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Characterization of two types of ribosomal gene transcription in Xenopus laevis oocytes

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When the germinal vesicle of Xenopus laevis oocytes is translocated into the vegetal hemisphere by centrifugation, the normally silent ribosomal spacer promoters are strongly induced. This induction correlates with the permeability of the nuclear envelope to dextran of molecular weight 70 kDa, thus raising the possibility that the transcriptional changes are due to mixing of nuclear and cytoplasmic components. This basic observation prompted a thorough investigation of ribosomal gene transcription in centrifuged oocytes which had the germinal vesicle either in the animal half (A-oocytes) or in the vegetal half (V-oocytes). Two types of ribosomal gene transcription were characterized: (1) in A-oocytes, spacer promoters remain silent, transcription initiation is dependent on the upstream terminator T3, and transcription is highly processive and recognizes sites of RNA 3′ end formation (like T2 and T3); (2) in V-oocytes, spacer promoters are induced, transcription initiation is independent of T3, but most transcripts terminate prematurely after less than 150 nt. Furthermore, the transcription machinery in V-oocytes does not respond to T2 or T3 signals. The implications of the present observations for our understanding of the regulation of the spacer promoters and of the function of the upstream terminator T3 are discussed.

The ribosomal gene repeat units of many species contain—in addition to the major gene promoter—additional promoters in the spacer (reviewed by Reeder, 1989). In the X. laevis rDNA clones studied so far, spacer promoters number from two to seven (Botchan et al., 1977). Their function and regulation has been the subject of numerous publications (e.g., Moss, 1983; Morgan et al., 1984; Pruitt and Reeder, 1984; De Winter and Moss, 1986). It is clear that their activity is modulated independently of that of the gene promoter. In oocytes, the spacer promoters are virtually silent; during gene activation at the mid-blastula transition, they are activated along with the gene promoter, but their relative activity decreases with progressing embryonic development (P. Labhart, unpublished data). High levels of spacer promoter activity are also found in RNA from a tissue culture cell line. Results with oocyte injection experiments suggested that the differential regulation of gene and spacer promoter is not due to sequence differences in the 150 bp promoter (Morgan et al., 1984).

In previous work, we had identified and characterized a terminator element, named T3, that is located about 60 bp upstream from the gene promoter (Labhart and Reeder, 1986 and 1987a). In addition to directing RNA 3′ end formation and termination of transcription, the T3 site was found to have a strong effect on initiation from the nearby promoter in certain transcription systems (McStay and Reeder, 1986). While a fraction of this promoter stimulation by T3

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can be ascribed to prevention of promoter occlusion by the terminator function of T3, there is evidence that the T3 element can also have a direct positive effect on the promoter, thus acting as a promoter element (McStay and Reeder, 1990). In addition, we had observed that T3 upstream from the promoter can have an effect on events downstream from initiation, like the recognition of sites for 3′ end formation (Labhart and Reeder, 1987b), suggesting that transcription from a T3-containing promoter is qualitatively different from transcription from a promoter lacking T3. Interestingly, spacer promoters do not possess a T3 element; they thus represent naturally occurring T3-less promoters. Whether their differential regulation is due—at least in part—to the lack of the T3 element has never been directly addressed.

Here I report the surprising finding that the normally silent spacer promoters in the X. laevis oocyte can be fully activated simply by centrifugation of the germinal vesicle into the vegetal hemisphere. I present evidence that in such centrifuged oocytes the nuclear envelope is no longer a barrier between nucleoplasm and cytoplasm. On the other hand, centrifugation of oocytes with the animal hemisphere up maintains the germinal vesicle in its native state. Two different types and patterns of ribosomal gene transcription can thus be "created" by translocating the germinal vesicles into either the animal or the vegetal hemisphere. The two types of transcription differ in their requirement of T3 for initiation, in their processivity, and in their ability to recognize signals for RNA 3′ end formation and termination. The results presented in this paper explain the previously described dependence of T2 function on the presence of T3 upstream of the promoter (Labhart and Reeder, 1987b) and add to our understanding of the regulation of spacer promoter activity and of the mechanism of promoter stimulation by T3.

Materials and methods

Plasmid constructs

For some of the experiments (Figs. 4A and 5), the same ribosomal minigenes that had been described in previous work were used. Those include the reference plasmid ψ52 (Labhart and Reeder, 1984 and 1985); plasmids ψ40-T2, 403-T2, BGL-T2, PA-112-T2, 403-T3D, and 403-T3E (Labhart and Reeder, 1987b).

For the experiments shown in Figures 2C, 4A, 6, and 7, a new type of ribosomal minigene was derived from the constructs used and described in a previous publication (Labhart and Reeder, 1990). To be able to analyze transcription of injected minigenes by an RNase protection assay, the BamH I-Hind III fragment in the minigene body, which contained 40S precursor and 28S rRNA sequences, was replaced with the 346 bp BamH I-Hind III fragment of pBR322. Furthermore, the entire minigene was cloned into the vector pGem4.

The wild-type version of this plasmid had unchanged ribosomal sequences at T3, the promoter, and at T2; a map of this minigene is shown in Figure 1B and at the bottom of Figure 7. Five mutations of this plasmid were used: (1) a 5′ promoter deletion truncated to the Sma I site at −90; (2) an EcoR I linker scanner mutant at −142/−133 (Reeder et al., 1987); (3) a Bgl II linker scanner mutant in T3 (McStay and Reeder, 1986); (4) the T2 point mutant C261, which con-

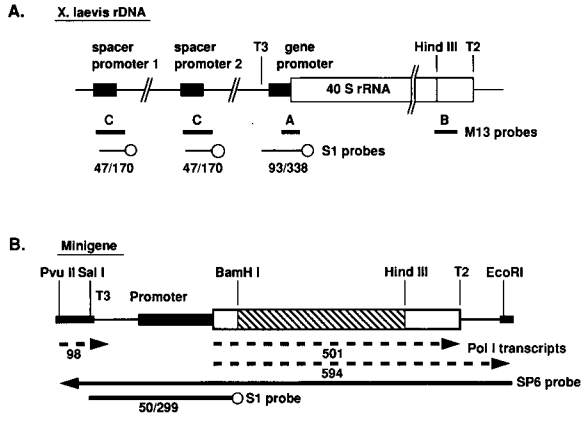

Figure 1. Gene maps and location of probes. **A.** Partial map of a typical X. laevis ribosomal repeat unit. Relevant sites and the location of single-stranded M13 probes for the "reverse"-RNase protection assay (A, B, and C) and of single-stranded restriction fragments used as S1 probes are indicated. The ^{32}P-label at the 5′ end of the S1 probes is shown as an open circle. The total length of the S1 probes and the expected protected length in nucleotides are given below the probes by the numbers after and before the slash, respectively. **B.** Map of the ribosomal minigene construct with the major functional sites and restriction sites. The SP6 probe for the RNase protection assay (solid arrow to the left) and the expected protected RNA fragments and their lengths in nucleotides (dashed arrows to the right) are shown. The S1 probe for detecting initiation at the minigene promoter is presented as in panel **A.**

verts T2 into a T3-like site; and (5) the T2 point mutant G255 (the sequence of both mutants is given in Labhart and Reeder, 1990). The plasmid containing a spacer promoter that was injected for the experiment shown in Figure 2B was similar to plasmid pXlr315 in Reeder et al. (1983), except that the spacer promoter sequences extended further 5′ to a Sma I site 127 bp upstream from the BamH I site.

Oocyte injections

Partial ovaries from X. laevis females were incubated overnight in modified Barth's solution containing 0.1% collagenase (type II, Sigma C-6885) and 100 μg/ml gentamicin sulfate (Whittaker). Large oocytes were selected and placed into small Petri dishes containing modified Barth's solution. The Petri dish had a grid with a mesh size of 1.25 mm attached to the bottom in order to immobilize the oocytes. Centrifugation was at room temperature in a table top centrifuge, typically at 1500–2000 rpm for 12 minutes (radius = 160 mm). After centrifugation, all oocytes which had the germinal vesicle at or close to the boundary between the animal and vegetal hemispheres were discarded (except for the experiment in Figure 2A, lanes 4–9). After injection, oocytes were sorted in A- and V-oocytes.

About 40 nl of DNA solution were injected per oocyte. The injection solution contained 25 μg/ml plasmid DNA, 50 mM NaCl, 5 mM Tris (pH 7.5), 0.1 mM EDTA, and 500 μg/ml α-amanitin. In some experiments, fluorescein isothiocyanate-dextran (FITC-dextran) of average molecular weight 70 kDa (Sigma FD-70) was added to the injection solution at a concentration of 20 mg/ml. For the in vivo labeling experiment, [α-^{32}P]CTP (20 mCi/ml, 800 Ci/mmol) was injected into oocyte nuclei (40 nl/oocyte).

Nuclear run-on transcription

Germinal vesicles from injected A-oocytes were manually isolated, and their homogenates

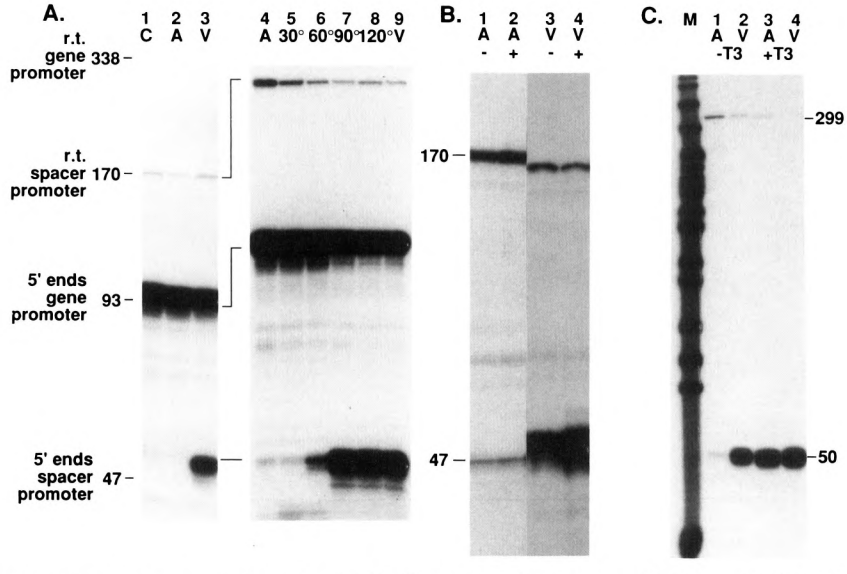

Figure 2. Transcription initiation is T3-dependent in A-oocytes and T3-independent in V-oocytes. **A.** Induction of the spacer promoter upon centrifugation of the germinal vesicle into the vegetal hemisphere of the oocyte. S1 analysis of RNA from oocytes that had been centrifuged with the animal pole up (A-oocytes, lane 2) or with the vegetal pole up (V-oocytes, lane 3). Lanes 5–8 show the analysis of oocytes centrifuged at intermediate positions between the extremes of animal pole up (0°, lane 4) and vegetal pole up (180°, lane 9). Lane 1 shows the result with uncentrifuged control oocytes. S1 analysis was with a mixture of a probe specific for the gene promoter and a probe specific for the spacer promoter (see Fig. 1A for location of probes). **B.** Injected cloned spacer promoters are co-regulated with the endogenous spacer promoters. S1 analysis of RNA from uninjected control oocytes ("minus" lanes) and from oocytes injected with a plasmid containing a spacer promoter ("plus" lanes). The S1 probe used (the same as in **A**) does not distinguish between the endogenous and the cloned spacer promoter. Note that in A-oocytes (lanes 1 and 2) the low level of spacer promoter activity is unchanged in the presence of the injected plasmid (lane 2), whereas in V-oocytes (lanes 3 and 4) the additional signal from the injected plasmid (lane 4) is clearly detectable over the signal from the induced endogenous spacer promoters. **C.** Transcription analysis of ribosomal promoters plus and minus T3 in A- and V-oocytes. Minigene plasmids with wild-type T3 (+T3 lanes) or mutated T3 (−T3 lanes) were injected into A- (lanes 1 and 3) and V-oocytes (lanes 2 and 4), and the RNA was analyzed with the S1 protection assay. The construct and the S1 probe used are shown in Figure 1B. Note that the promoter with the mutated T3 is only active in V-oocytes (lane 2), thus behaving like a spacer promoter.

were transcribed in vitro as described previously (Labhart and Reeder, 1987a).

RNA analysis

For RNA extraction, oocytes were homogenized in 0.3 M NaAc, 0.5% SDS, 10 mM EDTA, 50 mM Tris (pH 7.5), and 1 mg/ml proteinase K. After incubation at 37°C for 1–2 hours, the samples were extracted once with phenol and once with chloroform, and the total nucleic acids were precipitated with 2 volumes of EtOH.

For size-fractionation, oocyte RNA was denatured by boiling in 95% formamide, 0.1 mM EDTA and electrophoresed on a preparative 5% polyacrylamide gel containing 8 M urea. The gel was cut into four fractions, whereby the Xylene Cyanole FF dye marked the boundary between fractions C and D. A labeled Hpa II digest of pBR322 was fractionated on the same gel to determine the size-distribution of single-stranded nucleic acids present in the four fractions. The gel slices were eluted in 0.5 M NH_4Ac, 0.1% SDS, and 1 mM EDTA and in the presence of carrier E. coli RNA at 37°C overnight. The eluted RNA was then extracted once with phenol, once with chloroform, and precipitated with EtOH.

S1 protection assay was performed as described previously (Labhart and Reeder, 1986). The 5′ end-labeled, single-stranded DNA probes specific for the endogeneous gene and spacer promoter were described in an earlier publication (probes A_2 and B_1 in Labhart and Reeder, 1987c). S1 probes specific for the minigene constructs were prepared as described (Labhart and Reeder, 1985).

For the RNase T1 protection assay, the wild-type minigene construct was linearized with Pvu II and used to synthesize RNA with SP6 RNA polymerase in the presence of [α-^{32}P]CTP. The labeled RNA (907 nt) was isolated from a 6% polyacrylamide gel containing 8 M urea by elution, as described above for the size-fractionated RNA. The RNA probe was hybridized to oocyte RNA in 30 µl of 0.3 M NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA at 65°C for at least 3 hours. After chilling on ice, 60 µl of hybridization buffer containing 600 U RNase T1 (Sigma, R-8251) was added. RNase-digestion was at 37°C for 30 minutes and was stopped by the addition of 5 µl proteinase K (10 mg/ml) and 5 µl 10% SDS. After an additional 30 minutes at 37°C, the samples were extracted with phenol:chloroform, and precipitated with 3 vol of EtOH in the presence of 1.5 M NH_4Ac and 10 µg carrier E. coli RNA.

The "reverse"-RNase protection assay for the analysis of labeled RNA with cold single-stranded DNA probes (M13-clones) had been described previously in detail (Labhart and Reeder, 1987a and 1989). In the present paper it is called "reverse"-RNase protection assay to set it apart from the RNase T1 protection assay described in the previous paragraph. The in vivo labeled RNA was analyzed with the same probes, designated A, C, and D in Labhart and Reeder (1989). The RNA labeled during nuclear run-on transcription was analyzed with probe A only. As described in Labhart and Reeder (1987a), probe A yields two different sizes of protected RNA fragments, one for transcripts starting at the endogenous gene promoter and another for transcripts starting at the promoter on the injected plasmid.

Identification of RNase T1 protection bands

Several control experiments (not described in Results) were performed in order to identify bands seen on autoradiographs of RNase T1 protection assays (Figs. 6B and 7). Some residual undigested probe was always detected, especially since long exposure times were used for the low amount of RNA from V-oocytes. But real signals could be distinguished from this background due to their absence in control assays with non-homologous RNA (see for example Fig. 7, lanes 7–12).

The band of about 85 nucleotides (nt) and the doublet at about 280–290 nt in Figure 6B, lanes 2 and 4 (marked by arrows), were positively identified as transcripts starting at the ribosomal promoter by assaying the same RNA samples with a SP6 probe that ended at the BamH I site in the minigene (see map in Fig. 1B). With such a probe, the 85 nt band was no longer seen, and the 280–290 nt doublet was running at about 230–240 nt (data not shown). About 30 nt of the 5′ end of the endogeneous 40S precursor and about 75 nt of its 3′ end can also protect the SP6 RNA probe used. A series of bands from about 60–75 nt (visible at the very bottom of Figure 6B) probably are due to protection of the probe by the 3′ end of the precursor at T2. The following results support this interpretation: (1) these bands are also found with RNA from uninjected control oocytes, but they are

not found with E. coli RNA; and (2) they are also found when the SP6 probe is truncated at the BamH I site, but they are not found when an SP6 probe extending from the BamH I to the Sal I site is used (see map in Fig. 1B). The RNase protection assay shown in Figure 6B therefore confirms that the level of 40S precursor 3′ ends is lower in V-oocytes than in A-oocytes.

Results

Activation of spacer promoters upon centrifugation of the germinal vesicle into the vegetal hemisphere of oocytes

When X. laevis oocytes are centrifuged, their germinal vesicle moves to a location just below the surface of the oocyte. This technique is routinely used for injection experiments, since it makes the germinal vesicle visible and ensures a high yield of successful microinjections (Kressmann et al., 1977). In the experiment shown in Figure 2A, groups of 70–80 oocytes were put into centrifugation dishes and oriented so that about half were positioned with the animal pole up and about half with the vegetal pole up. After centrifugation for 12 minutes at 500–600 × g, oocytes that had their germinal vesicles translocated to the animal pole were separated from those that had their germinal vesicles translocated to the vegetal pole. After incubation for 18 hours, total RNA was extracted from the two samples and analyzed with a pair of S1 probes specific for transcripts starting at the gene promoter and transcripts starting at the spacer promoters (for probes, see Figure 1A). As can be seen in Figure 2A, control uncentrifuged oocytes show a strong signal for initiation at the gene promoter, whereas spacer promoter transcripts are not detectable (lane 1). Oocytes with their germinal vesicles at the animal pole (A-oocytes) gave the same result (lane 2). In contrast, centrifugation of the germinal vesicle into the vegetal hemisphere (V-oocytes) resulted in a drastic activation of the spacer promoter (lane 3), while the level of RNAs starting at the gene promoter is not much changed or—in most experiments—is somewhat reduced.

Figure 2A also shows an analysis of centrifuged oocytes that had the germinal vesicles in intermediate positions between the two poles (lanes 4–9). The result shows that the spacer promoters are already induced when the germinal vesicle is centrifuged to a location between the two hemispheres (90°, lane 7).

A promoter with a mutated T3 behaves like a spacer promoter

In order to test whether the differential behavior of spacer and gene promoter is also seen with injected cloned promoters, and in order to identify the DNA sequence(s) responsible for this regulation, various plasmid constructs were injected into germinal vesicles located either in the animal or in the vegetal hemisphere of the oocyte. First, cloned spacer promoters were injected into A- and V-oocytes. Even though the S1 probe used did not allow me to distinguish the endogenous from the injected spacer promoters, the result (Fig. 2B) clearly shows that the injected spacer promoters are co-regulated with the endogenous spacer promoters: injected A-oocytes (lane 2) showed no increase of the spacer promoter signal over the signal from uninjected oocytes (lane 1). On the other hand, V-oocytes injected with cloned spacer promoters showed a several-fold additional increase in the S1 signal (lane 4) compared to uninjected V-oocytes (lane 3).

I then speculated that the main or only reason for the differential behavior of the endogenous gene and spacer promoter in animal and vegetal hemispheres was the lack of a T3 site upstream from the spacer promoter. To test this idea, I compared the transcription of two minigene constructs, one containing a gene promoter including its upstream T3 site (245 bp upstream sequence), and a construct identical to the first one except for clustered point mutations in the T3 box (McStay and Reeder, 1986).

The result obtained with an S1 probe specific for 5′ ends of RNAs starting at the cloned promoter is shown in Figure 2C. The promoter plus T3 showed similar activity in both A- and V-oocytes (lanes 3 and 4). In contrast, the construct with the inactivated T3 showed very little initiation in A-oocytes (lane 1), but gave a strong signal in V-oocytes (lane 2). In fact, in V-oocytes there was no difference in the promoter activity plus or minus T3. It appears therefore that transcription initiation is very dependent on T3 in A-oocytes, but independent of T3 in V-oocytes. Thus, a T3-less gene promoter indeed behaves like a spacer promoter.

Cytoplasmic and nuclear components can mix in V-oocytes

While attempting to perform nuclear run-off experiments with V-oocytes, I found that it is extremely difficult to isolate germinal vesicles from V-oocytes manually, even though they are still recognizable as discrete structures. This observation suggested that in V-oocytes the germinal vesicles might be structurally changed. I therefore tested whether in V-oocytes nuclear and cytoplasmic components are still separated by the nuclear envelope, or whether they are free to move between the two compartments. To that end I injected FITC-labeled dextran of molecular weight 70 kDa. This size is known not to pass through the nuclear pore complex passively (Paine et al., 1975; Dingwall and Laskey, 1986). After overnight incubation, the oocytes were dissected and examined under regular light for the presence of the fluorescent label. It was found that in A-oocytes the injected dextran was still in the germinal vesicles. On the other hand, in the majority of V-oocytes the dye was clearly diluted or no longer visible, indicating that it was able to diffuse into the cytoplasm of the oocyte. In several FITC-dextran injection experiments, there were rare A-oocytes that had lost their green nuclear staining (especially after stronger centrifugation), and, alternatively, a few V-oocytes were found that had maintained the dextran in their germinal vesicle. Therefore, I analyzed individual V-oocytes for the induction of the spacer promoters. As shown in Figure 3, there was a clear correlation between the loss of FITC-dextran from the germinal vesicles (W lanes) and the induction of the spacer promoter (and an injected T3 mutant). Furthermore, analysis of strongly centrifuged A-oocytes that had lost the FITC-dextran from their germinal vesicle showed that the spacer promoters were induced (not shown). Since in a typical experiment, maintenance of FITC-dextran in the germinal vesicles was found in A-oocytes and loss of the dextran from the germinal vesicles in V-oocytes, the use of the terms A- and V-oocytes was maintained.

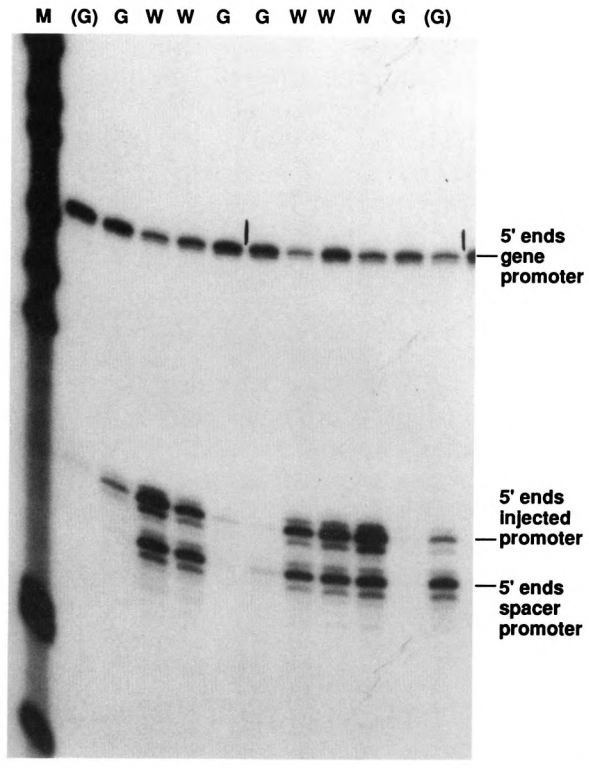

Figure 3. Spacer promoter activity in V-oocytes correlates with loss of nuclear location of injected dextran of 70 kDa. FITC-labeled dextran (along with a plasmid with mutated T3) was injected into nuclei of V-oocytes, and nuclear or cytoplasmic distribution was monitored after incubation for 18 hours. Individual oocytes were assayed with a mixture of S1 probes for the gene promoter, the spacer promoter, and the injected cloned promoter. G denotes presence of green color in the nucleus; W denotes white nucleus, i.e., loss of green color. (G) denotes light green color of the nucleus. Note the correlation between loss of nuclear distribution of the FITC-dextran and activation of both spacer promoter and a T3-less injected promoter.

V-oocytes are in a semi-stable transcriptional state

Since the experiments with FITC-dextran showed that the germinal vesicles in V-oocytes are no longer in their native state, it was important to determine whether V-oocytes were rapidly deteriorating, or whether they were in a stable state. The finding that spacer transcripts were synthesized in V-oocytes already suggested that transcription had to continue at least for some time. I further investigated this point with the following two experiments.

First, I examined whether the same stimulation of a T3-mutated promoter in V-oocytes would be observed if the plasmids were injected at different times after centrifugation. In this experiment, a T3-containing and a T3-mutated promoter were co-injected immediately after centrifugation, or 1, 3, and 6 hours later. Transcripts from the two plasmids could be distinguished with specific S1 probes. As shown in Figure 4A, the same result is obtained at all time

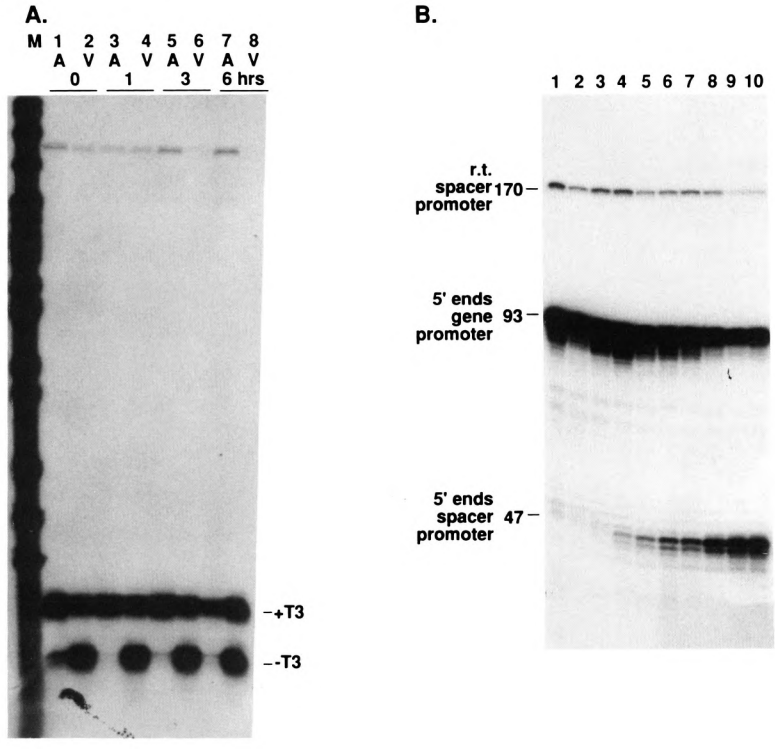

Figure 4. V-oocytes are in a stable transcriptional state for several hours. **A.** A pair of minigenes, one with a wild-type T3 and one with a mutated T3, were co-injected immediately after centrifugation (lanes 1 and 2), and 1 hour (lanes 3 and 4), 3 hours (lanes 5 and 6), or 6 hours after centrifugation (lanes 7 and 8). Transcription from the two plasmids can be distinguished by the specific S1 probes used. The probe for the T3 mutated promoter is the one shown in Figure 1B (50 nucleotide protection for correctly initiated transcripts), whereas the probe for the T3-containing promoter is similar but gives a protection of 62 nucleotides (see Labhart and Reeder, 1985 for details). Note that the activation of the plasmid with mutated T3 (−T3) in V-oocytes is seen even when injected 6 hours after centrifugation. **B.** Time course of the accumulation of the spacer promoter transcripts in V-oocytes. S1 assay was as in Figure 2A. Time points analyzed in lanes 1–10 are 0.5, 1, 2, 3, 4, 6, 9, 24, and 24 hours. Note that spacer transcripts continue to accumulate after 9 hours.

points: the T3-containing promoter is similarly active in A- and V-oocytes, whereas the T3-mutated promoter is weak in A-oocytes and strong in V-oocytes.

Second, the kinetics of the activation of the spacer promoters was studied by extracting and analyzing RNA from V-oocytes at different times after centrifugation. Figure 4B shows that transcripts starting at the spacer promoter are first detected 1–2 hours after centrifugation and continue to accumulate for more than 9 hours—indicating that the induction of the spacer promoters occurs shortly after centrifugation, and that the spacer promoters remain active in V-oocytes for more than 9 hours. This conclusion is further supported by the in vivo labeling experiment shown below (Fig. 6A).

Strong initiation in A-oocytes depends on correct spacing between T3 and the promoter

Since the T3 site had originally been identified as a terminator (Labhart and Reeder, 1986), the dependence of initiation on this site in A-oocytes may be due to prevention of promoter occlusion. In that case, initiation should remain undiminished if the T3 site is moved further upstream. If, on the other hand, T3 were a true promoter element, changing the distance between the promoter and T3 might be expected to affect initiation. I therefore tested whether for high promoter activity in A-oocytes, T3 had to be in its natural location 60 bp upstream from the promoter, or whether it could be moved further upstream. Two constructs were injected in which T3 and the promoter were "pushed apart" by insertion of either 112 bp or 3.7 kb of vector DNA. S1 analysis of RNA from injected A-oocytes showed that the full stimulatory effect onto the promoter is seen only when T3 is in its natural position upstream from the promoter (Fig. 5, lane 1; band labeled "5′ ends experiment"). The two push-apart clones gave a considerably weaker signal (lanes 4 and 5), albeit not as low as in the T3-mutant, -deletion, or -inversion (lanes 2, 3, and 6). As expected, the level of transcripts reading into the promoter (bands designated "r.t. plasmid") is lower in the lanes with a functional T3 in the right orientation, regardless of its distance from the promoter (lanes 1, 4, and 5). These results indicate that most of the T3-dependent promoter signal in A-oocytes is not due to prevention of promoter

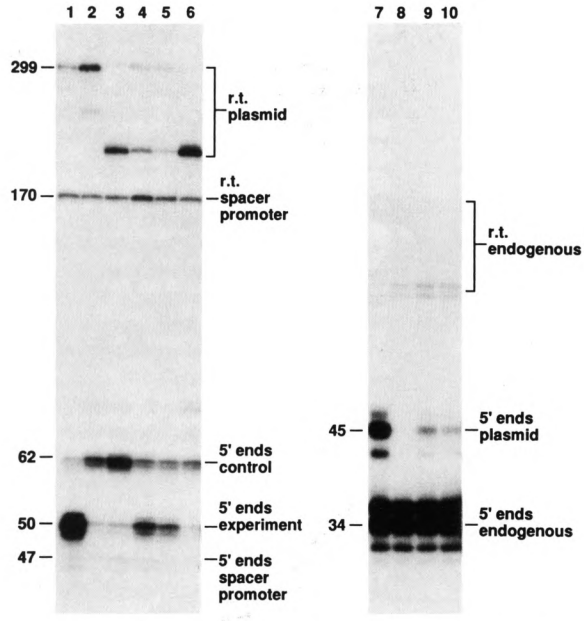

Figure 5. T3 needs to be in its natural location for high promoter activity in A-oocytes. The following minigene constructs were injected into nuclei of A-oocytes: wild-type construct with T3 in its natural location (lanes 1 and 7), T3 mutant (lane 2), T3 deletion (5′–158; lanes 3 and 8), 112 bp push apart clone (lanes 4 and 10), 3.7 kb push apart clone (lanes 5 and 9). The construct assayed in lane 6 had the T3 site cloned 3.7 kb upstream from the promoter but in inverted orientation. For the precise structure of these push apart constructs, see Labhart and Reeder (1987b). The injected oocytes were analyzed either by S1 protection of total RNA (lanes 1–6) or by "reverse"-RNase protection analysis of nuclear run-on assays (lanes 7–10). For the S1 assays, the experimental plasmids were co-injected with a T3-containing control plasmid, and the RNA was assayed with probes specific for the experimental (50 nt protection) and the control plasmid (62 nt protection), as well as with the probe specific for the endogenous spacer promoter. Note that the push-apart constructs give an intermediate signal both in vivo in A-oocytes and upon in vitro transcription of germinal vesicles from injected A-oocytes.

occlusion. In this experiment, a control plasmid containing T3 was co-injected with the experimental plasmids. Its transcription yielded a different S1 protection band at 62 nucleotides (bands labeled "5′ ends control"). Not surprisingly, there appears to be a competition effect between the promoter on the experimental plasmid and the control plasmid. This effect was not investigated further. The RNA from the injected oocytes was also hybridized simultaneously with the probe specific for the endogenous spacer promoter in order to confirm the A-type nature of the oocytes. The result shows that in all six lanes the spacer promoter activity is not induced above basal levels (spacer promoter transcripts would protect 47 nucleotides of the probe from S1-digestion; see Fig. 2A).

A-type promoter function is maintained in homogenates of isolated germinal vesicles

In an attempt to address the question whether the V-type of ribosomal transcription is due to loss of a nuclear factor or gain of a cytoplasmic factor, run-on assays with homogenates of manually isolated germinal vesicles were performed. In this system, cytoplasmic factors should be largely absent, and soluble nuclear factors are diluted into the isolation medium and/or reaction buffer. We had reported previously (Labhart and Reeder, 1989) that in this system initiation at the ribosomal promoter continues efficiently in vitro. As mentioned above, germinal vesicles could only be isolated from A-oocytes. In the experiment shown in Figure 5, lanes 7–10, A-oocytes were injected with various constructs, and after incubation for 16 hours germinal vesicles were isolated and transcribed in the presence of [α-^{32}P]CTP. The labeled RNA was analyzed with the "reverse"-RNase protection assay using probe A (see Fig. 1A). Due to the structure of the injected plasmids, RNA initiated at the plasmid promoters gives rise to a protected RNA fragment of about 45 nucleotides, whereas the endogenous transcripts yield a band at about 34 nucleotides (Labhart and Reeder, 1987a). The experiment shows that in the germinal vesicle homogenate the A-type of promoter function is maintained, i.e., initiation is strong in the presence of T3 in its natural location (lane 7), but barely detectable upon deletion of T3 (lane 8). Readdition of a T3 site either 112 bp (lane 10) or 3.7 kb (lane 9) upstream from a T3-less promoter gives rise to an intermediate signal. These results therefore support a model in which the T3-independent initiation seen in V-oocytes is brought about by the influx of cytoplasmic components.

Transcription is very polar in V-oocytes

In addition to the activation of T3-less promoters, there is one other major transcriptional change taking place in V-oocytes. In Figure 2A it can be seen that the readthrough signal obtained with the spacer promoter probe remains unchanged or decreases upon induction of the

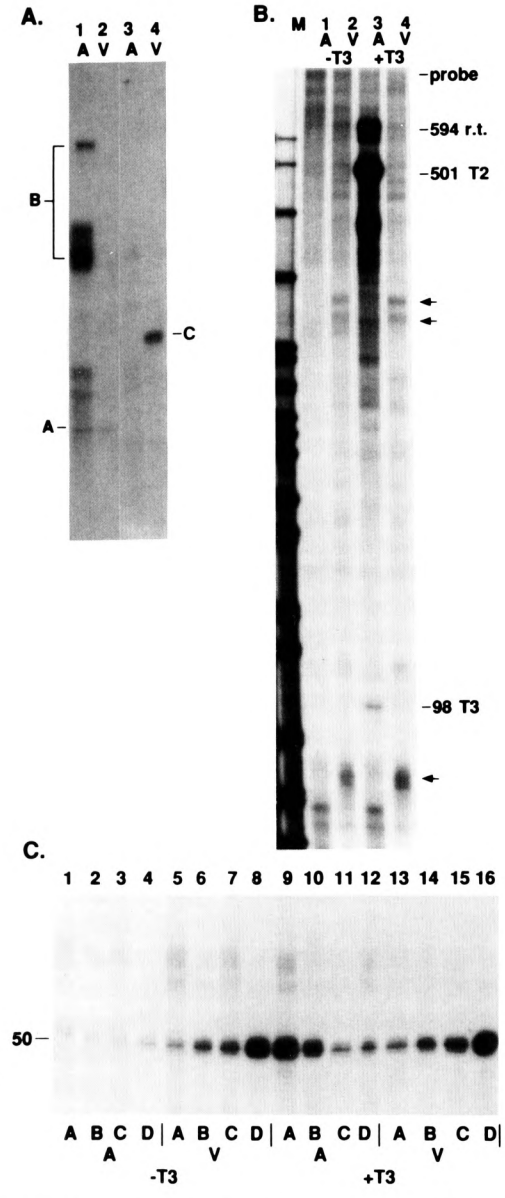

Figure 6. Transcription is very polar in V-oocytes. **A.** RNA was labeled in vivo by injection of [α-^{32}P]CTP (along with α-amanitin) into A- and V-oocytes and analyzed with a "reverse"-RNase protection assay using single-stranded DNA probes from three different regions of the ribosomal gene repeat. In lanes 1 and 2, a mixture of a probe from the very 5′ end (probe A) and from the 3′ end of the 40S precursor coding region (probe B) was used. In lanes 3 and 4, a probe specific for the region of the spacer promoters was used (probe C). See Figure 1A for the location of the probes. Note that the signal obtained at the 3′ end of the gene (B) is much lower in V-oocytes, while the signal at the 5′ end (A) remains essentially unchanged. The spacer promoter is turned on in V-oocytes, consistent with the data shown in Figure 2A. **B.** Transcription analysis of ribosomal promoters plus and minus T3 in A- and V-oocytes. Minigene plasmids with wild-type T3 (+T3 lanes) or mutated T3 (−T3 lanes) were injected into A- (lanes 1 and 3) and V-oocytes (lanes 2 and 4), and RNA was analyzed with an RNase T1 protection assay. The SP6 RNA-probe used is shown in Figure 1B. Lane M shows an end-labeled Hpa II digest of pBR322. Arrows denote RNA-protection bands characteristic of the short transcripts in V-oocytes. Only a T3-containing promoter in A-oocytes gives rise to large amounts of long transcripts (lane 3). **C.** Size-fractionation of RNA synthesized from ribosomal promoters injected into A- and V-oocytes. The lengths of single-stranded DNA are larger than 450 nt in fraction A, 250–450 nt in fraction B, 145–250 nt in fraction C, and 60–145 nt in fraction D. RNA from the same four samples analyzed in Figure 2C was used. S1 assay of the fractionated RNA was with the same probe used in Figure 2C. Note that in V-oocytes the majority of the transcripts starting at the promoter are shorter than 150 nucleotides (fraction D; lanes 8 and 16).

spacer promoter in V-oocytes. Since the probe used cannot distinguish between individual spacer promoters (see Fig. 1A), this result suggests that the transcripts starting at the most upstream spacer promoter do not extend as far as the next spacer promoter. Those spacer transcripts must therefore be shorter than about 1 kb. To investigate this phenomenon further—particularly to see whether this is also true for the transcripts originating at the gene promoter—the following experiments were performed.

After injection of [α-^{32}P]CTP into centrifuged oocytes and incubation for 18 hours, the labeled RNA was extracted from A- and V-oocytes. The labeled RNA was then hybridized to single-stranded DNA probes mapping to various sites along the rDNA repeat, and the RNase-protected RNA fragments were analyzed on denaturing polyacrylamide gels ("reverse"-RNase protection assay). A probe specific for the 5′ end of the 40S precursor rRNA (Fig. 1A, probe A) showed that in V-oocytes initiation continued at a similar rate as in A-oocytes (Fig. 6A, bands A in lanes 1 and 2). Consistent with the S1 analysis of Figure 2A, a probe specific for transcripts starting at the spacer promoter (probe C) detected no initiation in A-oocytes (lane 3), whereas V-oocytes showed an initiation rate at the spacer promoter which was similar or—as in the experiment shown—even greater than the one observed at the gene promoter (lane 4, band C). (At least a twofold higher signal from the spacer promoter can be expected because of the multiplicity of those promoters.) A probe to a region just downstream from the 3′ end

of the 28S coding sequence (Fig. 1A, probe B) yielded a strong signal for A-oocytes (Fig. 6A, lane 1, bands B), whereas it was greatly reduced or undetectable in V-oocytes (lane 2). (The multiple protected bands seen with probe B are probably due to known sequence microheterogeneities in that region.) Thus in V-oocytes the transcription initiating from the gene promoter is more polar than in A-oocytes. (The term "polarity" is used here to describe the reduced accumulation of promoter-distal RNA). This conclusion was also confirmed with an S1 analysis of size-fractionated RNA (the same assay as in Figure 6C; see below), which showed that in V-oocytes there is an increase in the amount of transcripts starting at the gene promoter that are only <150 nt long. In addition, this assay showed that virtually all of the spacer transcripts synthesized in V-oocytes are only <150 nt long (data not shown).

Only the combination of T3 plus promoter in A-oocytes gives rise to long ribosomal transcripts

In order to determine whether the difference in the lengths of the RNA synthesized in A- and V-oocytes is also seen with injected plasmids, two additional assay systems were employed. In Figure 6B, the same RNA samples that had been analyzed by S1 protection assay in Figure 2C were subjected to an RNase T1 protection assay using a labeled SP6 RNA probe encompassing the entire length of the minigene plus flanking sequences (see map in Fig. 1B). When RNA produced in the A-oocytes from a T3-containing promoter was analyzed (Fig. 6B, lane 3), the two major protected bands of 501 and 594 nt corresponded to RNA extending from the promoter to T2 at the end of the minigene and to RNA starting at the promoter and reading through T2, respectively. An additional protected RNA fragment of 98 nucleotides represents transcripts terminating at the T3 site upstream from the promoter. As expected from the S1 analysis (Fig. 2C), a T3-less promoter did not produce sufficient levels of RNA to be detected by the RNase T1 protection assay (Fig. 6B, lane 1). In contrast, the pattern of protected RNA fragments from injected V-type of oocyte RNA was very different, both with and without T3 (lanes 2 and 4). Despite the high levels of 5′ ends that were detected with the S1 assay (Fig. 2C, lanes 2 and 4), RNase protection bands corresponding to RNA extending from the promoter to T2 and beyond were virtually not detectable; instead, several shorter protected RNA species are seen, the most prominent of which are a fragment of about 85 nucleotides and a characteristic doublet running at around 280–290 nt (marked by arrows in Figure 6B). Further control experiments showed that those bands indeed represent transcripts starting at the ribosomal promoter (see Materials and Methods). Thus, in V-oocytes the majority of transcripts end after 100 to a few hundred nucleotides at heterogenous but discrete sites.

The interpretation of the RNase T1 protection assay was confirmed with the assay shown in Figure 6C. The same four RNA samples were fractionated on a denaturing polyacrylamide gel, and the gel was cut in four fractions. A labeled Hpa II digest of pBR322 was run in parallel in order to determine the approximate size-distribution of RNA in the four fractions. RNA was eluted from the four gel slices and subjected to S1 analysis to detect RNA 5′ ends starting at the promoter. The result shows that in A-oocytes, the majority of transcripts from a T3-containing promoter are longer than 450 nucleotides (= fraction A, lane 9). On the other hand, in V-oocytes predominantly short transcripts are produced (<150 nt, fraction D), from a promoter both with and without T3 (lanes 8 and 16). Interestingly, the S1 signal obtained with the RNA from A-oocytes injected with a T3-less promoter is about equally distributed in the four fractions (lanes 1–4). Since the four fractions do not represent four equal ranges of size-classes, this finding indicates that in A-oocytes and in the absence of T3 transcription is not only much weaker but also polar. Stimulation in the presence of a functional T3 does not affect all four size classes equally; instead, the longest size-class shows an estimated stimulation in the range of 50- to 100-fold (compare lanes 1 and 9), while the shortest two size-classes are stimulated only a few fold (compare lanes 4 and 12). Thus T3 appears not only to stimulate transcription, but also to confer high processivity to the initiating transcription complex.

The same basic RNA polymerase I promoter is recognized in A- and V-oocytes

The ribosomal promoter in X. laevis comprises a sequence from −142 to about +1 relative to the initiation site (Sollner-Webb et al., 1983;

Reeder et al., 1987). In order to see whether this same promoter is recognized in A- and V-oocytes, I injected two diagnostic mutations and analyzed the RNA extracted from the oocytes with both the S1 and the RNase T1 protection assay. As shown in Figure 7, both a 5′ −90 deletion and a linker scanner mutation in the important region at −142/−133 are silent in both types of oocytes (lanes 1, 2, 7, 8). Control injection of plasmids containing a promoter plus T3 and a promoter with mutated T3 show again the T3-dependent highly processive transcription in A-oocytes and the heterogenously terminated transcripts in V-oocytes (lanes 3, 4, 9, 10). The result with the promoter mutations, along with the α-amanitin resistance of all the transcription from injected plasmids, indicates that transcription in both A- and V-oocytes is by RNA polymerase I.

T2 and T3 sites are ignored in V-oocytes

In the experiment shown in Figure 7, two additional plasmids were analyzed that had point-mutations in the T2 box at the 3′ end of the minigene. One of them was mutation C261, which restores termination function to the otherwise termination-deficient T2 site but leaves 3′ end formation unchanged, and the other was G255, which abolishes 3′ end formation. The result shows that in A-oocytes, C261 (lane 5) is as efficient in 3′ end formation as the wild-type T2 (lane 4), while G255 abolishes 3′ end formation, leading to a strong read-through signal (band at 594 nt in lane 6). In this experiment, the average length of the transcripts produced in V-oocytes was greater than in the experiment shown in Figure 6B. This permitted better investigation of the 3′ end formation at T2. Interestingly, the result shows that those transcripts that reach T2 do not form correct 3′ ends at this site. Even though there are several RNase protection bands visible at around 500 nt, they do not align with the band expected for correct 3′ end formation at T2. Furthermore, there is no change in this band pattern with the G255 mutation. I conclude that V-type transcription is unable to recognize the T2 box signal.

The inactivity of the C261 mutant in 3′ end formation in V-oocytes suggests strongly that it is also inactive in transcription termination. We had previously shown (Labhart and Reeder, 1990) that while 3′ end formation can occur in the absence of termination, termination was seen only when 3′ end formation was occurring. I nevertheless tested directly for termination

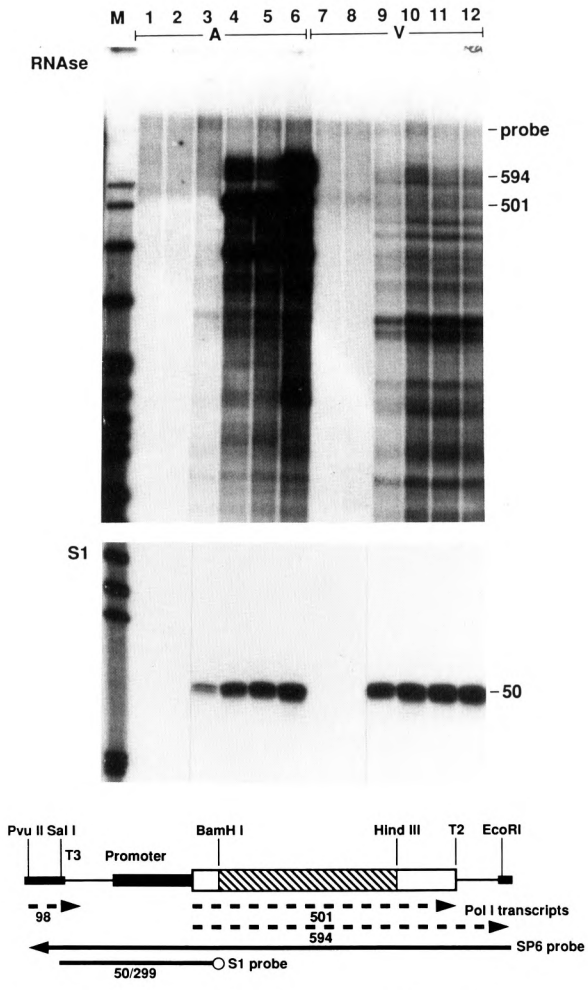

Figure 7. Further characterization of A- and V-type ribosomal transcription. Plasmids with the basic structure shown below the autoradiographs were injected into A- and V-oocytes and analyzed either by RNase T1 protection assay (upper panel) or by S1 protection assay (lower panel). The mutations analyzed were: lanes 1 and 7, 5′ −90 promoter deletion mutant; lanes 2 and 8, promoter linker scanner mutant −142/−133; lanes 3 and 9, T3 mutation; lanes 4 and 10, all wild-type construct; lanes 5 and 11, C261 mutation of T2; lanes 6 and 12, G255 mutation of T2. Note that the promoter mutations show no transcription in both A- and V-oocytes, and that the T2 site (protection of 501 nt in lanes 4 and 5) is not recognized in V-oocytes (lanes 10–12). The gel has been overexposed in order to show the low amount of long transcripts in V-oocytes. (Some of the heterogenous bands in the upper panel [lanes 3–6] and the relatively strong S1 signal of the T3 mutant in A-oocytes [lane 3] are probably due to contamination of the A-RNA samples with V-RNA.)

in V-oocytes. The assay for terminator function in our previous work involved looking for a drop in RNA levels downstream from a putative termination site. Since the general processivity in V-oocytes was found to be very low, I cloned the T3 site and the T3 mutation ~100 bp downstream from a transcription start site and measured RNA levels at a site ~350 bp downstream. The result showed—as expected—that in A-oocytes only a low level of RNA reads through the intact T3 site, while mutation of the T3 site causes this readthrough RNA to increase. In V-oocytes, on the other hand, there is about the same low level of RNA detectable downstream from both the intact and the mutated T3 site (data not shown). This low level is consistent with the general low processivity of transcription in V-oocytes. The finding, however, that the wild-type T3 site does not cause a further drop in the readthrough RNA indicates that the T3 site is non-functional in V-oocytes.

Discussion

What are V-oocytes?

Centrifugation of Xenopus oocytes has been widely used to make the germinal vesicles visible for successful injections. While the original papers recommended centrifugation of the oocytes with their animal pole up (Kressmann et al., 1977), it is not clear whether all subsequent studies using oocytes injection follow that recommendation, nor are any experimental data given why centrifugation with the vegetal side up should not be performed. Here I report that gentle centrifugation of oocytes with their animal pole up appears to maintain the germinal vesicle in its native state (A-oocytes), whereas even gentle centrifugation with the vegetal pole up, or stronger centrifugation at any orientation, causes structural changes of the germinal vesicle (V-oocytes). Those changes can be assayed for by monitoring the loss from the nucleus of FITC-dextran of molecular weight 70 kDa. While the state of the germinal vesicle in V-oocytes can be described pejoratively as being damaged or leaky, the present results show that there are interesting changes taking place in ribosomal gene transcription, thereby giving the V-type of transcription potential significance.

It should be emphasized that V-oocytes are not simply oocytes with a completely disintegrated germinal vesicle. It has been shown repeatedly that injection of ribosomal or other genes into the cytoplasm leads to no transcription at all (Sollner-Webb and McKnight, 1982; Mertz and Gurdon, 1977). In V-oocytes, some nuclear structure is still recognizable and, most importantly, my "delayed injection" experiment (Fig. 4A) showed that injection into a V-nucleus still leads to activation of the ribosomal promoter, even if performed several hours after the creation of V-oocytes. Furthermore, the ribosomal enhancer, which is thought to be involved in setting up active transcription complexes (Reeder, 1984; Labhart and Reeder, 1985), is functional both in A- and V-oocytes (data not shown).

The observed changes in transcription are very unlikely due to some localized "transcription factor" in the oocyte cytoplasm. I base this conclusion mainly on the finding that the V-type of transcription can also be induced when oocytes are centrifuged at higher force with the animal pole up. For the same reason, the changes are probably not caused by yolk platelets, which are localized primarily in the vegetal hemisphere. Nevertheless, the present observations seem to reflect some difference between the animal and vegetal hemisphere of the oocyte, since the germinal vesicle is much more susceptible to structural changes when translocated into the vegetal hemisphere.

Are the observed transcriptional changes in V-oocytes caused by loss of nuclear factors or influx of cytoplasmic factors? Run-on experiments with germinal vesicles isolated from A-oocytes suggest that both mechanisms may contribute to the V-type transcription pattern. With respect to promoter function, the A-type of transcription was observed in homogenates of isolated germinal vesicles, i.e., initiation was dependent on T3 in its natural location upstream from the promoter, and push-apart constructs gave intermediate initiation signals (Fig. 5). Thus, the A-type of transcription is maintained when any diffusible nuclear components, including transcription factors, are diluted and only low amounts of cytoplasm are present. This observation suggests an involvement of cytoplasmic factors in the induction of T3-less promoters. It will be interesting to see whether the V-type of promoter function can be created by the addition of cytoplasmic extract. On the other

hand, transcription in the germinal vesicle homogenate appears to be more V-like with respect to processivity, in that previous experiments showed a drastic drop in transcription rates from 5′ to 3′, especially on injected minigene constructs (Labhart and Reeder, 1990). The strong polarity of transcription in isolated germinal vesicles from A-oocytes suggests that a factor that confers high processivity to ribosomal transcription in vivo is easily lost in homogenates of germinal vesicles. This raises the possibility that the same phenomenon in V-oocytes is also due to a loss of some nuclear component.

Regulation and function of spacer promoters

The most striking change taking place in V-oocytes is the activation of the spacer promoters. Since the discovery of their existence and of their activity (Boseley et al., 1979; Moss, 1983), there has been much discussion about their potential function. As much as the endogenous genes are concerned, the oocytes and an X. laevis tissue culture cell line represent the two extremes of cell types with silent and active spacer promoters, respectively. Furthermore, my own unpublished observations show that during embryogenesis the spacer promoter is turned on at the midblastula transition, but that during further embryonic development the level of spacer transcripts gradually decreases with respect to the level of transcripts initiating at the gene promoter. From those combined observations, a striking correlation emerges between spacer promoter activity and the rate of cell division. If the function of the spacer promoter is to increase the polymerase loading at the gene promoter (De Winter and Moss, 1986), then one might propose that the activity of the spacer promoters is required only to re-start transcription at the gene promoter after each cell division. However, the induction of the spacer promoter could also be a sheer consequence of cell divisions and—in an extreme model—have no function at all. While the present observations do not let us decide between those models, they are consistent with the noted correlation between cell division and spacer promoter activity: as in V-oocytes, in rapidly cycling cells the nuclear structure is being dissolved during every mitosis. This could lead to a similar mixing of cytoplasmic and nuclear components.

One strategy for gaining insight into the function of spacer promoters was to inject various constructs containing cloned spacer promoters into oocyte nuclei. In those studies the injected spacer promoters were found to be as active as injected gene promoters; thus there appeared to be a discrepancy between the regulation of the endogenous and exogenous genes. Based on the experiments presented in this paper, I have to conclude that the use of V-oocytes contributed to the observed deregulation of the spacer promoter. For example, Morgan et al. (1984) found equal signals from injected spacer promoters and gene promoters, indicative of V-type transcription. Strong transcription from injected spacer promoters was also found by De Winter and Moss (1986) and in my own work (e.g., Labhart and Reeder, 1987b). Here I show that if injected into "native" germinal vesicles of A-oocytes, spacer promoters are virtually silent, like the endogenous spacer promoters.

With the identification and characterization of the T3 site upstream from the gene promoter and its demonstrated ability to act as a promoter element (McStay and Reeder, 1990), the absence of such a T3 site upstream from the spacer promoter has become a very notable feature. Is this lack of a T3 site the main or only functional difference between the spacer and gene promoter? I am not aware of any experiment, published or unpublished, in which a gene promoter with a mutated T3 could not substitute for a spacer promoter. Thus, it appears that all the data are consistent with such a notion.

T3 as a promoter element

Whether T3 (as well as similar terminators upstream from ribosomal promoters in other species) stimulates the nearby promoter by prevention of occlusion or by a positive effect on the promoter has been the subject of several studies (e.g., Bateman and Paule, 1988; Henderson et al., 1989; McStay and Reeder, 1990). The result obtained with the push-apart clones in A-oocytes (Fig. 5) confirms the findings of earlier in vitro transcription studies (McStay and Reeder, 1990) and further supports the notion that T3 can act as a true promoter element. It should be noted, however, that the push-apart constructs consistently showed a promoter signal intermediate to the signals obtained with the wild-type and with the T3 mutant. This finding can be explained by postulating that T3 can work at a distance, albeit with lower

efficiency; alternatively, there may be a promoter occlusion component in the T3 stimulation. The latter interpretation is supported by psoralen-crosslinking studies (Lucchini and Reeder, 1989), in which prevention of readthrough into the promoter by transcription-terminating crosslinks led to an increase in the promoter signal, but not to the levels seen with the T3 site upstream from the promoter.

Closer inspection of transcription without and with T3 in A-oocytes reveals a result with potential significance for the mechanism of promoter stimulation by T3. The size-fractionation analysis of the low level of transcription in the absence of T3 (Fig. 6C, lanes 1–4) shows that there is an almost equal distribution of the transcripts between the four size-classes. This means that transcription from a T3 mutated promoter is rather polar even in A-oocytes. Thus T3 not only stimulates initiation, but also appears to confer high processivity to the transcription complex. This is a very inconspicuous result, but it yields an important new insight into the mechanism of promoter stimulation by the upstream terminator T3.

Formation of short ribosomal transcripts

In recent years many cases have been described in which the expression of genes is regulated by transcriptional attenuation or premature termination (reviewed in Spencer and Groudine, 1990). In several instances, however, the significance of those short transcripts is not clear yet, or they could not be demonstrated in vivo. In the present work I show that similar short transcripts can also be demonstrated with genes transcribed by RNA polymerase I: in V-oocytes, transcription is very polar, with the majority of transcripts not exceeding 150 nt in length. The precise 3′ ends of those transcripts have not been mapped. Because such short transcripts are observed both on endogenous genes and on different plasmid minigenes, the endpoints are unlikely to be determined by specific sequence elements in the DNA. It is more likely that this premature RNA 3′ end formation is a function of the distance from the initiation site. The same type of short ribosomal transcripts has so far not been demonstrated in normal X. laevis cells; therefore their significance remains unclear.

Because nuclear run-off experiments could not reliably be performed with V-oocytes, the question whether those short transcripts are the result of actual termination or of instability of promoter-distal RNA could not be directly tested. In other experiments, however, we had found a lower transcription rate at the 3′ end of ribosomal genes compared to the 5′ end (Labhart and Reeder, 1989 and 1990). This drop in transcription rate from 5′ to 3′ was most drastic in nuclear run-on assays of injected minigene constructs. These results suggest that ribosomal gene transcription can be regulated at the level of elongation, but the relationship of those findings to the present V-type transcription is not clear.

Is the activation of spacer promoters and of T3 mutated promoters in V-oocytes just a consequence of relief from promoter occlusion due to the low processivity? As discussed above, the low promoter activity of T3-less promoters in A-oocytes can be ascribed only in part to promoter occlusion. Therefore, if low processivity is the only cause for the activation of T3 mutants in V-oocytes, such promoters should not be activated more in V-oocytes than the push-apart constructs in A-oocytes. However, several of the present experiments show that promoter activity in V-oocytes tends to be even stronger than the activity of a promoter with its upstream T3 in A-oocytes. Therefore, prevention of promoter occlusion may contribute to the activation of spacer promoters and of T3 mutated promoters in V-oocytes; but there appears to be a second—perhaps major—mechanism by which such promoters are activated. As discussed above, the present results suggest a model in which this second mechanism involves cytoplasmic factors. It should also be noted that a mechanism of spacer promoter activation with an occlusion component can only be correct if the short transcripts in V-oocytes are the result of actual termination and not of instability of promoter-distal RNA.

Evidence for a qualitatively changed elongation complex in V-oocytes

In addition to its terminator and promoter function, we reported previously that T3 upstream from the promoter is required for efficient 3′ end formation at T2 at the 3′ end of injected minigene constructs (Labhart and Reeder, 1987b). The present observations explain that phenomenon: a mixture of A- and V-oocytes must have been used. The situation is best illustrated by

superimposing corresponding A-and V-lanes in Figures 2C, 6B, and 7. In such a mixture, the effect of T3 on the promoter signal is at most twofold, but the 3′ ends at T2 are generated solely by transcription from the T3-containing promoter in A-oocytes. Transcription from a T3 mutated promoter in A-oocytes is too low to give a detectable signal at T2, and in V-oocytes the majority of the transcripts do not reach T2 on the minigene constructs used. Still, the minority of transcripts that do reach T2 in V-oocytes do not form correct 3′ ends at that site (Fig. 7). Based on the present result, our earlier data concerning an interaction between T3 and T2 (Labhart and Reeder, 1987b) should be reinterpreted in the following way. There are indeed two types of ribosomal transcription which differ in their ability to recognize site T2. But the dependence of T2 function on a T3 site upstream from the promoter is probably indirect: 3′ end formation at T2 is primarily dependent on A-type of transcription, though efficient A-type of ribosomal transcription is seen only in the presence of T3 upstream from the promoter. The elucidation of the molecular differences between A-and V-types of ribosomal gene transcription will require their reproduction in vitro and the identification of the factors causing those differences.

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