 5′ side of nucleotide (nt)+2 (relative to the cap site, nt +1) but retains all but one base pair (bp) of the 5′-untranslated leader, the entire coding region, the 3′-untranslated region, and approximately 300 bp on the 3′ side of the poly(A) addition site (23). A *Bgl* II–*Bam*HI fragment of Ad type 2 (Ad2), which spans 8904–10,685 bp and contains the 5′ 76 bp of the VA_I gene, was purified and inserted into the *Bam*HI site of S2 to create pVAtk2 (Fig. 1). The plasmid pϕtk (Fig. 1) was constructed by inserting the *tk* fragment from S2 into the vector pϕ4 (24), which contains 438 bp of Ad2 DNA from nt −405 to +33.

RNA Extraction and Analysis. Forty-eight hours after transfection of human 293 cells (25), RNA was extracted from the cytoplasm (26) and from the nucleus (27), and aliquots were selected on oligo(dT)-cellulose (28). Primer extension analysis was performed as described (26) with a 5′-end-labeled, 29-nt primer that was complementary to nt 143–172 from the VA_I transcription start site of pVAtk2. Products were electrophoresed through 5% acrylamide/8.3 M urea sequencing-type gels (29). The 3′ ends of tk-specific RNAs were mapped with S1 nuclease (Sigma) as described (30), except that hybridizations were carried out with 1–3 μg equivalents of total RNA and 2–5 ng of probe at 58°C, and the hybrids were digested at 45°C. Probes were 3′-end-labeled with T4 DNA polymerase and [α-^{32}P]dXTPs according to standard procedures (27). The S1-resistant products were electrophoresed through 8% acrylamide/8.3 M urea sequenc-

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Abbreviations: nt, nucleotide(s); bp, base pair(s); Ad, adenovirus.

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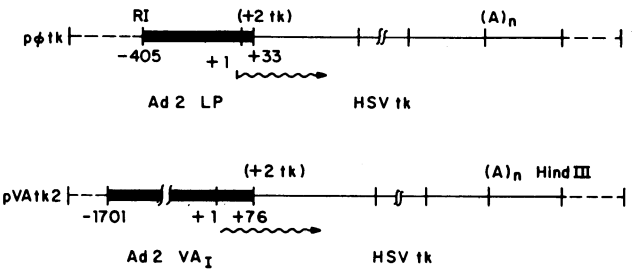

FIG. 1. Structures of the recombinant plasmids. Ad sequences are denoted by a thick line, herpesvirus (HSV) sequences by a thin solid line, and pBR322 sequences by a dashed line. Direction of transcription is indicated by a wavy arrow. LP, late promoter; tk, thymidine kinase. Numbers above the lines refer to herpesvirus *tk* sequences, and those below refer to the adenoviral nucleotides, relative to the respective RNA start sites, at nt +1.

ing-type gels or 1.4% agarose gels. Samples to be run on agarose gels were first denatured by reaction with glyoxal (31). Gels were dried and exposed to XAR-5 x-ray film without intensifying screens, and autoradiograms were scanned with a Gilford model 250 spectrophotometer.

RESULTS

Experimental Strategy. The object of the present study was to determine whether the formation of a correct mRNA 3′ end is obligatorily linked to transcription by RNA polymerase II *in vivo*. For these experiments, we constructed a chimeric plasmid, pVAtk2 (Fig. 1), designed to produce an RNA containing all the signals necessary for accurate 3′-end formation of an mRNA, but transcribed by RNA polymerase III. Because RNA polymerase III terminates transcription at nucleotide sequences of four thymidines in a G + C-rich environment (32), a protein-encoding gene lacking any possible RNA polymerase III termination sites was required. Unlike many genes transcribed by RNA polymerase II, the *tk* gene of HSV-1 has no cluster of four or more thymidines until approximately 110 bp downstream of the site previously shown to encode the 3′ end of the mRNA (23). The control region of the VA_I gene of Ad2 was selected to replace the promoter of the *tk* gene. The VA_I gene is transcribed by RNA polymerase III (33), and the sequences required for production of VA_I RNA are all located within 76 bp from the start sites (34, 35). All *tk* sequences on the 5′ side of and including the cap site were absent from pVAtk2 to preclude RNA polymerase II transcription. In several experiments, we failed to detect significant levels of transcription from the *tk* promoter, suggesting that this promoter is not active in 293 cells (unpublished results). Therefore, an RNA polymerase II control plasmid, pϕtk, was constructed that replaces the *tk* promoter with a strong RNA polymerase II promoter, the late promoter of Ad2. These chimeric plasmids were transfected into parallel cultures of human 293 cells (36) by calcium phosphate coprecipitation (37), and cytoplasmic and nuclear RNA were extracted 48 hr later. The 293 cells were chosen because they constitutively express gene products from early region 1A of Ad type 5 (Ad5) (38), which are known to stimulate RNA production from both the VA_I (39) and Ad late (26) promoters.

Transcription Initiation from the RNA Polymerase III Promoter. We first tested whether any *tk*-specific RNA was produced from pVAtk2. Primer extension analysis was performed on RNA extracted from 293 cells 48 hr after transfection with either pVAtk2 or pϕtk. The predominant extended products from the pVAtk2 template were 169 and 172 nt (Fig. 2). These bands are exactly the sizes predicted if tk-specific RNA was initiated at nt +1 and +4 of the VA_I gene, which have been shown to be the authentic VA_I transcription start sites (40). RNA extracted from both the nucleus and cytoplasm contained a considerable amount of these RNA polymerase III transcripts, suggesting that they were expressed efficiently, were stable, and could be transported to the cytoplasm (Fig. 2). These transcripts were also found in both the oligo(dT)-bound and -unbound fractions, indicating that a fraction of the RNA was polyadenylylated. Densitometry scanning of the autoradiogram shown in Fig. 2 revealed that approximately 10% of the tk-specific RNA was contained in the poly(A)$^+$ fraction for both nuclear and cytoplasmic RNA. The major extended product using RNA from cells transfected with pϕtk was 141 nt (Fig. 2), the predicted size for a correctly initiated transcript from the Ad2 late promoter (24). Greater than 95% of this RNA was contained in the oligo(dT)-selected fraction.

Mapping the 3′ Ends of the tk-Specific Transcripts. Quantitative S1 nuclease mapping with a 3′-end-labeled DNA

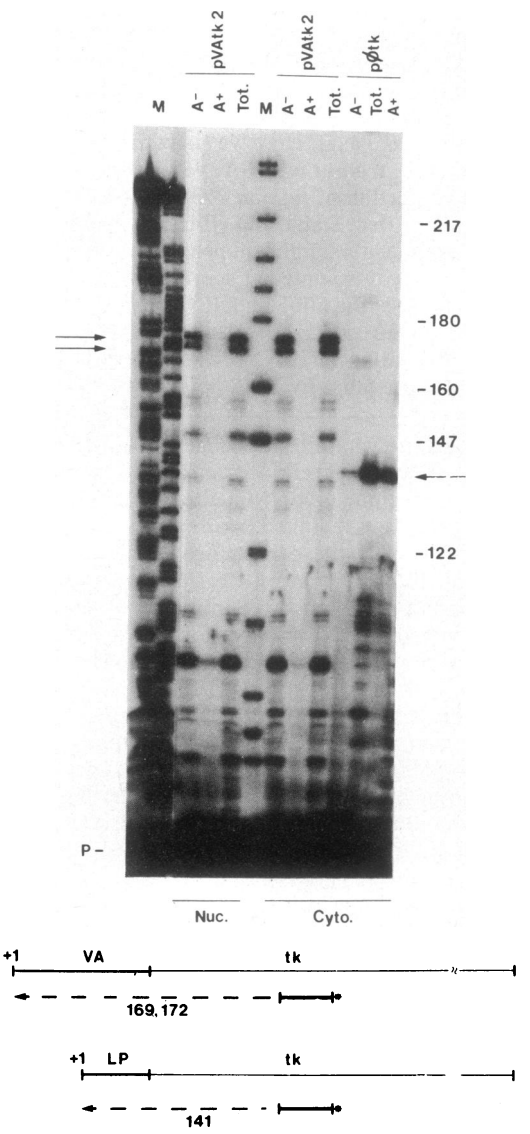

FIG. 2. Determination of RNA start sites. Cytoplasmic (Cyto.) or nuclear (Nuc.) RNA (3-μg equivalents) that had been selected on oligo(dT) following extraction from 293 cells transfected with either pVAtk2 or pϕtk was analyzed by primer extension. The reversed-transcribed products were electrophoresed through an 8% polyacrylamide/8.3 M urea sequencing-type gel. Extended products are indicated by arrows. The lower portion of the figure is a schematic drawing of the procedure. M, pBR322 DNA digested with *Hpa* II and 5′-end-labeled or A+G and C+T sequencing reactions of an unrelated DNA.

probe was used to define the 3′ ends of RNA from transfected cells. To identify most, if not all, possible 3′ ends, a probe covering all but 51 nt of the *tk* gene was used in initial mapping experiments, and the protected fragments were electrophoresed on an agarose gel under denaturing conditions. Somewhat surprisingly, four major bands, as well as several minor ones, were detected with both nuclear and cytoplasmic RNA fractions obtained from 293 cells transfected with pVAtk2 DNA (Fig. 3). However, only one of these protected fragments was observed when RNA bound to oligo(dT)-cellulose was analyzed (Fig. 3, pVAtk2, lane A+). Within the limits of the gel system, this protected fragment was the size expected (1250 nt) if produced by hybridization to an RNA with a 3′ end at the site corresponding to the *in vivo* poly(A)-addition site of tk mRNA. In addition, the fragment comigrated with the protected fragment obtained when poly(A)$^+$ RNA from pϕtk-transfected cells was used in S1 analysis (Fig. 3, pϕtk, lane A+). Based on densitometer scanning of the autoradiogram, the polyadenylylated RNA from pVAtk2-transfected cells was approximately 10% of the total tk-specific RNA, which is the same fraction of RNA that primer extension analysis indicated was polyadenylylated. No tk-specific RNA was detected when S2, a plasmid lacking any promoter sequences, was used in transfection experiments (results not shown).

A smaller DNA probe was prepared to identify the 3′ end of the polyadenylylated RNA polymerase III transcript more precisely. This probe, diagrammed at the bottom of Fig. 4, should yield protected fragments of 91–95 nt if the same 3′ ends mapped by McKnight and Gavis (23) were used in pVAtk2- or pϕtk-transfected cells. When S1-nuclease analysis was carried out on total cytoplasmic RNA from 293 cells transfected with pVAtk2, a series of protected fragments 85–95 nt were detected that exactly comigrated with the bands seen when RNA from transfected cells with pϕtk was used. Additionally, when oligo(dT)-fractionated RNA was used in S1 analysis, these species were detected with poly(A)$^+$ but not poly(A)$^-$ RNA (data not shown). The heterogeneity in the pattern may arise from the unstable hybrid formed between a second AAUAAA sequence in the RNA, which immediately precedes the 3′ ends, and its DNA complement in the probe. These results show that an RNA polymerase III-derived RNA can be accurately and specifically cleaved and polyadenylylated *in vivo*.

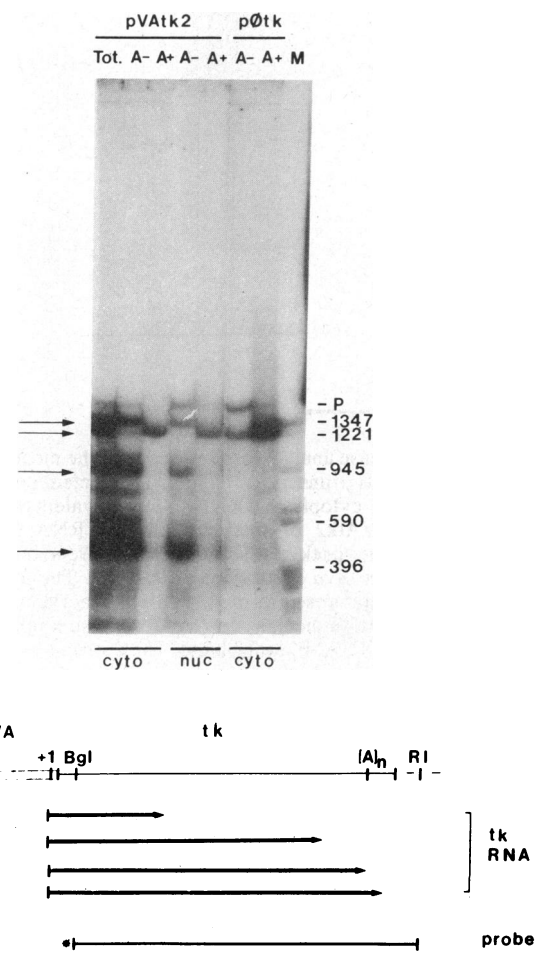

FIG. 3. S1-nuclease mapping of the RNA 3′ ends. Cytoplasmic (cyto) or nuclear (nuc) RNA (3-μg equivalents) that had been selected on an oligo(dT)-cellulose column after extraction from 293 cells transfected with either pVAtk2 or pϕtk was hybridized to a 3′-end-labeled 1580-bp *Bgl* II–*Eco*RI fragment of *tk* DNA. Following digestion with S1 nuclease, the protected fragments were denatured with glyoxal and electrophoresed through a 1.4% agarose gel. Arrows indicate the major 3′ ends. P, intact probe. A schematic diagram is shown in the lower portion of the figure. Tot., total RNA. A$^-$, poly(A)$^-$ RNA. A$^+$, poly(A)$^+$ RNA. M, DNA size markers.

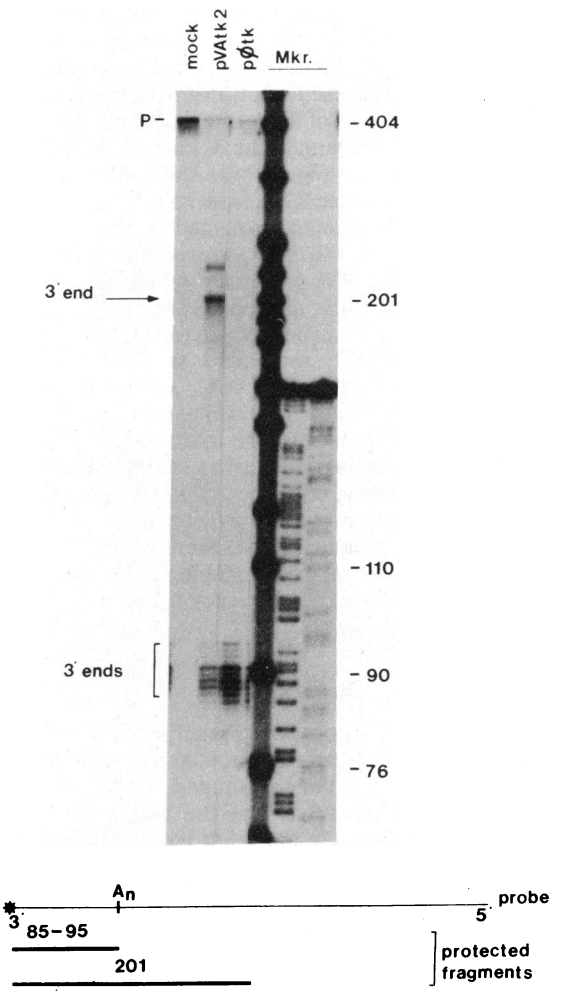

FIG. 4. Fine mapping of the 3′ ends of the largest RNAs. Cytoplasmic RNA (3 μg) from cells transfected with no DNA (mock), pVAtk2, or pϕtk was hybridized to a 3′-end-labeled 415-bp *Ava* I–*Eco*RI fragment of *tk* DNA at 58°C and digested with S1 nuclease at 30°C for 1 hr. The products were electrophoresed through an 8% polyacrylamide/8.3 M urea sequencing type gel. The 3′ ends of the polyadenylylated RNAs are denoted by a bracket, while the 3′ end of the RNA polymerase III product seen only in the pVAtk2 lane is marked by an arrow. The sizes in nt are shown. The probe is drawn at the bottom of the figure. Mkr., DNA size markers.

S1-Nuclease Mapping of RNA Polymerase III Termination Sites. One pVAtk2-specific transcript detected by the analysis shown in Fig. 3 appeared to be longer than the correctly polyadenylylated RNA. The size (about 1350 nt) of the protected fragment suggested that the RNA probably terminated in the thymine-rich region bounded by guanines and cytosines, which corresponds to the predicted size of RNA polymerase III transcription termination in this pVAtk2. The 415-bp probe used to determine the 3′ end of this polyadenylylated RNA was also used to position the 3′ end of this nonpolyadenylylated species. An RNA terminating at the first thymidine of the thymidine cluster should yield a protected product of about 202 nt. As shown in Fig. 4, a band of about this size is seen when RNA extracted from cells transfected with pVAtk2, but not pϕtk, was used in mapping experiments. This nucleotide sequence appears to act as an efficient RNA polymerase III termination signal, because no protected fragment longer than this was reproducibly observed. The band of about 220 nt apparently represents an artifact of the S1 analysis, because it was not detected when more S1 nuclease was used in digestions (data not shown).

Several transcripts that appeared to be prematurely terminated within the *tk* gene were also detected in pVAtk2 transfected cells (Fig. 3). We had tested pVAtk2 as a template in an *in vitro* transcription reaction and detected RNAs of similar sizes, (ref. 7 and unpublished results) supporting the idea that these species arose from transcription termination. To determine the positions of these internal termination sites, a smaller probe, which spanned the 3′ ends of the two most prominent bands seen in Fig. 3, was utilized. S1-nuclease analysis of total cytoplasmic RNA from cells transfected with pVAtk2 yielded two prominent protected fragments (Fig. 5). The smallest band, of 148 nt, corresponds to a species terminated with about 30% efficiency within the sequence GCCTTATGC, specifically, at the sequence CTT. The largest protected fragment was about 620 nt (just below the intact probe band) and probably represents an RNA terminated within the sequence CGTTATTTACC. Approximately 30% of the polymerase molecules also terminate at this signal (see Fig. 3). These data suggest that the interruption of a stretch of four thymidines by an adenosine, within a G+C-rich environment, reduces the efficiency of RNA polymerase III transcription termination by only a factor of three. None of these prematurely terminated RNAs were polyadenylylated, but all were apparently relatively stable, and transported to the cytoplasm with the same efficiency as the polyadenylylated species (see Fig. 3).

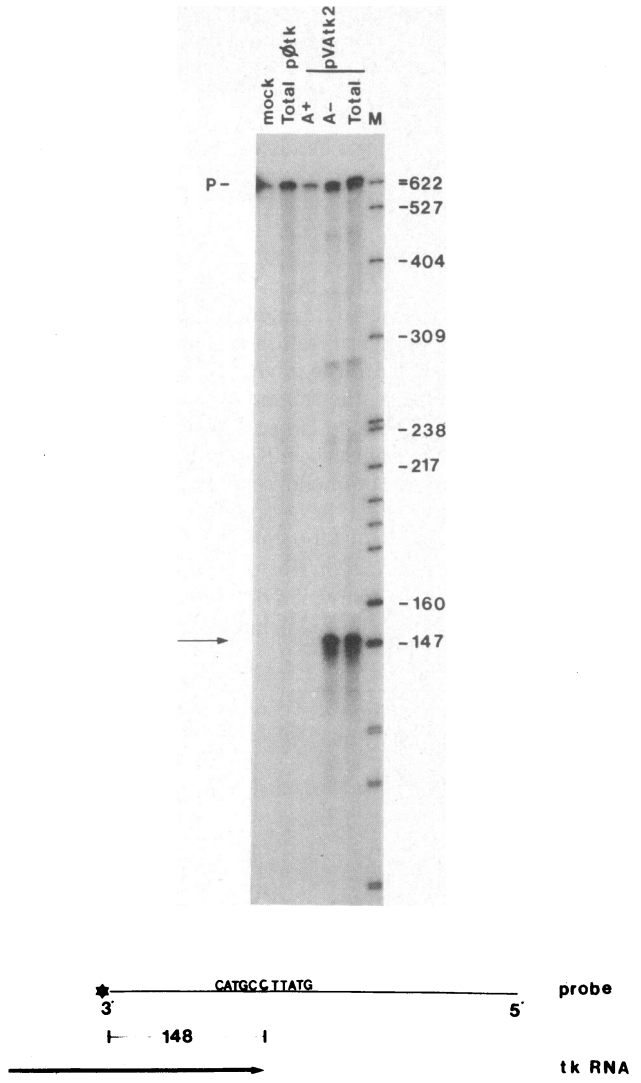

FIG. 5. S-1 nuclease analysis of the 3′ ends of the most abundant RNA polymerase III transcripts. Oligo(dT)-selected poly(A)$^+$ or -unselected poly(A)$^-$ cytoplasmic RNA (3-μg equivalents) from cells transfected with pVAtk2 or total cytoplasmic RNA from cells transfected with either pϕtk or pBR322 (mock) were hybridized to a 3′-end-labeled 640-bp *Ava* I fragment of *tk* DNA. The S1-resistant species were electrophoresed through an 8% polyacrylamide/8.3 M urea gel. The arrow indicates the band representing the most abundant protected RNA. P, probe band. The drawing at the bottom of the figure gives the nucleotide sequence of the probe in the region complementary to the 3′ end of this RNA. The sizes in nt are indicated.

DISCUSSION

In the present study the question of whether the formation of an mRNA 3′ end is obligatorily coupled to transcription by RNA polymerase II has been examined. Experiments with nuclear extracts have shown that the two events can be uncoupled *in vitro* (16). On the other hand, studies *in vitro* with a HeLa whole-cell extract (15) and *in vivo* with chimeric transcripts synthesized by RNA polymerase I (22) were consistent with a possible linkage between transcription by RNA polymerase II and processing. While *in vitro* systems have been useful for analyzing many aspects of transcription (see ref. 1 for review), they may lack components or structures found *in vivo* or preexisting enzyme complexes may be disrupted during extraction. On the other hand, interpretation of *in vivo* experiments may also be complicated. For example, endogenous RNA polymerase I transcription is probably localized within the nucleolus. Therefore, as noted by Smale and Tjian (22), the finding that RNA polymerase I-transcribed tk RNA was not polyadenylylated may simply reflect the fact that the transfected templates were probably transcribed in the nucleolus and the resulting RNAs were thus not accessible to the enzymes involved in mRNA 3′-end formation.

We have examined the fate of RNA produced from a protein-encoding gene transcribed by RNA polymerase III *in vivo*. Using a transient expression system, we showed that this RNA could be used as a substrate for cleavage and polyadenylylation. While only 20% of the total RNA polymerase III-transcribed RNA extended as far as the poly(A)-addition site, 50% of the RNA molecules that did (or 10% of the total tk RNA) were properly cleaved and polyadenylylated. While these numbers are based on analysis of transcripts accumulated at the end of transient expression assays, rather than on measurements of newly synthesized RNA, we believe that 50% is a good estimate of the fraction of full-length RNA polymerase III transcripts that were accurately cleaved and polyadenylylated. For this not to be the case would require that a significant fraction of the RNA

polymerase III-synthesized transcripts were unstable and, therefore, not detected in analysis of accumulated tk-specific RNA. This is unlikely for two reasons. First, the relative abundance of the terminated, polyadenylylated, and prematurely terminated transcripts, in both nuclear and cytoplasmic fractions, suggests that they were all stable. Second, quantitation of the tk-specific transcription in nuclei isolated from pϕtk- and pVAtk2-transfected cells indicated that the VA promoter gave rise to a similar level of tk RNA as did the Ad2 late promoter (data not shown). This is in good agreement with the amount of tk RNA detected by analysis of steady-state RNA (e.g., Fig. 3) and, therefore, provides strong support for the conclusion that approximately 50% of the full-length RNA polymerase III-transcribed RNA was cleaved and polyadenylylated.

Why was only 50% of the RNA extending past the poly(A) site processed? One explanation is that because RNA polymerase III apparently transcribes only about 110 nt past the poly(A)-addition site, some as yet unidentified sequence important for efficient cleavage may not be present in the chimeric RNA, or the secondary structure of the terminated RNA is different, and less favorable to cleavage/polyadenylylation than would be a longer precursor. Alternatively, the transcript terminated 100 nt downstream of the poly(A) site may not be a substrate for polyadenylylation at all. For example, it may be that the La antigen, which binds to precursor RNA polymerase III transcripts (41), perhaps to strings of uridine residues such as those encoded at RNA polymerase III termination sites (42), interferes with polyadenylylation.

We do not know whether the chimeric VA-tk-RNA was translated. However, experiments carried out in conjunction with K. Blackwell and S. Silverstein (unpublished results), failed to detect tk^+ transformants after transfection of mouse Ltk^- cells with pVAtk2, suggesting that the VA-tk RNA, if present, was not translated. We also note that the relative abundance of the polyadenylylated species relative to the nonpolyadenylylated RNAs was identical in the nuclear and cytoplasmic fractions (e.g., Fig. 3), suggesting that the poly(A) sequence does not significantly affect either the nuclear–cytoplasmic transport or the stability of the hybrid RNA.

The findings described here show that mRNA polyadenylylation is not obligatorily coupled to transcription by RNA polymerase II *in vivo* and argue against models proposing that pre-mRNA processing is catalyzed by transcription-processing multienzyme complexes. However, since we analyzed RNA present at the end of a transient expression assay, we cannot rule out the possibility that the rate of polyadenylylation of the RNA polymerase II-transcribed pre-mRNA was greater than the rate of polyadenylylation of the RNA polymerase III-transcribed precursor. Our findings also suggest that mRNA 3′-end formation *in vivo* does not require that the pre-mRNA contain a 5′ cap. Although we have not examined the structure of the 5′ ends of the chimeric RNA polymerase III transcripts studied here, all RNA polymerase III transcripts so far examined lack caps, perhaps because capping, in contrast to polyadenylylation, is tightly coupled to transcription by RNA polymerase II.

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