High initiation rates at the ribosomal gene promoter do not depend upon spacer transcription
(RNA polymerase I/Xenopus laevis/UV mapping/rRNA gene)

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ABSTRACT We report experiments that test the model that in Xenopus laevis, RNA polymerase I is "handed over" in a conservative fashion from the T3 terminator to the adjacent gene promoter. We have introduced transcription-terminating lesions into the ribosomal DNA repeat by irradiating cultured cells with ultraviolet light. We used isolated nuclei to measure the effect of such lesions on transcription. UV damage sufficient to prevent all elongating RNA polymerase from reaching T3 from upstream had no adverse effect on the density of RNA polymerase at the very 5' end of the gene. We conclude that high rates of transcription initiation at the gene promoter do not depend upon polymerase passing from one repeat to the next or on polymerase initiating at the spacer promoters.

The tandemly repeated ribosomal genes of Xenopus laevis have been shown to contain three types of transcriptional regulatory elements: promoters, enhancers, and at least one terminator (Fig. 1). The gene promoter directs transcription of the 40S ribosomal RNA precursor and is active in essentially all cells of the organism. The duplicated spacer promoters are active in many cells, including several lines of cultured cells, but are silent in oocytes (1, 2). Recent work from this laboratory has focused on characterizing sites of 3' -end formation and putative terminator elements in the ribosomal gene spacer (3). We described site T2 as the site where 3' ends of the 40S precursor are formed, presumably by a rapid RNA processing event. The only real terminator of ribosomal gene transcription, site T3, is found 215 base pairs upstream from the transcription start site at the gene promoter. The T3 site, in addition to its termination activity, also has the novel property of being able to stimulate the adjacent gene promoter (4). This observation, coupled with the fact that at least some RNA polymerase can reach T3 after traversing the entire repeat unit (3, 5), led to the speculation that T3 might be able to pass the terminated polymerase over to the adjacent promoter in a conservative fashion without losing it to the free pool (1, 3, 6). Such a "hand-over" mechanism would also provide a rationale for the existence of the spacer promoters. Polymerase initiating at the spacer promoters could be handed over to the gene promoter and thus contribute to the dense packing of polymerase routinely observed on the X. laevis ribosomal genes (1, 7). Here we report experiments which argue that passage of RNA polymerase I from one repeat to the next or from the spacer promoters to the gene promoter is not required to maintain the high density of polymerase packing that is normally observed. If polymerase hand-over does occur in these cells its effect is not detectable.

MATERIALS AND METHODS

Isolation of Nuclei. X. laevis A-6 cells were grown at room temperature in 50% L15 medium (GIBCO) supplemented with 10% fetal bovine serum. Nuclei were isolated essentially as described by Crampton and Woodland (8). Cells were detached from the culture flask by treatment with trypsin and washed once in phosphate-buffered saline. They were then resuspended at a concentration of 10^7 cells per ml in homogenization medium [0.3 M sucrose/2 mM Mg(OAc)2/3 mM CaCl2/10 mM Tris-HCl, pH 8.0/0.1% Triton X-100/0.5 mM dithiothreitol (DTT)]. Cells were disrupted in a glass–glass homogenizer (B pestle; Belco Glass). After 20–50 strokes, 1 volume of centrifugation medium [2 mM sucrose/5 mM Mg(OAc)2/10 mM Tris-HCl, pH 8.0/0.5 mM DTT] was added and the mixture was layered on 6 ml of centrifugation medium in a 15-ml Corex glass tube. The nuclei were pelleted by centrifugation in a Sorvall HB-4 rotor at 10,000 rpm for 30 min. The nuclei were washed once in nuclei freezing buffer [50 mM Tris-HCl, pH 8.0/40% (vol/vol) glycerol/5 mM MgCl2/0.5 mM DTT] and finally resuspended at a concentration of 1–4 × 10^7 per ml. Nuclei were fresh or were stored in aliquots at −70°C.

UV Irradiation of Cells. Cells were trypsinized, pelleted, and resuspended in the same (conditioned) medium at a concentration of about 10^7 per ml. Aliquots of this cell suspension were irradiated in plastic dishes with a solution depth of about 2 mm. The UV flux of our device is 2200 μW/cm^2 (254 nm) and irradiation was for the times indicated. After irradiation, more conditioned medium was added and the cells were incubated at room temperature for 60–90 min before isolation of the nuclei. The purpose of this incubation was to allow polymerases located downstream from UV-induced pyrimidine dimers to run off. Control cells were treated exactly the same except for the lack of irradiation. A similar protocol was used by Johnson et al. (9).

In Vitro Transcription of Nuclei. An aliquot of isolated nuclei was pelleted and resuspended at a concentration of about 1–5 × 10^7 nuclei per ml in 1.67× transcription buffer (1× transcription buffer is 6 mM MgCl2/80 mM KCl/20 mM Hepes-KOH, pH 7.9/0.1 mM EDTA/5% glycerol/5 mM DTT). A typical 20-μl reaction mixture consisted of 12 μl of nuclei, 1 μl of a mixture of ATP, UTP, and CTP (5 mM each), 3 μl of [α-32P]CTP (to give a minimal concentration of 2 μM), 2 μl of a-amanitin (1 mg/ml), and 2 μl of H2O. The reaction was stopped and the RNA was extracted as described (3). In some reactions, sarkosyl (N-lauroylsarcosine, 0.2% or heparin (0.5 mg/ml) was present during transcription in order to prevent reinitiation.

RNase Protection Assay. The labeled RNA was coprecipitated with an excess of single-stranded DNA probe and resuspended in 30 μl of 0.3 M NaCl/10 mM Tris-HCl, pH 8.0.
7.5/1 mM EDTA Hybridization was at 75°C for at least 5 hr. After the samples were chilled on ice, 60 μl of hybridization solution containing RNase T1 (5 units/ml) and RNase A (5 μg/ml) was added. The samples were incubated at 25°C for 1 hr and subsequently extracted with phenol/chloroform/isoamyl alcohol (50:24:1, vol/vol). RNA fragments protected by the probes were purified and analyzed by polyacrylamide gel electrophoresis under denaturing conditions as described (3). For quantitation of the protected RNA fragments, appropriate exposures of autoradiographs were scanned with a laser densitometer (2202 Ultrascan; LKB). Where necessary, the readings were corrected for the length of the protected RNA fragment.

Single-Stranded DNA Probes. For the RNase protection assays, various fragments from the X. laevis ribosomal gene repeat were cloned in the appropriate orientation in phage vector M13mp18 or 18-mp19. The following fragments were used (the sequence of the X. laevis intergenic spacer, and the coordinate system used here, is given in ref. 10; in this coordinate system +1 is at the HindIII site at the 3' end of the 28S coding region and transcription of the 40S precursor initiates at +3952): probe A, +3918 to +3985; probe B, +4374 to +4669; probe C, +1 to +160; probe D, +1690 to +1864; probe E, +3662 to +3829. Note that some of those probes have been used in previous work (3) but that they were given different designations.

RESULTS AND DISCUSSION

Rationale of the Experiment. Other workers have shown that mapping the sensitivity of transcription to UV irradiation is a useful method for determining the length of transcription units and apparent entry points for RNA polymerase (9, 11–13). In using the UV mapping method to test the hand-over model, we have found that there are two useful ways to analyze the data. One method of analysis is to determine the relative UV sensitivity of transcription at several points along the gene. The UV sensitivity will be at a minimum at the polymerase entry point (13). The second method of analysis is to simply increase the UV dosage until all polymerase is prevented from approaching the promoter from upstream. At that point one asks whether polymerase density at the 5' end of the gene has been affected. The experimental design was as follows. Cultured cells were irradiated with UV light for various times and then kept in the dark for 60–90 min. This recovery time allowed the cells to reach a new equilibrium and, in particular, allowed polymerase downstream from UV lesions to clear the template. Nuclei were then prepared from each batch of cells and the density of RNA polymerase I in various regions of the ribosomal gene repeat was measured by transcribing RNA from the isolated nuclei in vitro. The labeled RNA was assayed with an RNase protection assay. Since for our test the most crucial region was the very 5' end of the gene, it was important to ascertain that the signal in this region in fact reflected the in vivo polymerase density. This requirement is fulfilled either when the in vitro initiation rate in the nuclei is sufficient to maintain the in vivo polymerase density or if reinitiation in vitro is prevented. In the following we show that reinitiation in purified nuclei is in fact efficient enough to support in vivo polymerase density. We find that both of these approaches locate the entry point of polymerase to the promoter at the 5' end of the 40S coding region and we see no evidence that polymerase coming from the intergenic spacer contributes to initiation at the gene promoter.

Transcription Initiation in Isolated Nuclei. Transcription initiation was monitored by allowing isolated nuclei to make radioactive RNA, hybridizing the RNA to a single-stranded DNA probe that overlaps the 5' end of the gene by 34 nucleotides (probe A), treating this hybrid with RNase, and electrophoresing the protected fragments in a sequencing gel. As shown in Fig. 2A (lanes 2–4) and B, label accumulated in this protected fragment at a linear rate for at least 1 hr. In other experiments (data not shown) we measured the chain elongation rate in isolated nuclei by removing the nascent chains with RNase and measuring the rate at which they regrew. At the measured elongation rate, 2.5 nucleotides per second, it would take a polymerase only 13.6 sec to traverse 34 nucleotides and label would cease accumulating in this fragment unless continued initiation occurred. In fact, this is exactly what happened when initiation was prevented by addition of heparin at the beginning of the reaction (Fig. 2A, lanes 5–7). Since the signal obtained with probe A in the presence of heparin is barely detectable on this autoradiograph, we can conclude that virtually all the signal detected with probe A in the absence of heparin (lanes 2–4) represents transcripts initiated in vitro.

Initiation in vitro is efficient as compared to elongation. This was shown by comparing the radioactivity incorporated into the extreme 5' end of the 40S precursor with the radioactivity incorporated into the extreme 3' end during a 30-min reaction (Fig. 2A, lanes 14 and 16). Densitometric measurements of several experiments showed that the molar ratio of the 5' signal to the 3' signal varied over a range of 1.2: 1 to 3.2:1, independent of the reaction time. The transcription signal obtained at the 3' end of the gene presumably reflects the density of polymerase that was present in vivo, since it takes about 45 min for any polymerase initiated in vitro to travel 8 kilobases. This conclusion is also supported by the finding that heparin had little effect on transcription at the 3' end (lane 17), whereas it abolished incorporation at the 5' end of the gene by preventing initiation (lane 15). Since the rate of incorporation of label into the 5' end was linear for up to 1 hr in vitro, and since the molar ratio of 5' to 3' signal was found to be in the range of 1–3 (but not <1) even after 30 min of in vitro transcription, we can conclude that the initiation rate in vitro is in fact such as to support in vivo polymerase density on the ribosomal genes. This furthermore implies that the mechanism responsible for the high polymerase density continues to be active in vitro. Our data indicate that the limiting step during in vitro transcription of the isolated nuclei is chain elongation.

We also examined initiation at the spacer promoters in isolated nuclei, by using probe D (see Fig. 1). An RNA fragment of the expected length continued to accumulate...
region as a function of UV dose were determined by densitometric tracing of the resulting autoradiographs. Fig. 3 shows the results of two independent experiments using two different ranges of UV doses. In the first experiment (Fig. 3A), we found that 10 min of irradiation severely affected transcription detectable at the 3' end of the gene (probe C), whereas an intermediate UV sensitivity was observed about 700 base pairs from the 5' end of the gene (probe B). Initiation at the gene promoter (probe A), however, continued at control rates. This result is consistent with the gene promoter being the site of entry for polymerase. It further suggests that polymerase traversing across the entire repeat is not a significant factor in maintaining normal rates of initiation at the gene promoter.

We observed repeatedly (in three out of four experiments) that spacer promoter activity increased by a factor of 4–5 after UV irradiation (Fig. 3A, probe D). A potential explanation for this result is that, under normal circumstances, the spacer promoters are partially occluded by polymerase reading through from the gene region. UV lesions eliminate this readthrough and allow the spacer promoters to function better.

In the experiment of Fig. 3A, the UV doses applied were not high enough to significantly affect transcription at T3 (probe E). In the second experiment (Fig. 3B), we therefore applied higher doses of UV light in order to introduce terminating lesions between the spacer promoters and T3. After 20 min of UV irradiation, the transcription signal at T3 had decreased by a factor of 30 and the signal at the 3' end of the gene has dropped to undetectable levels. This is consistent with the larger target size of the gene region as compared to the transcription unit(s) in the spacer. However, the rate of initiation at both the gene and spacer promoters was still at control levels. Note that in this particular experiment the spacer promoters showed about 50% of the activity of the gene promoter. These results, together with our observation that the in vitro initiation rate in the nuclei is sufficient to maintain the in vivo polymerase density (Fig. 2), allow us to conclude that transcribing RNA polymerase I arriving from upstream is not required to maintain normal rates of transcription initiation on these promoters.

UV Sensitivity of in Vivo Initiation. In the experiment shown in Fig. 3, the in vivo polymerase density at the 5' end of the gene was measured only indirectly. Therefore, we repeated the experiment, using 0.2% sarkosyl to prevent initiation in

Fig. 3. UV sensitivity of ribosomal gene transcription. Tissue culture nuclei were irradiated with increasing doses of UV light (254 nm). Nuclei were isolated and incubated in transcription mixtures for 30 min, and the labeled RNA was analyzed with the RNase protection assay using probe A (gene promoter; ●), B (●), C (3' end of gene; ●), D (spacer promoter initiation; ○), or E (3' ends at T3; ●). Relative transcription rates in those different regions were determined by scanning of appropriate exposures of the gels. The natural logarithm of the ratio of the transcription rate in nuclei from cells irradiated with dose d to the transcription rate in nuclei from control cells (ln(control)/ln(irradiated)) is plotted against the time of irradiation.

Fig. 2. Transcription initiation in isolated nuclei. (A) Nuclei from cultured A-6 cells were incubated in transcription reaction mixtures for 5 min (lanes 2, 5, 8, and 11), 15 min (lanes 3, 6, 9, and 12), 60 min (lanes 4, 7, 10, and 13) or 30 min (lanes 14–17), either in the absence (lanes 2–4, 8–10, 14, and 16) or in the presence (lanes 5–7, 11–13, 15, and 17) of heparin at 0.5 mg/ml. Labeled RNA was analyzed with the RNase protection assay using probe A (lanes 2–7, 14, and 15), probe D (lanes 8–13), or probe C (lanes 16 and 17). Lane 1: end-labeled Hpa II-digest of plasmid pBR322. r.t., Readthrough. (B) Quantitation of 5'-end signal of the gene promoter (●) and spacer promoter (○) from A (lanes 2–4 and 8–10).

In addition, this probe protected longer RNA, which is due to readthrough from upstream (e.g., from the first spacer promoter into the second or from the gene region). Note that the probe used did not allow us to distinguish transcription initiation events at the individual duplicated spacer promoters. Initiation at the spacer promoters was abolished by heparin, whereas chain elongation, as measured by the readthrough signal, was largely unaffected (lanes 11–13). Densitometric measurements of the results of several experiments indicated that the activity of the spacer promoters was in the range of 10–50% of the activity of the gene promoter. We do not know the cause for this variation between different preparations of nuclei.

UV Sensitivity of in Vitro Initiation. Having characterized the initiation properties of the nuclei, we performed the UV irradiation experiment as outlined above and transcribed the isolated nuclei in vitro for 30 min. The labeled RNA was analyzed with the RNase protection assay using the probes shown in Fig. 1. The relative transcription rates in each probe
Fig. 4. In vivo polymerase density at the 5' end of the 40S precursor coding region. Cultured cells were irradiated with UV and nuclei were isolated as in Fig. 3. Transcription was for 30 min in the presence of 0.2% sarkosyl. The labeled RNA was analyzed with the RNase protection assay using probe A. (A) Autoradiography of the gel. Lanes: 1, molecular size markers; 2-5, UV irradiation for 0, 10, 20, or 30 min. (B) Transcriptional signals, quantitated and plotted as in Fig. 3. a, 5' end of 40S precursor; c, readthrough from upstream.

vitro. We have found that sarkosyl at this concentration inhibits initiation similarly to heparin (see Fig. 2A), but, in addition, sarkosyl also inhibits termination at T3 more strongly than heparin does. When using probe A, we expect a (weak) signal from RNA polymerases that were engaged in transcription of the first 34 nucleotides of the precursor at the moment the cells were broken open, and a readthrough signal indicating polymerases reading through T3 because of the presence of sarkosyl. Fig. 4A shows that while transcription from upstream, reading through T3, decreased drastically upon UV irradiation, the transcription signal derived from the very 5' end of the gene was unchanged. A quantitation of the data is presented in Fig. 4B. [We have found that the readthrough signal never reaches the intensity of the 5' signal that would be observed in the absence of sarkosyl (data not shown). This is consistent with our finding that the gene promoter shows a 2- to 10-fold higher activity than the spacer promoters. Note that we achieved this rather strong 5' signal in the presence of sarkosyl by using more nuclei in the reaction and a longer exposure time of the autoradiograph.]

What Is the Role of Spacer Transcription? We think that it is important to distinguish between two qualitatively different types of spacer transcription. One type of spacer transcription is that which arises due to failure of polymerase to release when it reaches the 3' end of the region coding for the large ribosomal RNA precursor. All species so far examined form a 3' end in the transcript at this point. However, whether or not the polymerase is released appears to be optional. In X. laevis (3), and possibly in Drosophila (15), the polymerase is not released. In mouse, rat, and human, the site at the 3' end of the precursor coding region has been identified as a true terminator, and therefore polymerase is probably released (16-19). Thus, there may be no great biological consequence of transcription continuing through into the spacer as long as there is a strong terminator upstream of the gene promoter to prevent transcription into the gene promoter (3, 20, 21).

Spacer transcription arising from initiation at the spacer promoters is probably a different matter. Active spacer promoters have now been identified not only in Xenopus but also in Drosophila (22, 23), mouse (24), and rat (25). This conservation of function strongly suggests that such transcription has an important role. Whatever that role may be, we think that such transcription is not required to maintain high initiation rates at the gene promoter.

A recent report (26) from this laboratory described a related set of experiments in which psoralen adducts were used to prevent polymerase chain elongation around a plasmid circle in injected oocytes. Those experiments also showed that initiation at the gene promoter was not dependent upon polymerase approaching the promoter from upstream. The present work is an important extension of those studies, because we are studying transcription of the endogenous ribosomal genes in a cell type where the spacer promoters are active.

The formal possibility still exists that when RNA polymerase encounters a UV lesion, it ceases polymerizing RNA but remains attached to the DNA and somehow "slides" until it finds the next promoter. If this scenario were true, we would be wrong in concluding that UV irradiation prevents polymerase approaching the promoter from upstream and the current experiments would not constitute a test of the hand-over hypothesis. We think this scenario is rather unlikely but it probably should be tested before completely dismissing the hand-over hypothesis. It is surprising that polymerase initiation is so efficient in isolated nuclei, given the rather large volume into which they are diluted during isolation. This implies that when polymerase terminates, it is not released into a truly free or soluble pool. Instead, it seems more likely that the polymerase is somehow sequestered in the vicinity of the initiation site. Our results make it seem unlikely, however, that the site of sequestration is in an elongation complex on the ribosomal gene repeat.

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