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A DNA-Binding Protein Is Required for Termination of Transcription by RNA Polymerase I in *Xenopus laevis*

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We describe a partially fractionated in vitro transcription system from *Xenopus laevis* for the assay of transcription termination by RNA polymerase I. Termination in vitro was found to require a specific terminator sequence in the DNA and a DNA-binding protein fraction that produces a footprint over the terminator sequence.

Xenopus laevis is typical of many eucaryotes in having two transcription units located on each repeating unit of its ribosomal DNA (rDNA). One transcription unit is initiated at the gene promoter and has its 3′ end at site T2, which forms the 3′ end of the 40S precursor RNA (13). T2 appears to be a terminator that has acquired a single-base mutation that allows polymerase to read through while retaining the ability to form 3′ ends (16). The other transcription unit initiates at one or more of the spacer promoters and terminates at T3, a full termination element that is located about 60 base pairs (bp) upstream of the gene promoter (13, 17, 18). T3 also appears to be the terminator for transcription that has read through T2. Mutants of T3, assayed by oocyte injection, have shown that full terminator activity at T3 requires only the sequence GACTTGCNC (T3 terminator), where N appears to be a neutral base (16). Changing the terminal C to a G abolishes termination while retaining 3′-end formation; it is this C-to-G mutation which occurs naturally at site T2 (the T2 sequence is GACTTGCNG) at the 3′ end of the 40S coding region. Both T3 and T2 specify 3′ ends 15 nucleotides (nt) upstream of the T3 and T2 elements, respectively.

Termination elements have also been identified in mammalian rDNA, particularly in the mouse genome. A series of tandemly repeated terminators (Sal boxes) are located at the 3′ end of the 45S precursor coding region (7), and a single terminator is present upstream of the gene promoter in homology with the location of terminator elements in *X. laevis* (6, 9). A major difference between mouse and frog genomes is that the terminators at the 3′ end of the 45S region are not mutated in the mouse and cause efficient termination. In addition, it has been shown in the mouse system that a specific DNA-binding protein, TTF1, interacts with the Sal box and is required for termination (1, 8).

In this report, we describe a partially fractionated in vitro system derived from *X. laevis* in which transcription termination by RNA polymerase I can be assayed. We show that in *X. laevis*, as in the mouse, a DNA-binding protein is required for termination of transcription by RNA polymerase I.

MATERIALS AND METHODS

Plasmid templates. The *Ssp*I site in the vector pGEM4 (Promega Biotec) was converted to a *Bgl*II site by the addition of *Bgl*II linkers (5′-CAGATCTG-3′). The −158 to +50 *Sal*I-*Bam*HI fragment from plasmid 5′,Δ-158 (17) and the −245 to +50 *Sal*I-*Bam*HI fragment from plasmid Ψ40 (12) were cloned into the *Sal*I and *Bam*HI sites of the above-modified pGEM4 vector to give pTV −158 and pTV −245, respectively.

Double-stranded oligonucleotides with *Bgl*II-compatible ends (T3-A, T3-B, T3-B mutant, and T3-C) were cloned into the *Bgl*II site of pTV −158 to give pTV −158 T3-A, pTV −158 T3-A (reverse), pTV −158 T3-B, pTV −158 T3-B (mutant), and pTV −158 T3-C (sequences of the oligonucleotides are shown in Fig. 1). Note that T3-A was cloned into pTV −158 in both orientations. T3-A was also cloned into the *Bgl*II site of pTV −245 to give pTV −245 T3-A.

*Bgl*II linkers (5′-CAGATCTG-3′) were ligated onto a 124-bp *Sma*I fragment that contained T2 (positions 159 to 282; 13). This fragment was then ligated into the *Bgl*II sites of both pTV −158 and pTV −245 to give pTV −158 T2 and pTV −245 T2.

Plasmid pGEM1 T2 (*Sma-Hin*f) was used as the source of the T2 footprinting probe and was constructed as follows. The *Hin*fI site at position 303, downstream of T2 (13), was converted to an *Eco*RI site by the addition of an *Eco*RI linker (5′-GGAATTCC-3′) so as to allow cloning of the *Sma*I-*Hin*fI T2 fragment (positions 159 to 303) into the *Sma*I and *Eco*RI sites of the vector pGEM1 (Promega).

S-100 extracts. S-100 extracts were prepared from the *X. laevis* kidney cell line Xl K2 (established by J. Roan, this laboratory) as previously described (17) except that cells were harvested with PBSA containing 1 mM EDTA in place of trypsin and the final extract was dialyzed into 0.1 M KCl column buffer (CB100; 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 100 mM KCl, 0.1 mM EDTA, 20% glycerol, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride, 1 mm sodium metabisulfite). Preparation of S-100 extracts and subsequent chromatography were done at 4°C.

S-100 extracts in CB100 were chromatographed over DEAE-Sepharose CL6B (Pharmacia). Typically, 35 ml of S-100 (18 mg/ml of protein) was loaded onto a 20-ml DEAE-Sepharose column equilabrated in CB100. After loading, the column was washed with 30 ml of CB100, and peak protein fractions were pooled (40 ml), quick-frozen in liquid nitrogen, and stored at −70°C. This fraction is called DEAE flowthrough and represents approximately 60% of the protein loaded onto the column. The column was then eluted with 40 ml of 0.35 M KCl column buffer (CB350). Protein-containing fractions were pooled (20 ml), dialyzed for 4 h against 50 volumes of CB100, clarified by centrifugation, quick-frozen in liquid nitrogen, and stored at −70°C. This

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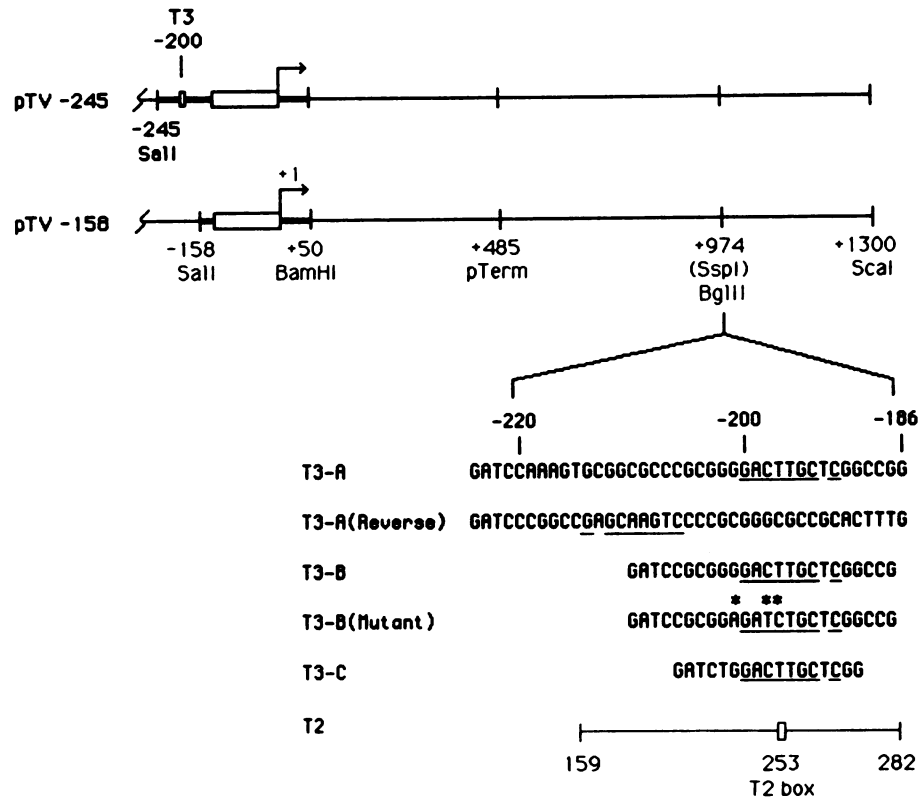

FIG. 1. Structures of transcription templates. Plasmids pTV −245 and pTV −158 contain, respectively, the *X. laevis* rDNA promoter sequences from −245 to +50 and −158 to +50 relative to the transcription initiation site at +1. Both sequences were cloned as *Sal*I-*Bam*HI restriction fragments into a modified form of the vector pGEM4 (Promega), in which the unique *Ssp*I site had been converted into a unique *Bgl*II site. Symbols: □, rDNA promoter (−142 to +6) and the T3 box; ▬, other rDNA sequences; —, vector sequences; →, site of transcription initiation (+1). Locations of the *Bgl*II site, the *Sal*I and *Bam*HI sites used for cloning, and the *Sca*I site used to linearize the templates for transcription assays are shown relative to the site of transcription initiation. The location of the fortuitous terminator in the vector sequence (pTerm; see text) is also shown. The sequences (noncoding strand) of double-stranded T3 oligonucleotides that were inserted into the *Bgl*II site of pTV −158 and pTV −245 to give plasmids pTV −158 T3-A, pTV −245 T3-A, pTV −158 T3-A (reverse), pTV −158 T3-B, pTV −158 T3-B (mutant), and pTV −158 T3-C are shown. Numbers above these sequences refer to their locations upstream of the site of transcription initiation. Nucleotides within the T3 terminator element are underlined, and the altered nucleotides in the mutated T3 terminator are marked by asterisks. The structure of the T2 fragment inserted into the *Bgl*II site of pTV −158 and pTV −245 to give pTV −158 T2 and pTV −245 T2 is shown. The numbers below refer to its location downstream of the 3′ end of the 28S (13), and the open box shows the location of the T2 element within this fragment.

fraction is called the DEAE 0.35 M fraction and represents approximately 30% of the protein loaded onto the column. We judge that the DEAE flowthrough represents a true fraction (as opposed to leakthrough from an overloaded column), since all of the RNA polymerase I activity adsorbed to the column and none appeared in the flowthrough. Likewise, adding an additional 200 mM KCl wash step between the CB100 and CB350 steps had no effect on the properties of either the DEAE flowthrough or 0.35 M fractions.

Transcription reactions. Transcription reactions with the unfractionated S-100 extract were performed as follows: S-100 extract (20 μl) was mixed with 400 ng of template plasmid (digested with *Sca*I) and incubated on ice for 10 min. Then 20 μl of reaction buffer (25 mM HEPES [pH 7.9], 80 mM KCl, 12 mM $MgCl_2$, 1.0 mM ATP, GTP, and UTP, 200 μM CTP, 10 mM creatine phosphate, 200 μg of α-amanatin per ml, 1 mM DTT) supplemented with 5 μCi of [α-^{32}P]CTP (800 Ci/mmol; Dupont, NEN Research Products) and 20 U of RNasin (Promega) was added. The reaction was then incubated at 25°C for 1 h.

Transcription reaction mixtures with the fractionated S-100 extract contained 20 μl of the DEAE 0.35 M fraction and a specified amount of DEAE flowthrough (usually 10 μl) or buffer. Combined DEAE 0.35 M and flowthrough was incubated with 400 ng of *Sca*I-digested template on ice for 10 min. After the addition of 20 μl of reaction buffer supplemented with [α-^{32}P]CTP and RNasin as described above, the reaction mixture was incubated at 25°C for 1 h.

All transcription reactions were stopped by the addition of 250 μl of 7 M urea–100 mM LiCl–0.5% sodium dodecyl sulfate–10 mM EDTA–10 mM Tris (pH 7.9)–100 μg of tRNA per ml. After phenol-chloroform and chloroform extraction, 20 μl of 7.5 M ammonium acetate was added. The reaction was then precipitated with 2.5 volumes of ethanol. This procedure removes most of the unincorporated isotope. The pellet was suspended in 10 μl of 10 mM sodium phosphate (pH 7.0). Then 25 μl of 70% dimethyl sulfoxide–1.5 M glyoxal (deionized)–10 mM sodium phosphate (pH 7.0)–0.05% bromophenol blue was added, and the tube was incubated at 50°C for 1 h. The glyoxylated RNA was electrophoresed overnight on a 1.5% agarose gel in 10 mM sodium phosphate (pH 7.0) with recirculation. An end-labeled *Taq*I digest of pBR322, glyoxylated as described above, was used as a size marker on all gels. Gels were dried

onto DE81 paper (Whatman, Inc.) and exposed on Kodak X-ray film.

Transcription signals were quantified by scanning autoradiographs with a Videk 1Kx1K CCD camera coupled with image analysis on a Sun 3/260 computer (Sun Microsystems) with Visage 2000 software (BioImage).

Nuclear extracts. (i) Fractionation of Rib 2. We initiated the fractionation of Rib 2 from S-100 extracts but switched to nuclear extracts as a more abundant source. Nuclear extracts were prepared from Xl K2 cells as described previously (19). Approximately 100 mg of nuclear protein in CB100 (50 ml) was loaded onto a 20-ml DEAE-Sepharose column equilibrated in CB100. The column was washed with CB100 until protein levels returned to base line. Protein-containing flowthrough fractions were pooled (60 ml) and loaded onto a 10-ml phosphocellulose P11 column (Whatman) equilibrated with CB100. The P11 column was washed with CB100 until protein levels dropped to base line. The column was then eluted sequentially with 15 ml of 0.3 M KCl column buffer (CB300), 0.65 M KCl column buffer (CB650), and 1.0 M KCl column buffer (CB1000). Protein fractions in the 0.65 M fraction were pooled (6 ml), dialyzed for 4 h against 50 volumes of CB100, quick-frozen in liquid nitrogen, and stored at −70°C. A T3 DNA affinity column was made with two annealed and polymerized oligonucleotides that represent the sequence −220 to −186, coupled by CNBr to Sepharose (10). The sequences of the oligonucleotides used were 5′-GATCCAAAGTGCGGCGCCCGCGGGGA CTTGCTCGGCCGG-3′ and 5′-GATCCCGGCCGAGCAA GTCCCCGCGGGCGCCGCACTTTG-3′. The dialyzed P11 0.65 M fraction (6 ml) was converted to 0.1 M KCl affinity column buffer (ACB100; 25 mM HEPES [pH 7.9], 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 1 mM DTT, 0.1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium metabisulfite) by adding Nonidet P-40 to 0.1% and $MgCl_2$ to 12.5 mM. Poly(dI-dC) (30 μg) was added, and the mixture was incubated on ice for 15 min. The protein was then applied to a 0.8-ml T3 DNA affinity column, and the flowthrough was applied two more times. The column was washed with 1 ml of ACB100 and 2 ml of 0.2 M KCl affinity column buffer (ACB200). Rib 2 was eluted from the column twice with 0.8 ml of 0.8 M KCl affinity column buffer (ACB800). The pooled ACB800 fractions (first-round affinity) were diluted to 0.1 M KCl by adding affinity column buffer without KCl, 5 μg of poly(dI-dC) was added, and the mixture was incubated on ice for 15 min. This mixture was then applied to a 0.5-ml T3 DNA affinity column, and the flowthrough was applied two more times. The column was washed with 1 ml of ACB100 and 1 ml of ACB200, and the Rib 2 was eluted twice with 0.5 ml of ACB800. The pooled ACB800 fractions (second-round affinity) were quick-frozen in liquid nitrogen and stored at −70°C.

(ii) Purification of xUBF. xUBF was purified from nuclear extracts as previously described (19).

DNase I footprinting. DNase I footprinting (5) was performed as previously described (21). Poly(dI-dC) was used as the nonspecific competitor for footprinting with crude Rib 2 fractions, and poly(dA-dT) was used for crude xUBF fractions. No competitor was used when footprinting with more purified fractions. The T3 footprinting probe used was the −245 to −166 *Sal*I-*Sma*I fragment (sequence shown in Fig. 1). For footprinting on the noncoding strand, this fragment was labeled on the 5′ end of the *Sal*I site with [γ-^{32}P]ATP (3,000 Ci/mmol; Dupont) and T4 polynucleotide kinase. For footprinting on the coding strand, the 3′ end of the *Sal*I site was labeled with [α-^{32}P]TTP (800 Ci/mmol; Dupont) and the Klenow fragment of DNA polymerase I. The T2 probe used was derived from plasmid pGEM1 T2 (*Sma-Hin*f) by digesting with *Eco*RI and labeling the 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase, followed by digestion with *Bam*HI.

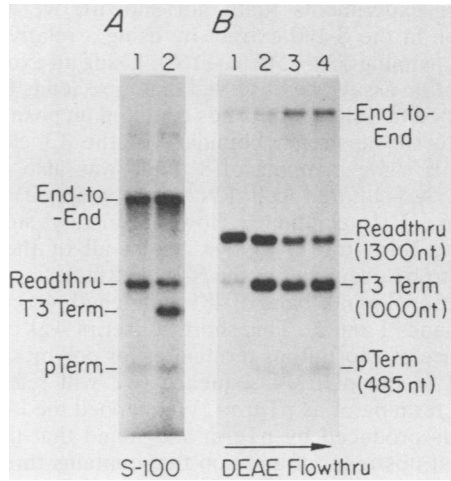

FIG. 2. Assay of termination by RNA polymerase I in vitro. (A) Termination in the S-100 extract. Plasmids pTV −158 and pTV −158 T3-A were linearized with the restriction enzyme *Sca*I and transcribed in vitro with unfractionated S-100 extract. Reactions were performed, and the RNA was analyzed on a 1.5% agarose gel as described in Materials and Methods. Shown are the results of transcribing pTV −158 (lane 1) and pTV −158 T3-A (lane 2). (B) Stimulation of termination by DEAE flowthrough. *Sca*I-digested pTV −158 T3-A was transcribed in 20 μl of the DEAE 0.35 M fraction supplemented with 0 (lane 1), 5 (lane 2), 10 (lane 3), or 15 (lane 4) μl of DEAE flowthrough as described in Materials and Methods. T3 Term, Transcripts initiated at +1 and terminated at the site of T3 insertion; Readthru, transcripts initiated at +1 that read through the site of T3 insertion to the end of the template; pTerm, transcripts initiated at +1 and terminated within the vector (see text). The approximate length, in nucleotides, of each transcript is shown. End-to-end transcripts are labeled as such.

RESULTS

An in vitro termination assay from *X. laevis* cells. S-100 whole-cell extracts, made from cultured *X. laevis* kidney cells, have the ability to specifically initiate and terminate transcription in vitro. An example of such a reaction is shown in Fig. 2A; the structure of the template is shown in Fig. 1. A fragment of *X. laevis* rDNA containing the gene promoter (sequences from −158 to +50) was inserted into the polylinker of pGEM4. When this construct was linearized at a unique *Sca*I site within the vector, specific initiation at the promoter resulted in a runoff transcript that was 1,300 nt long (Fig. 2A, lane 1). Initiation in the S-100 extract was also assayed by S1 nuclease protection and shown to occur at the identical location used in vivo (data not shown).

To assay for transcription termination, potential terminator sequences were inserted at a modified *Ssp*I site located 974 bp downstream of the transcription initiation site. When a fragment containing the T3 terminator (T3-A; Fig. 1) was placed in this location in the correct orientation, there was a decrease in the 1,300-nt transcript, and a shorter transcript of about 1,000 nt was produced (Fig. 2A, lane 2). Throughout this report, we will take the presence of this 1,000-nt transcript as evidence of termination.

In other experiments (data not shown), we examined termination in the S-100 extract by using a relatively large fragment spanning T3 (−245 to −168). Using an exonuclease VII protection assay, we observed that the 3′ ends formed in vitro mapped to a cluster of bands centered on position −219 (relative to the upstream boundary of the T3 element at −200). This same mapping of 3′ ends was also observed when in vivo-cultured cell RNA was mapped with the exonuclease VII technique. However, many steps were required to form the in vivo 3′ ends; all of these steps appeared to be occurring in the S-100 extract.

An additional minor band of 485 nt in length was observed (Fig. 2A, lanes 1 and 2). This short transcript was caused by the presence of a fortuitous terminator for polymerase I that is present in the pGEM4 sequence (we will refer to this fortuitous terminator as pTerm). We mapped the location of the 3′ ends produced by pTerm and found that they were located just upstream of a region that contains three copies of a sequence that is closely related but not identical to the GACTTGCNC motif that we believe is the active termination element in T3. The signal produced by pTerm responded to added protein fractions (Fig. 2B) and to oligonucleotide competition (see Fig. 6A) just like the authentic termination signal produced by the T3 element except that the signal produced by pTerm was considerably weaker. Therefore, it appears that pTerm is a fortuitous weak terminator in the vector. It will require further mutagenesis to completely establish which vector sequence elements are responsible for the 485-nt signal.

Strictly speaking, the termination assay shown in Fig. 2A measures 3′-end formation and makes no statement about release of the polymerase. In other experiments, however, we have found by S1 nuclease protection analysis that RNA synthesis downstream of the terminator site is reduced by about fivefold (data not shown). Furthermore, time course experiments revealed no precursor product relation between the 1,300-nt readthrough band and the 1,000-nt termination band of Fig. 2A (the 1,000-nt band appears slightly before or at the same time as the 1,300-nt band; data not shown). The T2 element, an RNA-processing site that is related in sequence to T3, behaved very differently from T3 in this assay system (see Fig. 7). Finally, the T3 element has been extensively characterized and shown to be a bona fide terminator in vivo (13). For these reasons, we will operationally define the event assayed in Fig. 2 as termination while being aware that the actual mechanism occurring at the terminator element remains to be completely defined.

Fractionation of the S-100 extract. The S-100 extract was applied to DEAE-Sepharose, and two fractions were collected, a flowthrough fraction and a fraction eluting with 0.35 M KCl. The 0.35 M fraction contained all of the components required for specific transcription initiation (Fig. 2B, lane 1). However, transcription termination was relatively deficient in the 0.35 M fraction (only about 5% of the specifically initiated transcripts were terminated). Addition of the flowthrough to the 0.35 M fraction stimulated termination (Fig. 2B, lanes 2 to 4) to the point where 80% of the specifically initiated transcripts were correctly terminated. Addition of the flowthrough had little effect on the overall amount of specific initiation, but it did cause an increase in the level of nonspecific, end-to-end transcription seen at the top of the gel. Formation of the 485-nt transcript was stimulated by the flowthrough in concert with the larger 1,000-nt transcript.

These results suggest that termination requires the presence of one or more proteins present in the DEAE flowthrough fraction in addition to those required for transcription initiation present in the DEAE 0.35 M fraction.

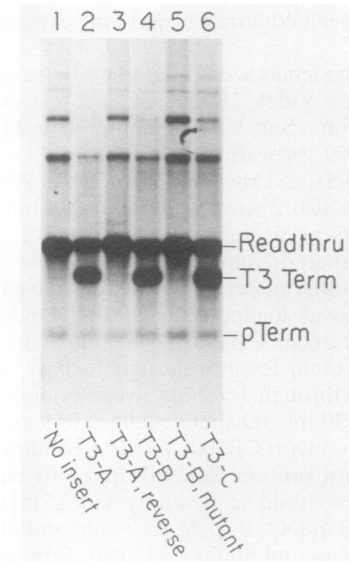

FIG. 3. Sequence requirements for termination. The following plasmids, with T3 oligonucleotides inserts, were cleaved with *Sca*I and transcribed in vitro with combined DEAE 0.35 M (20 μl) and DEAE flowthrough (10 μl) fractions: pTV −158 (lane 1), pTV −158 T3-A (lane 2), pTV −158 T3-A (reverse) (lane 3), pTV −158 T3-B (lane 4), pTV −158 T3-B (mutant) (lane 5), and pTV −158 T3-C (lane 6). The name of the T3 insert in each case is shown under the appropriate lane (see Fig. 1 for sequence). The various classes of transcript are labeled as in Fig. 2.

DNA sequence requirements for transcription termination in vitro. We used the reconstituted transcription system (flowthrough added back to the 0.35 M KCl fraction) to assay the effect of various DNA sequences (Fig. 1) on termination in vitro (Fig. 3). In the absence of any insert at the +974 test site, no termination was observed (Fig. 3, lane 1). Insertion of a fragment containing the T3 terminator (fragment T3-A; Fig. 1) resulted in termination and formation of a new band of approximately 1,000 nt in length (lane 2). Reversing the orientation of T3 (lane 3) abolished termination. A shorter fragment containing the T3 element (T3-B) still caused termination (lane 4), whereas a cluster of three point mutations (two within the T3 element) abolished termination (lane 5). The T3 element with only 1 additional bp on the 5′ side and 2 extra bp on the 3′ side was still capable of causing termination (T3-C; lane 6).

These results in vitro are in complete agreement with previous results obtained by oocyte injection (14, 16) which showed that the sequence GACTTGCNC, in the correct orientation, is necessary and sufficient for termination by *X. laevis* RNA polymerase I.

The transcription initiation factor xUBF is not required for termination. In preliminary experiments, we observed that adding an excess of a DNA fragment containing the T3 element inhibited transcription termination in vitro (a later version of this experiment is shown in Fig. 6A). These experiments suggested that termination required a protein with the ability to recognize and bind to the T3 element. Therefore, we began searching for such a protein.

One possible candidate for a termination protein is xUBF, a DNA-binding protein that has been purified in other work and is required for initiation by RNA polymerase I (19). xUBF was first detected because of its ability to produce

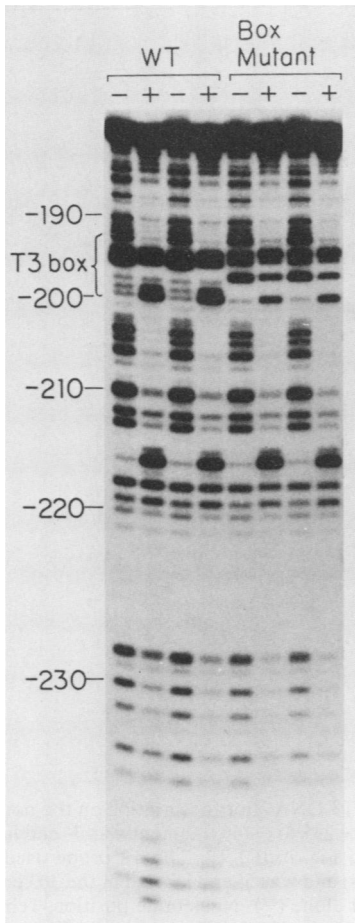

FIG. 4. Demonstration that xUBF binding to T3 is not impaired by mutations in the T3 terminator. xUBF (5 μl of 0.8 M Biorex 70 fraction; 20) was footprinted with DNase I onto wild-type T3 (WT) and mutant T3 (Box Mutant) as described in Materials and Methods. The wild-type probe was the −245 to −166 *Sal*I-*Sma*I fragment, from plasmid pGem40, 5′ end labeled at −245 (see Materials and Methods). The mutant probe was identical except that it was prepared from the T3 linker scanner mutant pGem T3 LS-7 (17). Mutant and wild-type probes differ in sequence, as do the oligonucleotides T3-B and T3-B (mutant) (see Fig. 1). The absence (−) or presence (+) of xUBF in the footprinting assay is shown above each lane as appropriate. Nucleotide positions relative to the site of transcription initiation and the location of the T3 element are shown alongside the footprint.

DNase I footprints on either side of the T3 element (Fig. 4), and only later was it realized that xUBF also footprints on the promoter and enhancer elements. Despite binding on either side of T3, it appears that xUBF is not directly involved in termination. This conclusion is drawn from the following observations.

First, the same cluster of three point mutations that abolished termination activity (Fig. 3, lane 5) had little or no effect on the DNase I footprinting of xUBF (Fig. 4). Since the mutations changed the sequence of T3, they also altered the DNase I digestion pattern, but hypersensitivity at −200 was still observed along with the same degree of protection upstream and downstream of the T3 element. Thus, there is no correlation between xUBF binding and termination.

Elsewhere we have shown the effect on termination of a series of linker scanner mutations that cover the T3 element plus regions on both sides (B. McStay and R. H. Reeder, submitted for publication). The only mutations that damage termination are those in the GACTTGCNC motif. Mutations in the xUBF-binding sites both upstream and downstream of that motif have no effect on termination. We note, however, that the ability of T3 to stimulate an adjacent polymerase I promoter is damaged by a linker scanner mutation just downstream of T3 (LS-11) which is in one of the xUBF-binding sites. Although xUBF may not be directly involved in termination, it may interact with the protein(s) that does bind to the terminator.

Finally, we show below (see Fig. 6B) that oligonucleotide T3-B (Fig. 1) competed against termination in vitro, whereas a mutated form of T3-B did not. However, both of these oligonucleotides competed with equal, albeit low, efficiency against footprinting of xUBF (data not shown).

Rib 2 an activity that binds to the T3 element. The DEAE flowthrough contained Rib 2, an activity that has been partially purified on the basis of its DNase I footprint directly over the T3 element. As described in Materials and Methods, Rib 2 has been partially purified by chromatography on DEAE-Sepharose, phosphocellulose P11, and two cycles on a DNA affinity column made with a T3 oligonucleotide.

We examined the characteristics of the Rib 2 footprint. Figure 5A is a comparison of the Rib 2 footprint on both strands of the DNA. The T3 terminator, GACTTGCNC, extends from −200 to −192. On the noncoding strand, Rib 2 protected from −213 to −193 with a number of DNase I-hypersensitive sites centered at −215 and −190. On the coding strand, Rib 2 protected from −215 to −195 with strong hypersensitive sites centered at −218 and −190. Overall, the Rib 2 footprints were almost identical on the two strands, protecting the terminator and approximately 15 bp upstream. It is interesting that the protected region upstream of the terminator includes the sequence that forms the 3′ ends specified by T3.

Rib 2 was also tested for its ability to footprint on a probe that is mutant in the terminator (Fig. 5B). Although Rib 2 gave rise to the same DNase I-hypersensitive sites on wild-type and mutant probes, there was no clear footprint on the mutant, suggesting that Rib 2 has a low affinity for this sequence. This was confirmed by subsequent competition experiments (Fig. 6).

Footprinting of Rib 2 on T2 is discussed below.

Rib 2 is required for termination. We have shown that the DEAE flowthrough from S-100 extracts stimulated termination (Fig. 2B). Because of interference by other DNA-binding proteins and the relatively low abundance of Rib 2, it is difficult to detect Rib 2 footprinting in the DEAE flowthrough. However, further chromatography of the DEAE flowthrough on phosphocellulose results in a fraction in which the specific footprint is clearly observed and which also stimulates termination in vitro (data not shown). Beyond this step, purification can be monitored by footprinting, but the fractions become increasingly inhibitory of transcription initiation. Therefore, they cannot be directly monitored for the ability to stimulate termination in vitro. Despite the transcription inhibitory properties of purified Rib 2 preparations, indirect oligonucleotide competition experiments argue strongly that Rib 2 is required for termination by *X. laevis* RNA polymerase I. Figure 6 shows an experiment in which a polymerized, double-stranded oligonucleotide containing the T3 element (T3-B; Fig. 1) was used to compete in the DNase I footprinting reaction. The oligonucleotide that contains the wild-type, functional T3 element (T3-B) competed very effectively against the Rib 2 footprint (Fig. 6B, lanes 2 to 6). In contrast, the T3-B mutant oligonucleotide,

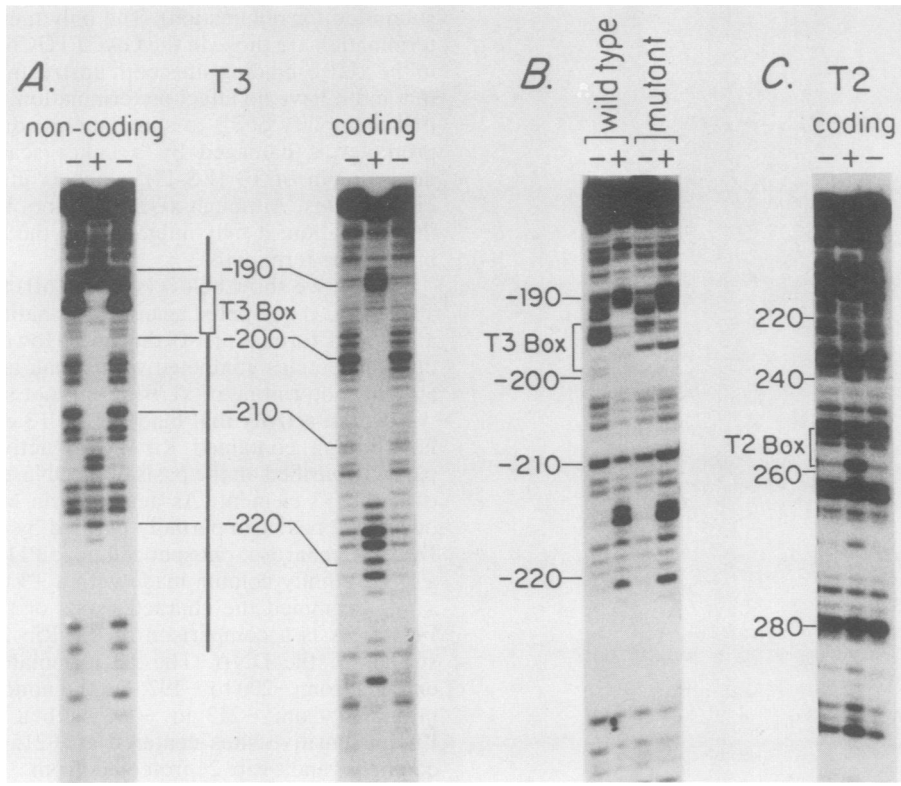

FIG. 5. Characterization of the Rib 2 footprint. (A) Noncoding and coding strands of T3 DNA. For footprinting on the noncoding strand, the same T3 probe was used as in Fig. 4. For footprinting on the coding strand, the same −245 to −166 fragment was 3′ end labeled at −245. (B) T3 mutant. Wild-type T3 and mutant T3 probes were as described in the legend to Fig. 4. (C) T2. The T2 probe used contained the sequences form nucleotides 159 to 303 downstream of the 28S (see Materials and Methods) and was 5′ end labeled at the 303 end. In all cases, footprinting reaction mixtures contained either 8 μl of affinity purified Rib 2 (+) or buffer alone (−). Nucleotide positions relative to the site of transcription initiation and the location of the T3 element are shown alongside T3 footprints. Nucleotide positions relative to the 3′ end of the 28S and the location of the T2 element are shown alongside the T2 footprint.

which has three point mutations that make it inactive as a termination site (Fig. 3, lane 5), was a poor competitor in the footprinting reaction (Fig. 6B, lanes 8 to 12).

Figure 6A shows the effect of the same two oligonucleotides when used as competitors in the in vitro termination assay. Oligonucleotide T3-B effectively eliminated termination, with a concomitant increase in readthrough (Fig. 6A, lanes 1 to 4), whereas the T3-B mutant oligonucleotide had little effect (Fig. 6A, lanes 5 to 8).

We conclude that Rib 2 is required for termination of transcription by *X. laevis* RNA polymerase I. Furthermore, Rib 2 must bind to the T3 element in the template to effect termination.

Characteristics of the T2 element in vitro. T2 is the element that forms the 3′ end of the 40S rRNA precursor. In oocyte injection experiments, T2 behaves as though it were a mutated version on T3 which has lost the ability to cause polymerase release but retains the ability to form 3′ ends (16). In the in vitro assays used in this study, T2 displayed slightly different characteristics but clearly behaved differently from T3.

A large fragment containing T2 was placed in the assay site and tested in the in vitro runoff assay (Fig. 7A, lanes 3 and 4). A T3 element assayed as a control gave a strong termination signal (lanes 1 and 2). In this in vitro system, T2 gave a very weak, almost undetectable termination signal (lanes 3 and 4). In oocyte injection experiments, we have previously found (15) that the ability of T2 to form 3′ ends is strongly dependent on the presence of a copy of the T3 terminator in its normal position upstream of the promoter. We did not see such a requirement in vitro. In one case the promoter driving transcription had a copy of T3 immediately upstream in its normal position (Fig. 7, lane 4), whereas in another this upstream copy of T3 was deleted (lane 3). In neither case did T2 function in this in vitro system.

The T3-B oligonucleotide competed against termination, presumably because of the binding of Rib 2 (Fig. 7B, lanes 1 to 3; this is the same result shown in Fig. 6A, lanes 1 to 4). Under identical conditions, the T2 oligonucleotide had no effect on termination (Fig. 7B, lanes 4 to 6). These results suggest that T2 has a much lower affinity for binding of Rib 2 than does T3.

Weak interaction of Rib 2 with T2 was also indicated by the results of DNase I footprinting. Rib 2 yielded no DNase I protection over the T2 element (Fig. 5C). However, it did cause hypersensitive bands to appear in the same relative locations on either side of the element as seen for T3. Thus, the footprint of Rib 2 on T2 was similar to the footprint of Rib 2 on a mutant version of T3 (Fig. 5B).

The data in Fig. 5C and 7 support the conclusion that Rib 2 interacts very weakly with T2 in vitro. Since T2 also is a poor terminator both in vivo and in vitro, the data support the further inference that binding of Rib 2 is required for termination. What these experiments do not tell us is how T2 can efficiently form 3′ ends in vivo and what role, if any, Rib 2 may play in that process.

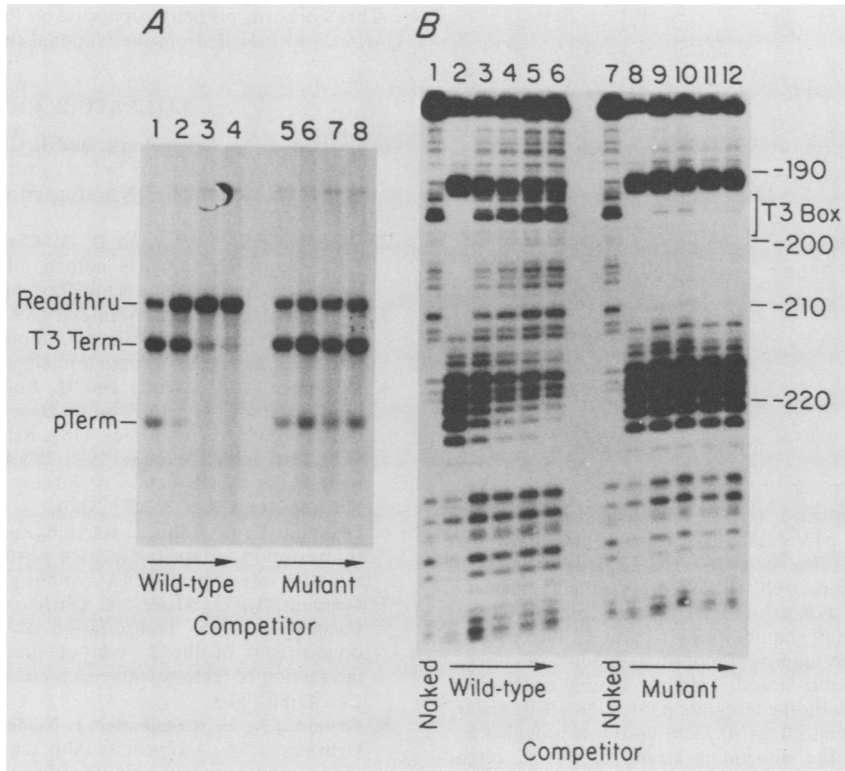

FIG. 6. Competition with oligonucleotides containing the T3 sequence. (A) Effect on termination. Plasmid pTV −158 T3-A was linearized with *Sca*I and transcribed in vitro with combined DEAE 0.35 M (20 μl) and DEAE flowthrough (10 μl) fractions in the presence of increasing amounts of either wild-type or mutant double-stranded T3 oligonucleotides that had been polymerized by ligation up to a length of 2 kilobase pairs. The sequences of competing wild-type (T3-B) and mutant (T3-B [mutant]) oligonucleotides are shown in Fig. 1. Lanes: 1 and 5, no added competitor; 2 to 4, 200, 400, and 600 ng, respectively, of wild-type competitor; 6 to 8, 200, 400, and 600 ng, respectively, of mutant competitor. The various classes of transcript are labeled as in Fig. 2. (B) Effect on Rib 2 footprint. Increasing amounts of wild-type or mutant T3 competitor (as described above) were added to Rib 2 footprinting reaction mixtures that contained 8 μl of affinity-purified Rib 2 and the 5′-end-labeled −245 to −166 T3 probe. Lanes: 1 and 7, naked DNA; 2 and 8, no added competitor; 3 to 6, 2.5, 5, 10, and 20 ng, respectively, of wild-type competitor; 9 to 12, 2.5, 5, 10, and 20 ng, respectively, of mutant competitor. Nucleotide positions relative to the site of transcription initiation and the location of the T3 element are shown alongside the footprints.

DISCUSSION

A DNA-binding protein is required for termination of transcription of *Xenopus* RNA polymerase I. We have previously described an in vitro system (S-100 extract from *X. laevis* culture cells) that supports accurate initiation of transcription on plasmid templates that contain the *X. laevis* ribosomal gene promoter (17). Here we show that this extract also supports efficient termination at the T3 terminator. In oocyte injection assays, we have found that the sequence GACTTGCNC is necessary and sufficient to direct termination (16). It is probable that the same element is sufficient to direct termination in vitro, since an oligonucleotide containing only 3 additional bp is an efficient terminator (T3-C; Fig. 3, lane 6). In addition, termination in vitro is eliminated by the same mutation and requires the same orientation of the element as is observed in the oocyte.

We have identified an activity, which we call Rib 2, that is present in the DEAE flowthrough fraction that gives rise to a DNase I footprint over the T3 terminator. Unfortunately, we have been unable to test directly the involvement of Rib 2 in termination, since our more purified Rib 2 preparations are inhibitory to initiation. One way in which a transcription inhibitor could be generated in our purification scheme is through partial proteolysis of Rib 2 itself, which might generate aberrant DNA-binding fragments. It is also possible that we have concentrated some nonspecific inhibitor. We are now trying to develop fractionation schemes for Rib 2 in which this inhibitory activity is removed.

Even though we have no direct functional test, the following correlations between Rib 2 binding and termination strongly suggest that Rib 2 is required for transcription termination at T3. First, a mutation in the terminator that abolishes termination has a severe effect on Rib 2 binding to T3. Second, both termination and footprinting are competed for by a wild-type but not by a mutant T3 oligonucleotide. Finally, Rib 2 does not footprint on T2, which can be considered a naturally occurring mutation that gives rise to a loss of termination function.

Polymerase I terminates via similar mechanisms in both mouse and frog genomes. Beginning with the earliest studies of polymerase I termination, it has been clear that the mouse and frog systems share many similarities. This study extends this comparison and suggests that the similarities extend to the level of molecular mechanism. In both organisms, termination requires a short DNA sequence element that must be present in the correct orientation. In the mouse this element is called the Sal box (7), and in *X. laevis* it is called the T3 terminator (16). Presumably this sequence is required in the DNA (not in the RNA transcript), since termination correlates with DNA binding and is abolished by competition with excess double-stranded DNA containing the terminator sequence (8; Fig. 6). The terminator sequence is the

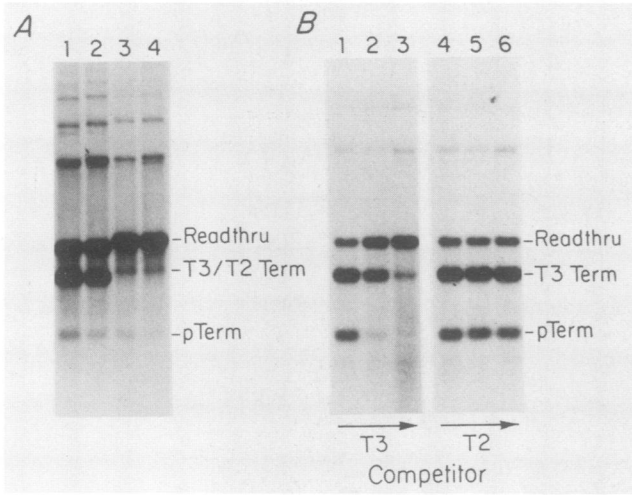

FIG. 7. Assay of T2 in vitro. (A) T2 end formation. Template plasmids with T3 inserts, pTV −158 T3-A (lane 1) and pTV −245 T3-A (lane 2), and with T2 inserts, pTV −158 T2 (lane 3) and pTV −245 T2 (lane 4), were linearized with *ScaI* and transcribed with combined DEAE 0.35 M (20 μl) and DEAE flowthrough (10 μl) fractions. (B) Comparison of the ability of T3 and T2 oligonucleotides to compete for T3 termination. Increasing amounts of either a T3 (T3-B; Fig. 1) or T2 double-stranded oligonucleotide were added to transcription reactions with the template pTV −158 T3-B (linearized with *ScaI*) in combined DEAE 0.35 M and flowthrough fractions. The sequence for the noncoding strand of the T2 oligonucleotide was 5′-CCGGGGGAGGCCTGACTTGCAGGCCCG-3′. Oligonucleotides that were used for competition were polymerized by ligation up to a length of 2 kilobase pairs. Lanes: 1 and 4, no competitor; 2 and 3, 200 and 400 ng, respectively, of T3 competitor; 5 and 6, 200 and 400 ng, respectively, of T2 competitor. The various classes of transcript are labeled alongside the gel as in Fig. 2.

binding site for a protein whose presence is also required for termination. In the mouse, DNA affinity column fractions containing this protein (TTF1) have been shown to stimulate termination when added to a crude extract that is deficient in termination (1). As discussed above, in the frog we deduce that a similar binding protein (Rib 2) is also required.

The mouse and frog ribosomal genes are among the very few cases for which it has been documented that a DNA-binding protein is involved in transcription termination. In most of the systems that have been examined, termination involves interaction between the RNA transcript and one or more proteins (reviewed recently by Platt [20]). Other instances in which a DNA-binding protein may be involved in termination include a report on procaryotes that binding of *lac* repressor upstream of the *lac* operon may cause termination (4). Another instance is termination in human mitochondria, in which a DNA-binding protein is thought to be involved (2, 11). For polymerase II, there is a report that a CCAAT box is an essential part of a termination sequence, presumably as a protein-binding site (3). At present there is no clear indication of how a DNA-binding protein causes termination in any of these instances.

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