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Mapping of transcription initiation and termination signals on *Xenopus laevis* ribosomal DNA

(promoter/terminators/electron microscopy/oocyte injection)

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ABSTRACT **We have injected cloned derivatives of *Xenopus laevis* ribosomal genes into *X. laevis* oocyte nuclei and examined the resulting transcription complexes in the electron microscope. From this work we conclude that the promoter lies somewhere within a region between −320 nucleotides upstream and +113 nucleotides downstream from the site of transcription initiation. This assignment agrees with inferences based on sequence conservation. It further suggests that the duplicated initiation region sequences located further out in the spacer ("*Bam* islands") are not required for the normal high densities of RNA polymerase loading seen on ribosomal genes. Concerning termination, the cluster of four Ts that forms part of the *Hin*dIII restriction site at the 3′ end of the gene appears to be part of the normal termination signal. Termination still occurs when only three Ts are present, but reduction to two Ts damages termination. Because clusters of three Ts appear at several sites within the gene, it is likely that sequences adjacent to the T cluster also are required for normal termination. In addition, we present evidence for a fail-safe termination site just upstream from the site of transcription initiation.**

The genes coding for the large ribosomal RNAs (the rDNAs) from the frog *Xenopus laevis* have been the subject of intensive study during the last decade. This work has culminated recently in determination of the nucleotide sequence of large parts of the repeating unit (1–4), identification of the primary transcript (5), and localization of the precise sites of initiation and termination of transcription (2). In this paper we begin the mapping of the nucleotides involved in promoter and terminator function in the ribosomal genes.

Mapping of promoter and terminator sequences requires a transcription system in which to measure the activity of genes with suitable deletions or other mutations. The approach that we have used is to inject cloned ribosomal genes into oocyte nuclei and to assay transcription of these genes by spreading the nuclear contents for electron microscopy, using the methods of Miller and Bakken (6). Such direct visualization of transcribing ribosomal gene plasmids, as reported by Trendelenburg and Gurdon (7), allows one to observe certain features of transcriptional behavior that would be difficult to study by biochemical methods.

In this paper we compare the specificity and frequency of initiation on a plasmid that bears a complete ribosomal gene and spacer repeat with that on a ribosomal gene from which much of the DNA surrounding the initiation site has been deleted. We also examine the efficiency of termination in recombinant plasmids with altered sequences at the 3′ end of the ribosomal gene.

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MATERIALS AND METHODS

Oocyte Injection. Stage V–VI *X. laevis* oocytes (8) were used for injections. Biochemical measurements have shown that these stages are still highly active in ribosomal RNA synthesis (9, 10). We have noticed, however, that the structure of the ribosomal genes can be quite variable from female to female, possibly due to variations in hormonal state. In the oocytes of some individuals the ribosomal genes show reduced polymerase density and short or absent nascent RNA chains. Such oocytes are very poor in initiating transcription on injected rDNA plasmids. In contrast, injection of oocytes that have large numbers of normal-appearing, well-packed ribosomal genes usually yield several hundred well-spread and analyzable plasmids per electron microscope grid. To increase our success rate, therefore, animals were screened for the state of their endogenous ribosomal genes before using them for plasmid injection experiments.

Circular plasmid DNA for injection was dissolved in modified Barth's solution (11) at 0.5 μg/μl, and in most experiments 5–10 ng was injected into the nuclei of individual oocytes as described by Kressman and Birnstiel (12). Injected oocytes were incubated at 18°C for 3–12 hr. In some experiments α-amanitin was mixed with the DNA solution at 200 μg/ml. Injection of 10–20 nl of α-amanitin at this concentration was enough to greatly reduce transcription from an injected plasmid carrying *X. laevis* 5S genes (data not shown).

Electron Microscopy. Nuclei of injected oocytes were manually isolated in 0.08 M KCl and transferred to a drop of pH 9 water (1 nucleus per drop), and their nuclear membranes were removed with forceps (6). The nuclear contents were spread for electron microscopy essentially as described by Miller and Bakken (6), except that we have found it necessary to centrifuge preparations at 25,000 × *g* to obtain greater recovery of transcribing plasmid molecules. Preparations were stained in 1% phosphotungstic acid.

Cloning of rDNA Fragments. All rDNA plasmids used in this work are described in Fig. 1. Recombinant DNA work was done using P2–EK1 containment procedures required by the current National Institutes of Health guidelines.

RESULTS

Transcription Initiation on pXlr 101A. Fig. 2 shows examples of micrographs obtained after injection of pXlr 101A into oocyte nuclei. pXlr 101A contains a full repeating unit of *X. laevis* rDNA inserted at the *Hin*dIII site of pBR322 (see Fig. 1). The large majority of the injected DNA remains as inactive beaded circles, but a small fraction displays gradients of nascent tran-

Abbreviations: kb, kilobase pairs; bp, base pairs; rDNA, ribosomal DNA.

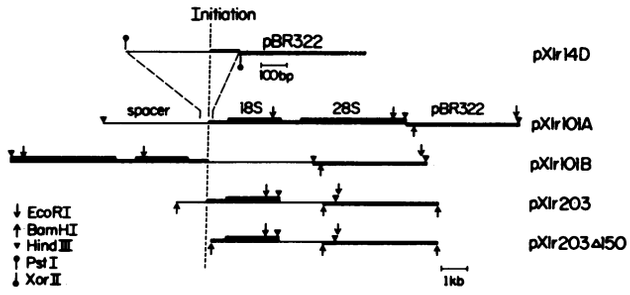

FIG. 1. Structure of various plasmids containing fragments of ribosomal genes. bp, Base pair; kb, kilobase pair. pXlr 14D: A 433-bp fragment bounded by a *Pst* I site at −320 and an *Xor* II site at +133 was removed from the parent pXlr 14 (13) and inserted between the *Pst* and *Xor* II sites in pBR322. pXlr 101A: This plasmid contains a single full repeat unit of *X. laevis* rDNA inserted at the *Hin*dIII site of pBR322. The insert in this plasmid was originally cloned from a *Hin*dIII digest of amplified rDNA inserted into pMB9 and was termed pXlr 101. pXlr 101 is the plasmid that was used by Trendelenburg and Gurdon (7) for oocyte injection. We subsequently removed the insert from pMB9, put it in pBR322, and renamed it pXlr 101A. The inserts in both plasmids are in the same orientation relative to the tetracycline resistance gene. pXlr 101B: This plasmid is identical to pXlr 101A except that the rDNA insert is in the opposite orientation relative to the tetracycline resistance gene. pXlr 203: This plasmid contains a minigene that should produce a transcript about 2.75 kb in length. It was constructed by linking an *Eco*RI/*Bam*HI fragment from the 5′ end of the gene to an *Eco*RI/*Bam*HI fragment from the 3′ end of the gene and inserting the result at the *Bam*HI site of pBR322. The fragments were derived from pXlr 14 (13) and the initiation and termination sites are the ones whose sequences were determined by Sollner-Webb and Reeder (2). pXlr 203Δ150: This is a deletion mutant of pXlr 203 in which a segment from the *Bam*HI site at −1150 before initiation to +150 after initiation has been eliminated by *Bal* 31 nuclease digestion. A *Bam*HI linker was added to the truncated end to allow recircularization of the plasmid.

scripts that are morphologically identical to the gradients seen on the endogenous ribosomal genes (Fig. 2). In good oocytes, we routinely see several hundred actively transcribing plasmids per electron microscope grid. Often the transcribed region is preceded by a nonbeaded spacer region, whereas the pBR322 portion of the circle remains beaded, in agreement with previous observations (7). Table 1 tallies the RNA polymerase packing density for both the injected and the endogenous ribosomal genes and shows that they are indistinguishable by this criterion. All transcription units on pXlr 101A molecules were of normal length and polymerase density.

Fig. 2*b* clearly demonstrates that, when pXlr 101A is injected with α-amanitin, the transcription complexes on the plasmids are completely normal—i.e., resistant to this inhibitor of RNA polymerases II and III. These data show that transcription on the injected rDNA is the result of specific initiation by RNA polymerase I and that this electron microscopy assay can be used to study the DNA sequences required for polymerase I promoter function.

Transcription Initiation on pXlr 203 and pXlr 14D. To begin identification of the rDNA sequences involved in promoter function we injected pXlr 203. This plasmid, which contains sequences from −1050 nucleotides before and +2250 nucleotides after the initiation site (see Fig. 1), reproducibly initiates transcription upon injection into oocytes (data not shown). In order to further map the promoter sequence we constructed pXlr 14D, a plasmid that contains a 433-bp rDNA insert extending from a *Pst* I site at −320 bp to an *Xor* II site at +113 bp (see Fig. 1). As shown in Fig. 3, pXlr 14D is transcribed upon injection. The density of RNA polymerases on pXlr 14D was as high or higher than that seen on endogenous ribosomal genes

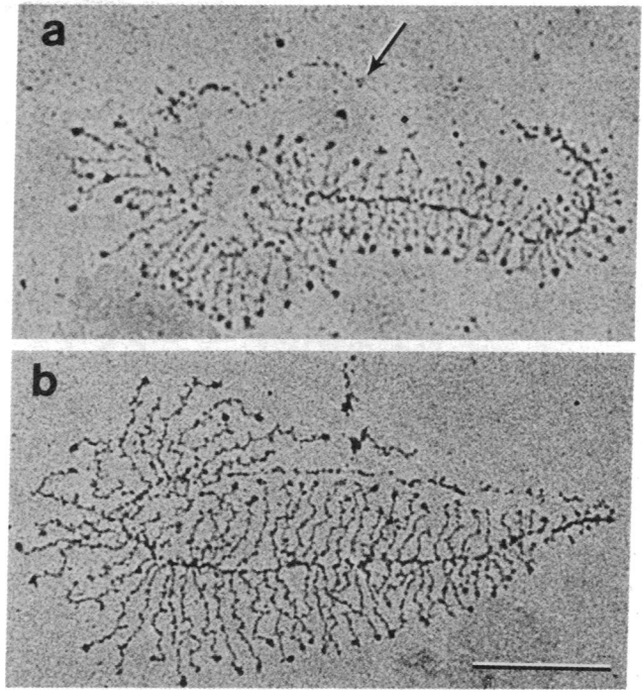

FIG. 2. Electron micrographs of pXlr 101A. This plasmid carries a full repeating unit of rDNA (see Fig. 1) and exhibits a transcribed region of mean (±SD) length 2.66 ± 0.32 μm ($n = 24$) which is the same as that seen on endogenous ribosomal genes (2.48 ± 0.36 μm, $n = 26$). (*a*) Example that clearly shows that the nontranscribed spacer (to the right of the arrow) is smooth, while the pBR322 vector (left of the arrow) is in a beaded configuration. (*b*) Example in which α-amanitin was injected with the DNA. Scale bar is 0.5 μm.

(Table 1). Also, this transcription was not affected by α-amanitin. Thus the RNA polymerase I promoter resides within a 433-bp region surrounding the initiation site.

Two pieces of evidence make it highly unlikely that the specific initiation observed on pXlr 14D is occurring in the plasmid vector rather than in the 433-bp rDNA insert. Injection of pBR322 alone on several occasions has never resulted in any transcription complexes visible in the electron microscope. Similarly, we have never seen any polymerase I transcription from pXlr 203Δ150, a plasmid that lacks the rDNA initiation site. The latter plasmid (diagrammed in Fig. 1) has had the 5′ end of the gene deleted for a distance extending 150 bp into the gene but it still contains about 4.6 kb of rDNA in addition to the pBR322 vector.

About 60% of the transcribing pXlr 14D plasmids show a single gradient of nascent ribonucleoprotein fibrils that begins at a discrete point on the circle (see the arrows in Fig. 3 *a* and *b*, which point to newly bound polymerases) and ends with the longest fibrils, which are attached immediately next to the initiation site. The other 40% show a different pattern, in which

Table 1. RNA polymerase density on endogenous ribosomal genes and recombinant plasmids

Source of rDNA	RNA polymerase density, molecules/μm
Endogenous ribosomal genes	35.8
pXlr 101A	34.5
pXlr 101B	35.1
pXlr 14D	42.4

At least 20 molecules of each type were measured.

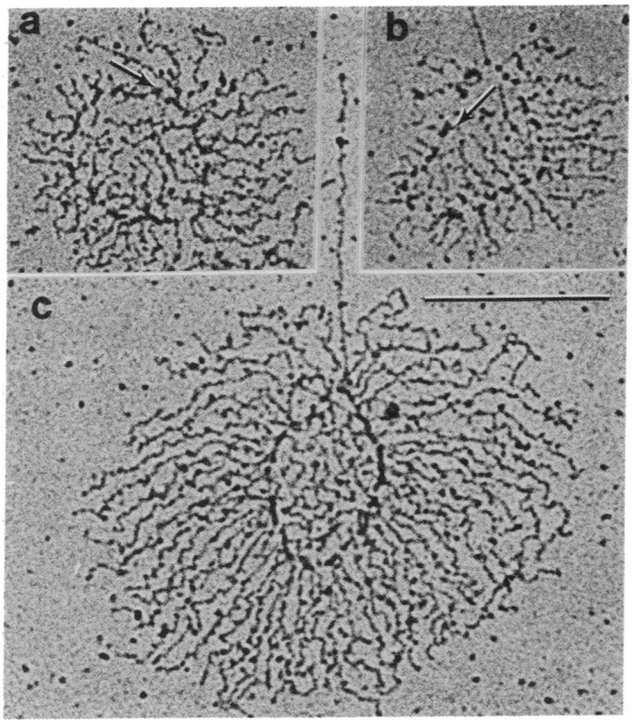

FIG. 3. Electron micrographs of pXlr 14D showing two patterns of transcription. In *a* and *b* there is a distinct gradient of fibril lengths, implying that transcription began at the arrow and proceeded counterclockwise until termination occurred just before the initiation site. In *c* there is no discernible gradient and the length of the fibrils suggests that transcription has proceeded several times around the circle without terminating. All plasmids were injected with α-amanitin. Scale bar is 0.5 μm.

it appears that transcription has continued around the circle several times. This second group is discussed more fully in the section on transcription termination.

Mapping the Terminator. *X. laevis* rDNA has a single *Hin*dIII site that is located at the 3′ end of the gene in each repeat unit. Sollner-Webb and Reeder (2) proposed that the *Hin*dIII recognition sequence may actually be part of the termination signal. This proposal was based on two observations: (*i*) the 3′ end of the 40S rRNA maps within the *Hin*dIII recognition sequence by S1 nuclease analysis; (*ii*) the *Hin*dIII recognition sequence supplies two of the T residues in a cluster of four Ts that is the largest T cluster within hundreds of nucleotides (Fig. 4). T clusters are part of the termination signal in other systems—i.e., in prokaryotic RNA polymerase and in RNA polymerase III (15–17). Thus, because the T cluster associated with the 3′ *Hin*dIII site was inevitably disrupted during the construction of pXlr 101A (Fig. 1), we were surprised that ribosomal transcription on the plasmid seemed to terminate correctly in the injection assay (Figs. 2 and 6). We reasoned that the rDNA insert brings in only two of the four Ts from the putative rDNA termination signal, and therefore one might expect the shortening of the T cluster to disrupt termination. In fact, the insertion of the rDNA into the *Hin*dIII site of the tetracycline resistance gene of pBR322 (14) resulted in the vector fortuitously supplying one additional adjacent T to make a cluster of three (Fig. 4). Apparently, as shown by pXlr 101A, three Ts are sufficient for termination to occur.

When the rDNA is inserted into pBR322 in the opposite orientation from pXlr 101A, the plasmid nucleotide now adjacent to the two Ts at the 3′ end of the gene is an A (plasmid pXlr 101B in Fig. 4). Injection of pXlr 101B into *X. laevis* oocytes shows that when the T cluster is reduced to two T residues, termination is impaired (Figs. 5 and 6). Only about one-fourth of the transcribing pXlr 101B plasmids terminate at the normal site; in the remaining three-fourths, transcription proceeds through this site and terminates either within the pBR322 sequence or in the rDNA spacer—often just prior to the initiation site. These results support the hypothesis that the cluster of T residues adjacent to the *Hin*dIII site is an essential part of the normal *Xenopus* RNA polymerase I termination signal.

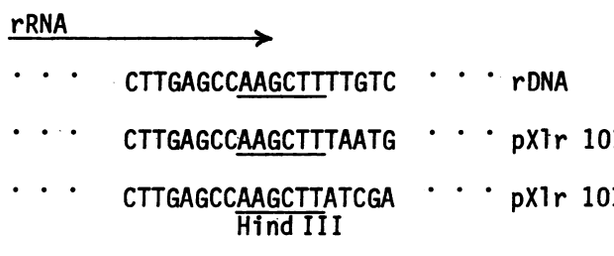

FIG. 4. Comparison of nucleotide sequences at the 3′ end of various ribosomal genes surrounding the *Hin*dIII restriction site. (i) *Endogenous rDNA*. S1 nuclease mapping shows that the 3′ end of the 40S precursor RNA protects within the *Hin*dIII recognition site (underlined) to the first T of the cluster of four Ts (2). (ii) *pXlr 101A*. In pXlr 101A the *Hin*dIII site contributes two Ts and a third T is contributed by the pBR322 vehicle (14). (iii) *pXlr 101B*. In pXlr 101B the pBR322 vehicle does not contribute any Ts to the T cluster (14).

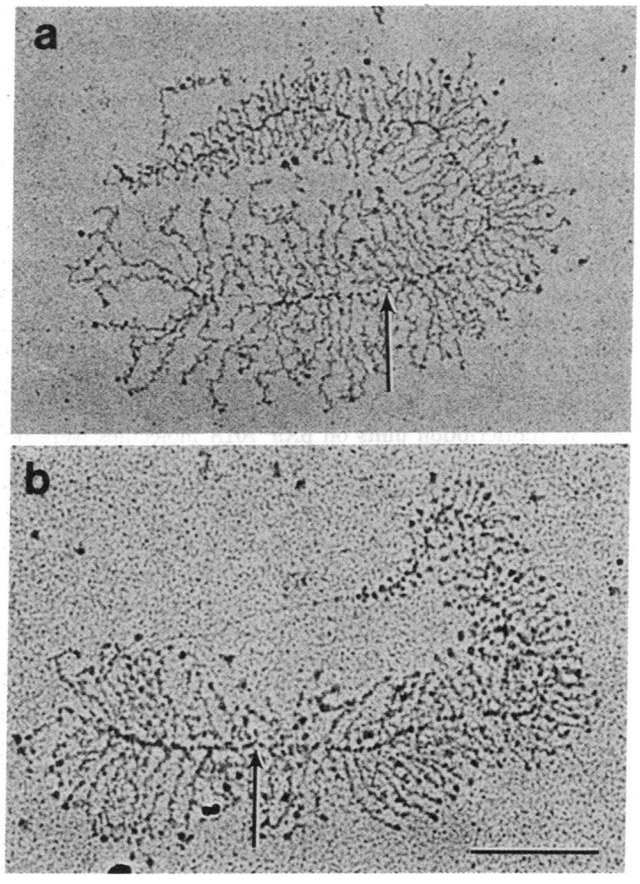

FIG. 5. Electron micrographs of pXlr 101B, showing transcription units of various lengths that are terminated within the pBR322 vector or the rDNA spacer. The arrows indicate where termination would normally occur, 2.5 μm away from initiation. The average length of 22 transcription units is 3.21 ± 0.5 μm. Scale bar is 0.5 μm.

Even in plasmids that exhibit heterogeneity in the location of the termination site (such as pXlr 101B), we note that all of the nascent ribonucleoprotein fibrils on a particular transcription unit stop at the same site. This site varies among different DNA molecules of the same injected plasmid. Such "all-or-none" stopping is consistent with the hypothesis that a factor is required for termination (18) and that the factor is present on only a fraction of the 3′ *Hin*dIII sites of pXlr 101B.

The results from injecting pXlr 101B into *X. laevis* oocytes also suggest that there may be a fail-safe termination signal upstream from the normal initiation site. Even though the terminator at the 3′ end of the gene is damaged in 101B, transcription *never* runs around the circular plasmid more than once. All RNA polymerases stop before reaching the initiation site (Fig. 6). A similar conclusion may be drawn from the transcription patterns of pXlr 14D: this plasmid is missing the 3′ end of the gene and transcription runs clear around the circle, but, as noted above, in over half of the observed cases, transcription stops after only one circuit just prior to the initiation site (Fig. 3 *a* and *b*). Unlike the situation with pXlr 101B, however, termination at this "fail-safe" site on pXlr 14D appears to be leaky and in many instances the polymerases appear to transcribe around the circle several times (Fig. 3*c*).

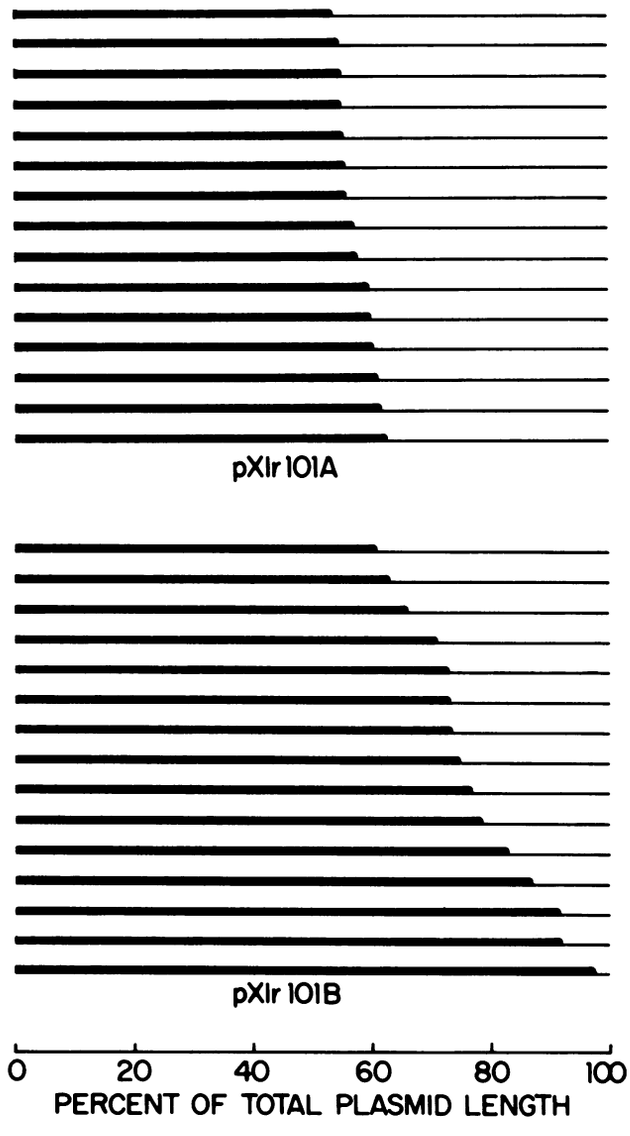

FIG. 6. Comparison of transcription unit length on pXlr 101A and pXlr 101B. The total length of each plasmid circle has been normalized to 100%. The thick line represents in each case the percent of total length that was covered by RNA polymerases.

DISCUSSION

We have shown that electron microscopy of injected recombinant plasmids is a workable method for determining the functional capacity of partially deleted or rearranged ribosomal genes and thus will allow us to map specific sequences required for promoter and terminator signals *in vivo*. Evidence that we are observing specific initiation at the RNA polymerase I promoter can be summarized as follows: (*i*) The transcription units we observe on injected plasmids have the same high density of nascent transcripts as is seen on endogenous ribosomal genes. (*ii*) Injection of pXlr 101A results in a transcribed region of the correct length for the known distance between the polymerase I promoter and termination. (*iii*) Transcription is observed with pXlr 14D, a plasmid that carries the *in vivo* rDNA initiation site but has only 433 bp out of the approximately 11 kb of an average ribosomal gene repeating unit. Transcription is not seen when pBR322, the plasmid vehicle, is injected alone. Nor is it seen when pXlr 203Δ150, a rDNA plasmid lacking the *in vivo* initiation site, is injected. (*iv*) The observed transcription is resistant to α-amanitin and therefore is due to polymerase I.

From these results we also conclude that the RNA polymerase I promoter must lie within the region between −320 bp and +113 bp relative to the initiation site. This assignment agrees with inferences made from sequencing data. First, sequence comparisons among three *Xenopus* species show no conservation of sequences in the initiation region except for 15 bp located from −11 to +4 bp on the *X. laevis* sequence (M. Crippa, personal communication). In addition, the region from −145 to +4 is duplicated several times in the middle of the "non-transcribed" spacer (2, 19), always in association with a *Bam*HI restriction site. Occasionally transcription is initiated in the spacer (20–22) and, in fact, does start within these "*Bam* islands" (unpublished observations). This implies that promoter sequences lie in the region from −145 to +4 surrounding the normal initiation site. Finer deletion and mutation studies will be required to further delineate the polymerase I promoter sequence.

It is still unknown whether the *Bam* islands serve any function in the frog's physiology or if they are only by-products of recombination events within the ribosomal locus. pXlr 101A has two *Bam* islands in its spacer, but we have never observed any evidence of initiation at these particular *Bam* islands. One speculation is that they somehow help polymerase to achieve the high packing densities seen on ribosomal genes, perhaps by serving as "loading zones" for polymerase (23). Our results with pXlr 14D make it unlikely that the *Bam* islands have any mandatory function in normal initiation. pXlr 14D has no *Bam* islands at all and yet polymerase I still loads on this DNA at the normal density.

Regarding termination, present evidence indicates that the cluster of four Ts that overlaps the *Hin*dIII site is part of the normal termination signal for polymerase I. S1 nuclease mapping studies have shown that both the 40S precursor RNA and mature 28S RNA terminate within the *Hin*dIII recognition sequence (2). The experiments with pXlr 101A and pXlr 101B reported in this paper suggest that the T cluster is not only the termination site but also the 3′ boundary of the functional termination signal. These experiments further suggest that only three Ts at this site are needed for termination, and that two Ts at this site (as in pXlr 101B) are insufficient to specify termination.

It is important to note that termination of pXlr 101A transcription is most likely occurring at the natural rDNA termintion site rather than at a fortuitous stop site in the adjacent pBR322 sequences. If such a fortuitous, high-efficiency termination site in pBR322 formed the 3′ boundary of the pXlr 101A transcription unit, it would have to reside in the tetracycline resistance gene, downstream from the *Hin*dIII insertion site. Yet, this exact region is present in the same orientation relative to the rDNA in pXlr 14D—800 nucleotides downstream from the initiaion site (see Fig. 1)—and in this plasmid transcription always proceeds completely through the entire tetracycline resistance gene without terminating (Fig. 3).

In pXlr 101A it appears that a cluster of three Ts at the *Hin*dIII site is sufficient for termination. However, it is very likely that some of the preceding nucleotides also are needed to specify termination in pXlr 101A, because clusters of three Ts found at other, internal sites in the gene do not cause termination (2, 4).

The results with pXlr 101B and pXlr 14D further suggest the presence of a fail-safe termination signal(s) in front of the normal initiation site as noted above. The presence of a fail-safe terminator is supported also by observations of endogenous genes that occasionally show transcription complexes in the spacer region (20, 22). These "prelude complexes" always seem to terminate before running into the 5′ end of the gene. Examination of the DNA sequences upstream from initiation shows two T clusters that are likely candidates for termination signals, one beginning at nucleotide −27 and the other at −228 (2).

In this study, the intact rRNA gene found in pXlr 101A [as well as in its parent plasmid, pXlr 101 (data not shown)] produces *only* transcription units with a length identical to that of the endogenous ribosomal transcription units. In previous studies in which pXlr 101 was injected into *X. laevis* oocytes (7) or into *Triturus* oocytes (24, 25), transcription units of other lengths were visualized also. At present, we can offer no explanation for these conflicting results. Regarding the frequency of transcription, we cannot make an accurate estimate of the fraction of injected plasmids that are transcribed. Even in the best preparations both active and inactive plasmids are often seen in large unresolvable aggregates. In addition, control experiments (unpublished) show that less than 10% of the injected DNA is deposited on the electron microscope grid. It is possible that plasmids densely packed with transcription complexes are preferentially deposited during centrifugation. However, because we detect transcription only when a bona fide rDNA promoter is present in the plasmid, we are confident that the electron microscope provides a reliable assay for promoter functions.

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