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Premature Termination of Transcription Can Be Induced on an Injected α-Tubulin Gene in *Xenopus* Oocytes

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The *Xenopus laevis* α-tubulin gene XαT14, which is highly expressed during oogenesis, exhibits accurate and efficient transcription initiation when microinjected into *X. laevis* oocytes. However, we found previously in nuclease protection assays of transcripts from injected XαT14 that many protected fragments that were shorter than expected could be produced. We show here by exonuclease VII mapping, Northern (RNA) blotting, and gel fractionation of RNA that these fragments were caused by truncated transcripts that share the same initiation sites as mature transcripts but whose 3′ ends are located in the 5′ leader just 45 to 72 nucleotides downstream. We present evidence from the analysis of in vitro pulse-labeled RNA that these truncated transcripts are formed by premature transcription termination rather than by RNA processing. At low template levels, very little premature termination occurred, but as more DNA was injected, the proportion of transcripts that were prematurely terminated increased steadily, even at template levels at which the initiation machinery was unsaturated. At high template levels, most transcripts were prematurely terminated. These results suggest that some sort of saturable antitermination function operates in oocytes in a manner that is dependent on the number of appropriate templates available rather than on the number of polymerases that initiate transcription. They also suggest that measures of initiation frequency may not always be a reliable means of assessing the amount of transcription of injected genes in oocytes.

It is well established that the control of mRNA production in eucaryotes is often accomplished by modulation of transcription initiation rates. However, it is becoming clear that the subsequent phases of transcription that are needed for the production of complete primary transcripts, namely, elongation and termination, do not always follow predictably from initiation. In particular, it has been estimated that in some cells nearly half of all polymerase II initiation events end in premature termination (25), and specific examples of premature termination have recently been described in several genes, including the proto-oncogenes c-*myc* (3, 6, 21), *c-myb* (1), and c-*fos* (7), the simian virus 40 (9) and adenovirus (19) late transcription units, the long terminal repeat of the human immunodeficiency virus (HIV [12]), and *Drosophila hsp70* (24). Since in some instances the level of premature termination appears controllable, it has been suggested that phenomena analogous to the elongation controls described for procaryotic genes may play an important role in the overall regulation of eucaryotic gene activity (22).

Detailed consideration of some of these examples suggests that several different mechanisms can cause premature termination. Upon differentiation of promyelocytic leukemia cells, intragenic termination is induced in the c-*myc* gene several hundred base pairs downstream of initiation at the exon 1-intron 1 border, just upstream from a site that becomes hypersensitive to DNase I (3). In the case of the HIV long terminal repeat, the block to elongation is much closer to the initiation site and results in the accumulation of truncated RNAs of about 60 (HIV type 1) or 125 (HIV type 2) nucleotides (12, 26). The HIV *tat* gene product allows RNA polymerase to overcome the transcription block and to synthesize full-length transcripts, suggesting that transcription from these promoters requires an antitermination activity. Finally, it has been shown that before heat induction, the *Drosophila hsp70* gene synthesizes nascent RNAs of about 25 nucleotides in length as a result of the operation of a transcriptional block in the 5′ leader (24). This block, which results in the transcription complex and transcript remaining engaged on the template, is released by heat shock such that the stalled polymerase can proceed through the rest of the gene.

Studies of premature transcription termination have primarily been carried out by using either nuclear run-on analysis or in vitro transcription systems based on cell extracts or purified RNA polymerase (e.g., references 13 and 23). However, it has recently been shown that the premature termination events that occur in the human and mouse c-*myc* genes are recreated when cloned copies of these genes are injected into *Xenopus* oocytes (4). One advantage that oocytes possess for this type of work is that a variety of novel nonadenylated RNA molecules, ranging from transcripts initiated in injected ribosomal DNA spacers (20) to synthetic sense and antisense transcripts (8), are stable in oocytes. Hence, it proved possible to map the termini of the truncated c-*myc* transcripts simply and accurately from samples of steady-state RNA (4).

We are studying the transcription of XαT14, a *Xenopus laevis* α-tubulin gene that is expressed during oogenesis and that exhibits accurate and efficient initiation of transcription when injected into oocytes at widely different DNA concentrations (18). We noticed previously in our nuclease protection assays of transcripts from injected XαT14 that a large number of unexpectedly short protected fragments could be found. We show here that even though transcription of injected XαT14 is initiated correctly and efficiently, it is prone to frequent premature termination in the 5′ leader. We also show that premature termination is rare at low template levels, that the ratio of premature termination to apparent elongation increases steadily with template amount even as initiation of both types of transcript rises, and that at high

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template levels most transcripts are prematurely terminated. In addition, the relative production of prematurely terminated transcripts increases with time during incubation. We interpret our data as evidence for the existence of a novel antitermination function in oocytes, the saturation of which is determined by the amount of available template rather than the number of polymerases initiating transcription. We do not know whether this phenomenon has any regulatory significance for oocytes or tubulin genes, but it offers a way of dissecting a potentially important aspect of the mechanism of transcription and illustrates the desirability of considering both initiation and elongation when studying transcriptional control experimentally in oocytes.

MATERIALS AND METHODS

Plasmids. The *X. laevis* α-tubulin gene XαT14 (27) was cloned into pBR322 as described previously (18) to form plasmid XX15. In this construct, the gene (6.8 kilobase pairs) is flanked by 5 kilobase pairs of 5′ DNA and 3 kilobase pairs of 3′ DNA. A derivative of this clone, ΨXX15, that produces a slightly longer transcript was constructed by insertion of the *Sma*I-*Hin*cII region of the M13mp18-M13mp19 polylinker into position +87 of the 5′ leader via a filled-in *Dde*I site. Plasmid DNA was processed through two sequential CsCl gradient centrifugations before use in oocyte injections.

Oocyte injections. Plasmid DNA was injected into *X. laevis* oocytes as described previously (18) in 20-nl volumes at various concentrations, as indicated. After incubation at 18 to 20°C for the periods stated, healthy oocytes were either homogenized and processed immediately for RNA, manually dissected into nucleus and cytoplasm before processing, or frozen in liquid N_2 and stored at −70°C.

Preparation and fractionation of steady-state RNA. RNA from whole injected oocytes was obtained by homogenizing 10 to 20 oocytes in 0.3 M sodium acetate–0.5% sodium dodecyl sulfate–1 mg of proteinase K per ml and incubating them at 37°C for 30 min. After extraction with phenol-chloroform, nucleic acids were precipitated with ethanol at −25°C and suspended in distilled water.

To obtain nuclear and cytoplasmic RNA fractions, groups of 10 injected oocytes were manually dissected in 0.1 M KCl–20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.7)–0.1 mM EDTA–6 mM $MgCl_2$, and the nuclei and cytoplasms were frozen separately in liquid N_2. RNA was prepared from cytoplasms as described above for whole oocytes, but nuclei were added to a proteinase K-digested homogenate of 10 uninjected oocytes before RNA extraction in order to equalize RNA recoveries.

Size-fractionated RNA was obtained by running total RNA from injected oocytes on thin (0.4 mm) 5% polyacrylamide–8 M urea gels and cutting out the appropriate gel slices with a razor blade, using coelectrophoresed DNA fragments as a molecular weight guide. RNA was recovered from gel slices by diffusion overnight into 0.5 M ammonium acetate–10 mM $MgCl_2$–1 mM EDTA–0.1% sodium dodecyl sulfate at 37°C before phenol-chloroform extraction and ethanol precipitation.

Nuclease protection assays. (i) RNase protection. A uniformly labeled antisense RNA probe (Fig. 1, probe 1) was produced by T7 RNA polymerase from a 146-base-pair (bp) *Hpa*II-*Dde*I fragment containing the XαT14 transcription start site that we had subcloned into the *Sma*I site of the transcription vector pSPT19 (Boehringer Mannheim Biochemicals). Transcription reactions were performed as described previously (14), using either [α-^{32}P]ATP or [α-^{32}P]CTP to obtain probe specific activities of 1×10^7 to 2×10^7 dpm/pmol. RNA samples from the number of oocytes noted in the figure legends were hybridized with 10 fmol of probe at 75°C in 40 μl of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate). Controls for probe excess were routinely included in these assays. For RNase digestion, we optimized the enzyme concentrations used to 4 μg of RNase A and 3 U of RNase T_1 per ml; otherwise, digests were performed as described previously (14). Protected fragments were resolved on 8% polyacrylamide–8 M urea gels before autoradiography with Fuji RX film.

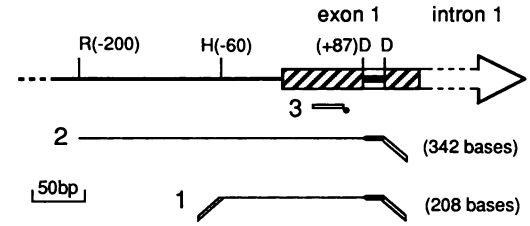

FIG. 1. Derivation of XαT14 probes. The 5′ region of XαT14 is indicated at the top of the diagram, with the black bar in exon 1 representing the polylinker sequences inserted into the *Dde*I site of ΨXX15. Locations of sites relevant to probe construction are given in parentheses (relative to the most upstream of four possible transcription initiation sites) for the restriction enzymes *Eco*RI (R), *Hpa*II (H), and *Dde*I (D). Probes 1 and 2 are nuclease protection probes (see Materials and Methods); lines off the horizontal represent sequences in these probes that are not complementary to transcripts of injected constructs. Probe 3 is a synthetic oligonucleotide (see Materials and Methods) used in primer extension assays that was 5′ end labeled (●).

(ii) S1 protection. A single-stranded DNA probe (Fig. 1, probe 2) was uniformly labeled by the prime-cut procedure (2), using a 17-base universal sequencing primer to prime synthesis of the 286-bp *Eco*RI-*Dde*I region of XαT14 that we had cloned into the *Eco*RI-*Sma*I sites of mp18. This antisense DNA probe was labeled with [α-^{32}P]dATP or [α-^{32}P]dCTP to specific activities of 3×10^7 to 7×10^7 dpm/pmol and was purified on low-melting-point agarose minigels before use. Hybridization of 10 fmol of probe to RNA samples was carried out as described above, and digestion was performed with 22 U of S1 nuclease (Amersham Corp.) per ml added in 360 μl of 0.25 M NaCl–30 mM sodium acetate (pH 4.6)–1 mM $ZnCl_2$–5% glycerol at 37°C for 30 min. Reactions were stopped by ethanol precipitation, and samples were suspended in 95% formamide–10 mM EDTA before being loaded on polyacrylamide-urea gels.

(iii) Exonuclease VII protection. Details of probe and hybridization were as for the S_1 assays. Unhybridized probe was digested by incubation with 18 U of exonuclease VII (Bethesda Research Laboratories, Inc.) per ml in 360 μl of 20 mM Tris (pH 8)–50 mM NaCl–10 mM EDTA at 37°C for 1 h.

Primer extension assays. A 28-nucleotide synthetic primer (Fig. 1, probe 3) was 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP as described previously (17). A 10-fmol sample of the probe (2×10^6 dpm/pmol) was hybridized to each RNA sample at 55°C in 0.4 M NaCl–10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES; pH 6.4) for 5 h. The primer was extended by avian myeloblastosis virus reverse transcriptase (Bio-Rad Laboratories) as described previously (30), and the products were resolved on 16% polyacrylamide–8 M urea gels.

Northern (RNA) blotting. Total RNA from injected oocytes was run on an 8% polyacrylamide–8 M urea gel,

after which the gel was soaked in 25 mM Tris (pH 10.4) for 30 min before transfer of the RNA to Zeta-Probe membrane (Bio-Rad). Semidry blotting was carried out by electrophoresis at 1 mA/cm^2 for 1 h in the presence of 40 mM ε-amino *n*-caproic acid (Sigma Chemical Co.). After transfer, the membrane was floated on 0.4 M NaOH for 10 min, dried, and then prehybridized for 2 h at 42°C in 50% formamide–1.5× SSC–1% sodium dodecyl sulfate–0.2 mg of tRNA per ml–0.5 mg of sheared herring sperm DNA per ml–0.5% dried fat-free milk. Hybridization was carried out overnight at 42°C in prehybridization buffer containing about 5×10^6 dpm of the same synthetic RNA probe as used in RNase assays per ml. After hybridization, the blot was washed in 0.1× SSC–0.5% sodium dodecyl sulfate at 65°C for 4 h.

Preparation and RNase protection analysis of in vitro pulse-labeled RNA. The method we have used is based on that described by Labhart and Reeder (15). Oocytes each injected with 3 ng of XX15 were incubated for 12 to 16 h, and then 20 to 40 nuclei were isolated manually in 0.1 M KCl–20 mM HEPES (pH 7.7)–5 mM dithiothreitol–5% glycerol–0.1 mM EDTA–6 mM $MgCl_2$. After all nuclei had been collected on ice, they were centrifuged briefly, resuspended in 10 to 15 μl of the same buffer, and added to a mixture of three unlabeled nucleoside triphosphates (final concentration of each, 250 μM) and either [α-^{32}P]CTP or [α-^{32}P]UTP (400, 800, or 3,000 Ci/mmol; final concentration, 7 to 2 μM) to give a final volume of 20 to 40 μl. Nuclei were incubated for 10 min at room temperature, and then 20 U of RNase-free DNase (Boehringer) was added to stop the reaction. After incubation at 37°C for 15 min, 80 μl of proteinase K buffer (1 mg of proteinase K per ml, 0.3 M sodium acetate, 0.5% sodium dodecyl sulfate, 10 mM EDTA, 100 μg of *Escherichia coli* tRNA per ml) was added, and incubation continued for 30 min. The reaction was then extracted with an equal volume of phenol-chloroform, the interphase was back-extracted with 0.3 M sodium acetate–10 mM EDTA, and the combined aqueous phases were reextracted with phenol-chloroform. After passage over P-60 (Bio-Rad), RNA was precipitated by addition of ammonium acetate to 2 M, 2.5 volumes of ethanol, and 10 μg of tRNA.

For analysis, the pulse-labeled nuclear RNA was suspended in 30 μl of 2× SSC together with 1 μg of single-stranded DNA from M13 clones that contained either the sense or the antisense strand of the *Eco*RI-*Dde*I fragment used to produce probe 2 (Fig. 1). The sample was hybridized at 75°C overnight, and then 60 μl of 4 μg of RNase A per ml–3 U of RNase T_1 per ml (in 10 mM Tris [pH 7.5], 5 mM EDTA, 30 mM NaCl) was added, and the sample was incubated at 30°C for 30 min. Then 1.25 μl of a 10-mg/ml solution of proteinase K was added, and incubation continued for 15 min at 37°C before extraction with phenol-chloroform. To reduce contamination of the sample by double-stranded nuclear RNA, KCl was added to the aqueous phase to 0.5 M, and the sample was applied to nitrocellulose (0.45-μm pore size). After 15 min, the filter was copiously washed in 0.5 M KCl–10 mM Tris (pH 7.5), and the hybridized RNA was then eluted by heating at 65°C in distilled water. RNA was ethanol precipitated, and the pellet was dissolved in formamide before being run on a polyacrylamide-urea gel.

RESULTS

Identification of truncated XαT14 transcripts in injected oocytes. We have injected into oocytes two different forms of the α-tubulin gene XαT14; plasmid XX15 contains the wild-type gene, whereas plasmid ΨXX15 contains in addition a 21-bp insert in the 5′ leader. Since the nuclease protection probes that we have used (Fig. 1) are uniformly labeled and include the first 16 nucleotides of the insert, the Ψ–wild-type transcript generates protected fragments that are 16 nucleotides longer than those of the wild-type transcript. Figure 2a shows an RNase protection assay of total RNA from two oocytes, each injected with 1 ng of either XX15 or ΨXX15. As found previously (18), transcription was initiated accurately and efficiently from the injected XαT14, resulting in the expected protected fragments of about 87 and 103 nucleotides for XX15 and ΨXX15, respectively. The number of transcripts produced by this amount of injected DNA exceeds that of the endogenous oocyte XαT14 genes by several orders of magnitude (18). However, we have repeatedly noticed in assays of such injected oocytes a prominent cluster of smaller protected fragments of 45 to 72 nucleotides

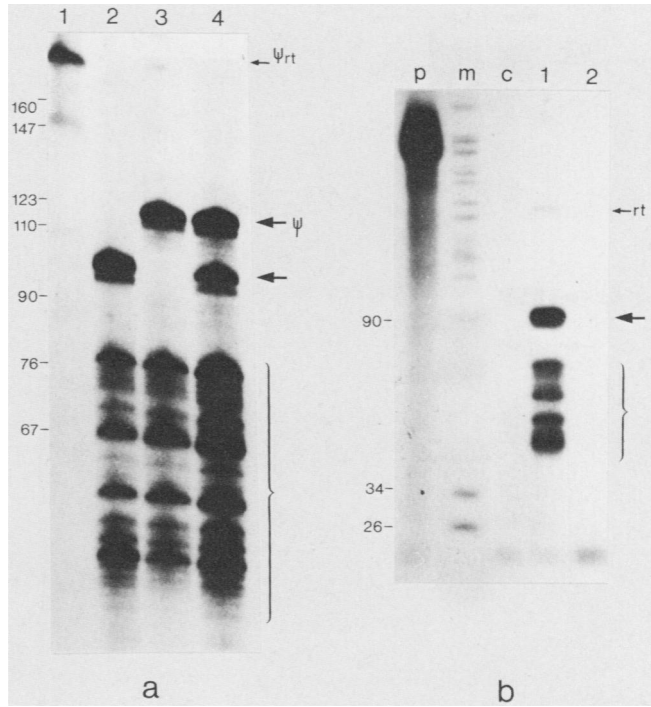

FIG. 2. Production of short RNase-protected fragments from XαT14 transcripts. (a) RNase protection of probe 1 by total RNA equivalent to two oocytes, each injected with XαT14 constructs as follows: lane 1, 1 ng of ΨHX9.5 plus 1 ng of pUC19; lane 2, 1 ng of XX15 plus 1 ng of pUC19; lane 3, 1 ng of ΨXX15 plus 1 ng of pUC19; lane 4, 1 ng of XX15 plus 1 ng of ΨXX15 (no pUC19). ΨHX9.5 (18) contains the same transcription unit as ΨXX15 but has an inactive promoter due to the deletion of sequences upstream of the *Hpa*II site at −60. Readthrough transcripts containing the 5′ region of this clone arise from initiations in the vector or elsewhere in the insert. (b) RNase assay of transcripts from one oocyte-equivalent of a batch of oocytes injected with either 2 ng of XX15 (lane 1) or 2 ng of XX15 resuspended in 2 μg of α-amanitin per ml (lane 2). Lane p, Probe taken through hybridization and digestion procedures with 30 μg of *E. coli* tRNA but without RNases added; lane c, as lane p but with RNases added; lane m, pBR322 marker fragments. Short protected fragments are indicated by braces, and protected fragments resulting from correctly initiated and elongated transcripts of XX15 and ΨXX15 (Ψ) are indicated by large arrows. Fragments resulting from readthrough transcripts (rt) are also indicated. The molecular size estimates shown in nucleotides were obtained from coelectrophoresed *Msp*I fragments of pBR322.

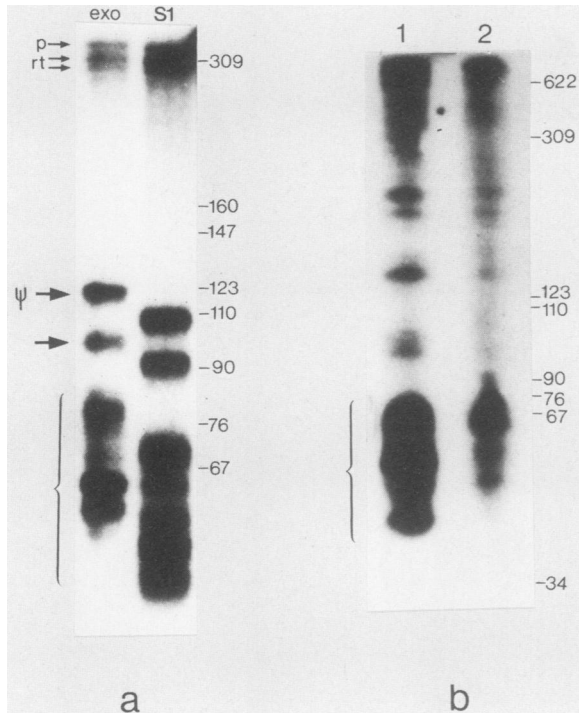

FIG. 3. Presence of short XαT14 transcripts. (a) Nuclease protection of probe 2 by total RNA from two oocytes, each injected with 1 ng of XX15 and 1 ng of ΨXX15. Protected fragments found after either exonuclease VII or S1 endonuclease digestion are shown. Exonuclease VII-protected fragments repeatably appeared several bases longer than the equivalent S1- or RNase-protected fragments. (b) Northern blot of total RNA from four injected oocytes electrophoresed on an 8% denaturing acrylamide gel and hybridized with probe 1. Oocytes were injected with either 2 ng of XX15 (lane 1) or 2 ng of XC5, a derivative of XX15 constructed by deleting all sequences downstream of a *Cla*I site at +250 (lane 2). Truncated XαT14 transcripts are indicated by braces. In panel a, the expected positions of protected fragments resulting from the correctly initiated and elongated transcripts of XX15 and ΨXX15 (Ψ) are indicated by large arrows. Fragments resulting from readthrough transcripts (rt) and undigested probe (p) are also indicated. The molecular size estimates shown in nucleotides were obtained from coelectrophoresed *Msp*I fragments of pBR322.

(brace in Fig. 2a). These short RNase protection products fell into two subgroups, often with particularly prominent bands at 45, 55, 60, and 70 nucleotides, although their precise pattern and number varied with oocyte batch and conditions such as DNA amount (see below).

We deduced that the small protected fragments were caused by RNAs initiated by the injected XαT14 promoter because they were not produced in oocytes injected with a 5′ deletion mutant that has an inactive promoter (Fig. 2a, lane 1). Production of the RNAs giving rise to both the expected protected fragments and the small fragments was abolished by injecting plasmid DNA in 2 μg of α-amanitin per ml and so presumably was due to transcription by RNA polymerase II (Fig. 2b). The small protected fragments were apparently not caused by transcripts starting from initiation sites downstream of the normal cap sites because the protection patterns were the same for transcripts of both wild-type and Ψ–wild-type constructs (Fig. 2a). We tested the possibility that the small protected fragments were the result of internal cleavage of RNA-RNA duplexes by RNases during the assay. Very similar populations of small protected fragments were found when a DNA probe (Fig. 1, probe 2) was used in S1 and exonuclease VII protection assays (Fig. 3a). Since exonuclease VII should not cleave heteroduplexes internally (5), this result suggested that the small protected fragments were caused by preexisting short RNA molecules.

We demonstrated the existence of such RNAs by running total RNA from injected oocytes on a denaturing acrylamide-urea gel, transferring the RNA to a nylon membrane electrophoretically, and then probing the blot with the synthetic RNA probe used in the RNase assays (Fig. 3b). A major portion of the transferred XαT14 RNA fell into the size class predicted for short transcripts that would give the observed nuclease protection patterns. To confirm the existence of these short RNAs and to try to map their termini, we size fractionated total RNA from oocytes injected with XX15 on a thin acrylamide-urea gel and, using coelectrophoresed DNA marker fragments as a guide, cut out the region between 40 and 80 bp. RNA was eluted from the gel slice and assayed by RNAse protection. The low-molecular-weight fraction of injected RNA was indeed able to produce a pattern of protected fragments very similar to that of the small fragments seen in the unfractionated sample (Fig. 4a). As would be expected, protected fragments due to the full-length transcripts that we know from Northern blots (data not shown) are present in the unfractionated sample were not produced from the low-molecular-weight fraction. We conclude from the combined results of exonuclease VII mapping, electroblotting, and gel fractionation that there are a large number of short, promoter-proximal XαT14 RNAs in injected oocytes.

It seemed possible that these RNAs might be produced by the premature formation of 3′ ends in transcripts that have bona fide cap sites. We have shown that this is the case by primer extension of fractionated RNA, using an oligonucleotide primer (Fig. 1, probe 3) that is complementary to the 5′ leader from +25 to +54 (relative to the most upstream cap site of XαT14 RNA). Analysis of the extension products derived from the RNAs in the low-molecular-weight fraction demonstrated the same four closely spaced 5′ ends found for XαT14 transcripts in unfractionated and poly(A)$^+$ RNA of injected oocytes (Fig. 4b) and in total tissue culture cell RNA (18). It therefore seems that the truncated XαT14 transcripts are initiated correctly but have premature 3′ ends. Knowing the position of their 5′ ends and the overall length of the protected fragments from uniformly labeled probes, we can localize the 3′ ends of the truncated RNAs to between +45 and +76 in the 5′ leader, i.e., taking into account all four possible start sites (see Fig. 8).

Analysis of pulse-labeled RNA from injected XαT14 genes. Since the assays described above were carried out on steady-state RNA, it was unclear whether the truncated transcripts resulted from premature transcription termination or from the cleavage of longer transcripts during incubation of the oocytes. The latter seemed a reasonable possibility because β-tubulin mRNAs are known to be subject to an autoregulated instability (31). To try to resolve this question, we analyzed in vitro pulse-labeled XαT14 RNA from injected oocytes as follows. After overnight incubation of oocytes injected with 3 ng of XX15, per nucleus, 20 to 40 nuclei were manually isolated and homogenized before pulse labeling for 10 min with [α-^{32}P]CTP or [α-^{32}P]UTP. Under these conditions, labeled nuclear RNA may be synthesized both by run-on transcription from polymerases already bound to templates and by polymerases initiating transcription de novo (11). Pulse-labeled XαT14

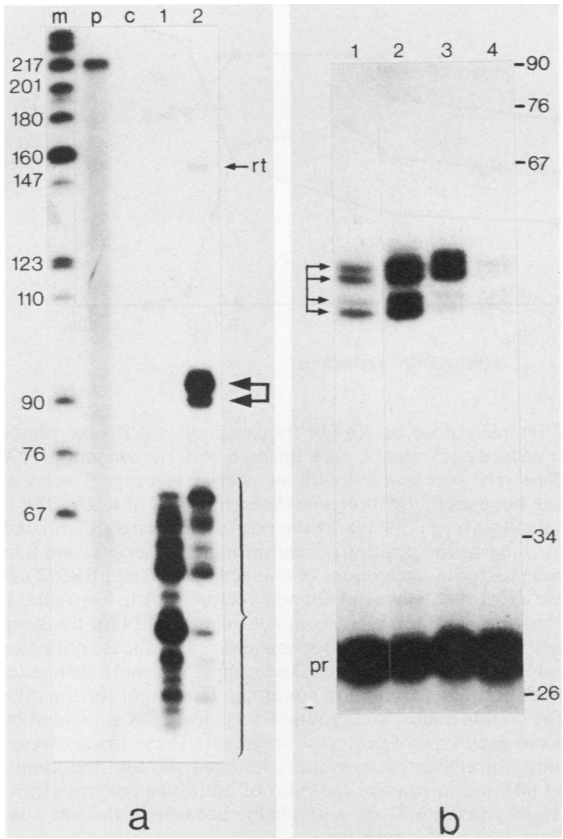

FIG. 4. Presence of truncated transcripts in size-fractionated RNA. (a) RNase protection assay. RNA from 20 oocytes injected with 2 ng of XX15 was run on a denaturing polyacrylamide gel, a low-molecular-weight fraction was recovered (see Materials and Methods), and an amount equivalent to about three oocytes was assayed by RNase protection (lane 1). Unfractionated RNA from the same injection batch equivalent to 0.5 oocyte was assayed in parallel (lane 2). Protected fragments resulting from truncated fragments are indicated by a brace; those expected from correct initiation and elongation are indicated by linked arrows, and those from readthrough transcripts (rt) are also shown. Lane p, Probe taken through hybridization and digestion procedures but without added RNA or RNases; lane c, as lane p but with RNases added; lane m, *Msp*I-digested pBR322 marker fragments (sizes are indicated in nucleotides). (b) Primer extension analysis. A 5′-end-labeled 28-nucleotide primer was hybridized to either three oocyte-equivalents of the low-molecular-weight RNA (lane 1) or two oocyte-equivalents of the unfractionated RNA (lane 2) used in panel a. The same four extension products are seen for both samples (linked arrows) and also for unfractionated RNA from oocytes injected with 25 ng of ΨXX15 (two oocyte-equivalents; lane 3) and poly(A)$^+$ RNA isolated from oocytes injected with XX15 (lane 4). (RNase protection analysis of the sample used in lane 3 is shown in Fig. 5a and demonstrated that this RNA consists almost entirely of truncated transcripts.) Differences in the relative usage of the four initiation sites seem to be animal dependent. Sizes in nucleotides of coelectrophoresed pBR322 marker fragments are shown. pr, Unextended primer.

RNA was selected by hybridization to single-stranded M13 clones containing either the sense (RNA-like) or antisense strand of the fragment used to provide probe 2 (Fig. 1). After digestion with RNases, the protected RNA fragments were compared with those produced in a standard RNase protection assay of steady-state RNA isolated from oocytes of the same injection batch.

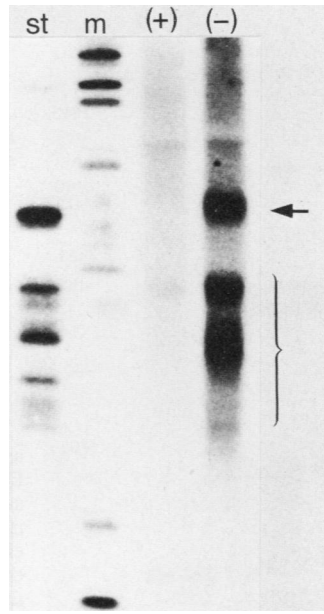

FIG. 5. RNase protection assays of pulse-labeled and steady-state RNAs. In vitro pulse-labeled RNA was prepared from 40 nuclei that were first dissected from oocytes injected with XX15 and then incubated for 10 min with [α-^{32}P]CTP (3,000 Ci/mmol). Equal amounts of RNA were hybridized to either a sense (+) or antisense (−) single-stranded DNA probe, and the fragments protected from RNase digestion were compared with those produced in a standard RNase assay of steady-state RNA from 0.2 oocytes of the same injection batch (st). The arrow shows the expected position of correctly initiated and elongated transcripts; the brace indicates the truncated transcripts found in both samples. Lane m, *Msp*I-digested pBR322 fragments.

The protected fragments obtained from pulse-labeled RNA that hybridized to the antisense probe but not to the sense probe were indeed similar to those derived from steady-state RNA (Fig. 5). It appears that both transcripts elongated beyond the terminus of the probe and 3′-truncated transcripts were produced during the 10-min pulse. The abundance of truncated transcripts relative to elongated transcripts proved to be similar for pulse-labeled and steady-state RNAs, as assessed by counting the radioactivity in the excised gel bands. Thus, the production of the truncated transcripts was not due to a cytoplasmic processing activity, although we think that their size was reduced slightly by exonucleases in the cytoplasm (see below), which may account for the relative underrepresentation of the lower set of truncated transcripts in the pulse-labeled sample. We also think it unlikely that rapid degradation in the nuclear homogenates could produce the truncated pulse-labeled transcripts, since we were unable to observe degradation of a labeled synthetic sense-strand transcript taken through a mock pulse-labeling reaction (not shown). However, on the basis of these results alone, we cannot exclude the possibility that the truncated transcripts were caused by transcription-linked RNA processing.

Effect of template dose on premature termination. As mentioned above, we found some variability in the absolute and relative numbers of small protected fragments in assays of RNA from different injections. In particular, we noticed a definite trend in that the ratio of truncated transcripts relative to elongated transcripts was markedly less when the amount of injected DNA was low (10 to 60 pg) than when

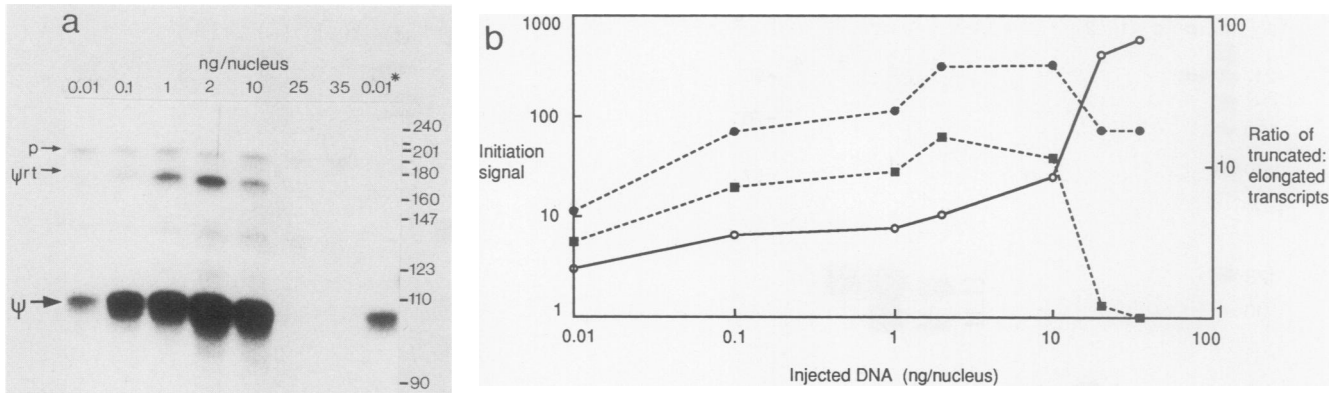

FIG. 6. Effect of injected template dose on XαT14 transcription. (a) RNase protection assay of total RNA from two oocyte-equivalents, each injected with the amount of ΨXX15 DNA indicated above each lane. The lane marked with an asterisk is a repeat assay of the sample assayed in the first lane but containing three oocyte-equivalents of RNA. The brace indicates prematurely terminated transcripts, Ψ marks the position of correctly initiated and elongated ΨXX15 transcripts, rt marks the position of readthrough transcripts, and p marks the position of undigested probe. Sizes in nucleotides of coelectrophoresed pBR322 marker fragments are shown. (In these experiments, we did not add vector DNA to keep total DNA per nucleus equal because at high ratios vector DNA competes with XαT14 for transcription [data not shown].) (b) Quantitation of the RNase protection assay. Gel slices containing the elongated or truncated fragments from all but the asterisked sample in panel a were excised, and their radioactivity was measured by scintillation counting. To correct for the different lengths of probe protected, the counts minus background for each slice was divided by the average number of C residues in each type of protected fragment. These figures were then used as estimates of the number of elongated transcripts initiated (■) and the number of truncated transcripts initiated (●) and to provide the ratio of both types of transcript (○). (The estimate for truncated transcripts at 0.01 ng is unreliable because of the low absolute numbers of counts and the large size of the gel slice.)

more DNA (2 to 10 ng) was injected. (We have used the term elongated here to describe transcripts showing protection to the end of the probe, although we do not know that all such transcripts extend to the end of the gene.)

To examine this template effect more rigorously, we carried out a titration experiment in which we altered the amount of ΨXX15 DNA injected into the oocytes of a single female from 10 pg to 35 ng per nucleus (i.e., from about 5×10^5 to 2×10^9 plasmid molecules) and assayed the transcripts by RNase protection. After injection of 10 pg of DNA, the only clearly visible protected fragments corresponded to correctly initiated and elongated transcripts (Fig. 6a). The amount of elongated transcript produced increased steadily with injected DNA up to 2 ng per nucleus, but less was produced at 10 ng and none was produced at 35 ng. Such a reduction in initiation at high template levels has been described for other injected polymerase II promoters (29) and may be due to interplasmid competition for the individual components of multimeric initiation complexes. In contrast, truncated transcripts were hardly detectable at 10 pg per nucleus, but they increased to extremely high levels in the 2- and 10-ng injections and then fell in number in the 25- and 35-ng injections. We excised the protected fragments from this gel to estimate the relative numbers of elongated versus truncated transcripts produced. At the optimal template level for initiation (2 ng in this animal), there were five times more truncated than elongated transcripts, whereas at 35 ng all transcripts were truncated. Even as the absolute amount of both types of transcript increased and then decreased with DNA concentration, the proportion of transcripts that terminated prematurely rose continuously (Fig. 6b). We confirmed these findings in a titration series using oocytes from a second animal (data not shown). Overall, we found the increased prevalence of termination with template dose in oocytes from many different frogs, although in some individuals a small number of prematurely terminated transcripts could be seen at the lowest DNA concentrations used, and in others a small number of elongated transcripts were produced even at very high amounts of injected DNA.

Cytoplasmic localization of truncated transcripts. To determine whether truncated transcripts were associated with paused polymerases that remained engaged on their templates, we manually separated the nucleus and cytoplasm from oocytes injected with XX15. Figure 7 shows the results of an RNase protection assay of steady-state RNA from one such experiment in which nuclear dissections were carried out 2 and 18 h after injection. After 18 h, the majority of the truncated transcripts in the oocyte had accumulated in the cytoplasm, although one band at 60 nucleotides was present in higher amounts in the nucleus. Interestingly, although some truncated transcripts had accumulated in the nuclear fraction after 2 h, there seemed far fewer truncated transcripts relative to elongated transcripts in these oocytes as a whole than in those incubated for 18 h. We confirmed the cytoplasmic accumulation of truncated transcripts in two other injections and noted their paucity relative to elongated transcripts at early time points in a further time course experiment (data not shown).

Although the three largest of the main truncated transcripts were detectable in the nuclear fraction, the small 45-nucleotide group was clear only in the cytoplasm. This finding suggests that the latter transcripts might have resulted from exonucleolytic trimming of the longer truncated RNAs. Even though truncated transcripts were cytoplasmi-

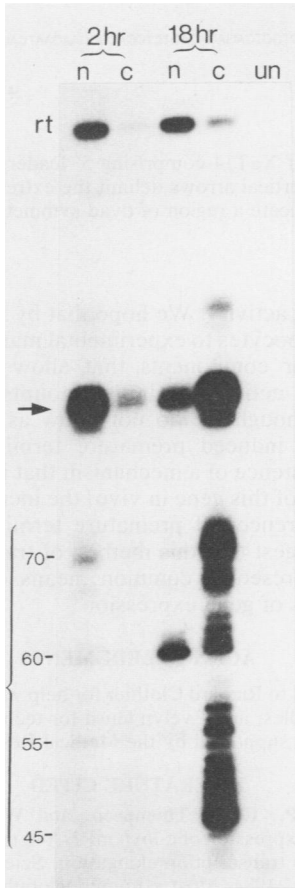

FIG. 7. Nuclear-cytoplasmic localization of transcripts. RNase protection assays of nuclear (n) and cytoplasmic (c) RNA from five oocytes injected 2 h previously and three oocytes injected 18 h previously with 1 ng each of XX15. Lane un shows protection of RNA from five uninjected oocytes. Positions of truncated transcripts (brace) and sizes in nucleotides of major truncated transcripts are marked. The arrow indicates protected fragments from correctly initiated and elongated transcripts; rt indicates read through transcripts.

cally localized, they were present in greatly reduced amounts compared with elongated transcripts in the poly(A)$^+$ fraction of transcripts from injected XαT14 (data not shown).

DISCUSSION

We have shown that transcription of an injected *Xenopus laevis* α-tubulin gene in oocytes can generate many transcripts that are correctly initiated but possess premature 3′ ends. Two findings, taken together, argue against RNA processing as an explanation for the production of these short transcripts. First, the extent of premature 3′-end formation in steady-state RNA accumulated after overnight incubation was equivalent to that observed in pulse-labeled nuclear RNA formed in vitro in just 10 min. Second, truncated transcripts were rare when small amounts of DNA were injected, became increasingly prevalent as more template was injected, and were the predominant type of transcript formed at large template doses, although transcript number was similar to that at low template doses. It seems unlikely that a nuclear processing activity would deal with the same amount of transcript in a completely different manner simply as a result of the amount of template injected. We have therefore concluded that the truncated transcripts are produced by the premature termination of transcription. In addition to premature termination being experimentally inducible through alterations in template dose, we also found that truncated transcripts were rare at early stages of incubation, suggesting that premature termination can also be induced as a result of prolonged incubation periods.

Despite the large number and variety of genes whose transcription has been studied in injected oocytes, we are aware of only one other proven case of premature transcription termination in this system, namely, the c-*myc* gene of humans and mice (4). Although the rarity with which the phenomenon has been observed may well indicate that it is a feature of only a few genes, it may also be that a particular combination of fortuitous circumstances is required to observe it. For one, the type of assay used to measure the transcription of injected genes is crucial for the recognition of the very short and nonadenylated truncated transcripts produced by XαT14. With use of unfractionated oocyte RNA, such transcripts would be either undetectable or indistinguishable from full-length transcripts in primer extension assays or in nuclease protection assays using 5′-end-labeled probes, and they would be detectable only by uniformly labeled probes if the termination event was upstream of the 5′ end of the probe. In addition, this type of transcript would not be seen by translation-dependent assays or in assays of poly(A)$^+$ RNA and could well be missing from Northern blots aimed at identifying transcripts in the kilobase pair size range. In addition, if premature termination was a feature of other injected genes, its recognition might depend on the particular levels of template injected.

An unusual feature of XαT14 that has been important here in understanding the generation of truncated transcripts is that after injection into oocytes, it exhibits accurate and efficient transcription initiation and the absence of nonspecific transcription even when the number of injected templates is at least 100 times lower than those commonly used for genes transcripted by RNA polymerase II. We think that the exemplary transcriptional behavior of the injected gene at low template doses is due to the fact that XαT14 is an *X. laevis* gene and that it is naturally expressed in oocytes. Clearly, without the ability of XαT14 to be faithfully and efficiently transcribed at low template doses, and hence our observation that truncated transcripts are relatively rare at these doses, we would have been led to the conclusion that premature termination observed at normal doses was a constitutive feature of the injected promoter or transcription unit. Instead, our results suggest that premature termination is an experimentally inducible phenomenon that may be useful in trying to understand the control of transcription in oocytes.

What might be inducing the premature termination of transcription on injected XαT14? As mentioned in our introductory comments, several different causes of premature termination appear feasible; one, described for the *Drosophila hsp70* genes (24), involves stalling of polymerase and nascent transcript in the 5′ leader and results in their prolonged engagement on the template until release by heat shock allows continued elongation. However, since truncated XαT14 transcripts accumulate at high levels in the cytoplasm during incubation, it seems likely that here the mechanism comprises a true termination event including transcript release rather than polymerase stalling. A second possible mechanism may be represented by the transcriptional block that operates first at the intron-exon boundary of

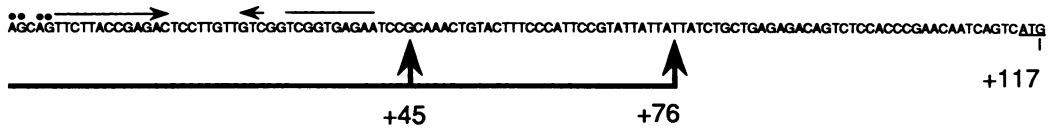

FIG. 8. DNA sequence of XαT14 5′ leader. The sequence of the 117-bp first exon of XαT14 comprising 5′ leader and ATG is shown numbered relative to the first of the four possible transcription initiation sites (●). The vertical arrows delimit the extremes of the region in which the 3′ ends of prematurely terminated transcripts form. The horizontal arrows indicate a region of dyad symmetry in which a stable 13-bp stem–8-base loop structure could form.

c-*myc* genes in mammalian cells and *Xenopus* oocytes (4). Since the block is situated well downstream of initiation and is correlated with the induction of a DNase I-hypersensitive site nearby (3), it may be caused by the regulated binding of a positive termination factor. If so, it would appear to represent a different type of mechanism than that operating in the case of XαT14, since we would predict that premature termination caused by binding of a positive termination factor would be more rather than less common at low template doses. A third type of explanation, and one that we favor for injected XαT14, is that perturbation of a cellular antitermination mechanism could induce premature termination. The existence of antitermination functions in eucaryotic cells analogous to those of procaryotes is suggested by the situation described for HIV promoters (12), in which transcription is blocked and short transcripts produced in the absence of the viral transactivator protein Tat. It has been suggested that Tat may act directly or indirectly as an antiterminator (26). The transcription block that Tat overcomes seems dependent on a stable stem-loop structure in the 5′ leader very close to the site of initiation (26, 28); interestingly, the proximal region of the XαT14 leader also could potentially form such a structure (ΔG, −16 kcal/mol) (Fig. 8). It should also be noted that the region in which the 3′ ends form in XαT14 is rich in A and T residues.

If induction of premature termination on injected XαT14 results from the perturbation of an antitermination activity, what can we deduce about the nature of this activity and how it has been perturbed? Investigations in several experimental systems have shown that the formation of normal and of premature 3′ ends can be intimately connected to the nature of either the promoter or the preinitiation complex from which RNA polymerases initiate transcription (4, 10, 16, 24). Consideration of the template titration and time course effects on premature termination in XαT14 leads us to suggest that in this case too the induction of premature termination may be due to alterations in the nature of transcription initiation. What is particularly intriguing about the effect of increasing template dose on transcription of XαT14 is that even while the tendency toward premature termination rises, the absolute number of elongated transcripts produced can also increase. Thus, it seems that our results cannot simply be explained by the titration of an antitermination factor acting at the level of transcribing polymerases or transcripts. Rather, we interpret our results as indicating the existence of an antitermination activity that can bind to promoters and whose presence at a crucial period during the initiation process can enable those promoters with assembled preinitiation complexes to drive the production of elongated transcripts. Conversely, the absence of this activity from such promoters would result in abortive, prematurely terminated transcription. It would follow that those templates injected at low DNA concentrations and those that assemble their initiation complexes earlier than others would be the most likely to acquire this antitermination activity. We hope that by making use of the amenability of oocytes to experimental manipulation, we can identify cellular components that allow the depletion of antitermination activity by large amounts of XαT14 to be overcome. Although we do not know as yet whether this experimentally induced premature termination in XαT14 reflects the existence of a mechanism that is used to regulate the expression of this gene in vivo, the increasing number of reported occurrences of premature termination in specific genes does suggest that this method of transcriptional regulation may represent a common means of profoundly affecting patterns of gene expression.

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