Control of 4–8S RNA transcription at the midblastula transition in *Xenopus laevis* embryos

Elsebet Lund and James E. Dahlberg

Department of Biomolecular Chemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706 USA

Transcription of *Xenopus laevis* U1 snRNA genes is subject to a precise program with respect both to the timing of activation at the midblastula transition (MBT) and to the relative levels of the two embryonic U1 RNAs (xU1b1 and b2) that are made. Here, we demonstrate that exogenous xU1b genes injected into developing *X. laevis* embryos come under the same controls as the endogenous genes. Injected U1 genes, unlike exogenous RNA polymerase III genes, remain quiescent until MBT and their activation at MBT requires protein synthesis during the early cleavage stages. Significantly, the onset of 4–8S RNA transcription occurs at the normal time, even when the DNA content of the embryo has been increased by injection of exogenous DNA or reduced through cleavage arrest, indicating that transcriptional activation at MBT is independent of the ratio of DNA (nucleus) to cytoplasm. In cleavage-arrested (coenocytic) embryos, the reduced level of DNA at MBT results both in a decrease in snRNA and tRNA synthesis (reflecting the lower gene dosage) and in a prolonged synthesis of large amounts of unusual RNA polymerase III transcripts, OAX RNAs. In normally cleaving embryos, small amounts of these unstable OAX RNAs (encoded by satellite I DNA) are synthesized only briefly at MBT. Our demonstration that RNA and DNA metabolism is aberrant in cleavage-arrested embryos requires reevaluation of previous experiments on transcriptional activation that utilized such coenocytic embryos.

[Key Words: Midblastula transition; U1 snRNA; *Xenopus laevis* embryos; transcription; satellite I DNA; OAX RNA]

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The first major developmental transition in early *Xenopus laevis* embryos occurs at the midblastula stage, about 7 hr after fertilization. It is characterized by a lengthening of the cell cycle, the onset of cell motility, and the activation of zygotic gene expression (Signoret and Lefresne 1971; Gerhart 1980; Newport and Kirschner 1982a). Control of these changes, collectively referred to as the midblastula transition (MBT), depends to a large degree on the ratio of nucleus to cytoplasm in the embryo (Newport and Kirschner 1982a,b).

When transcription is activated at MBT, the synthesis of small (4–8S) RNAs (Newport and Kirschner 1982a; Forbes et al. 1983a) is normally dominated by U1–U5 small nuclear RNAs (snRNAs) (made by RNA polymerase II) and by tRNA, 7S RNA, and U6 snRNA (made by RNA polymerase III). During the period from MBT to neurula, primarily two forms of embryonic U1 snRNAs accumulate, called xU1b1 and xU1b2 (Forbes et al. 1984; Lund and Dahlberg 1987). These two U1 RNAs are encoded pairwise in large tandem arrays (Lund et al. 1984; Ciliberto et al. 1985); however, more xU1b1 RNA is transcribed at MBT (Forbes et al. 1984) due, at least in part, to differences in the 5'-flanking regions of the xU1b1 and xU1b2 genes (Lund et al. 1987).

In their characterization of the MBT, Newport and

Kirschner (1982a,b) proposed that the onset of embryonic transcription was triggered by a critical ratio of DNA to cytoplasm. Subsequently, it was demonstrated that injected DNA promotes the formation of nuclear structures (Forbes et al. 1983b) and that stockpiled mitotic factors in the cytoplasm become limiting in the presence of high numbers of nuclei (Newport et al. 1985). Taking these findings into account, Kimelman et al. (1987) proposed that increasing the ratio of nucleus to cytoplasm by DNA injection should lead to a lengthening of the cell cycle, which, in turn, could result in premature activation of transcription. In those studies, the timing of transcriptional activation was analyzed in cleavage-arrested (coenocytic) eggs rather than in cleaving embryos, based on the assertion that synthesis of both DNA and RNA proceeds normally in coenocytic embryos during the early stages of embryogenesis (Newport and Kirschner 1982a).

Here, in an effort to understand transcriptional activation at MBT, we focus on the activation of embryonic snRNA genes. To do so, we have injected cloned xU1b genes into early embryos and monitored their expression as a function of time after fertilization. We show that in normally cleaving embryos, such exogenous genes are subject to correct developmental controls, remaining

quiescent until MBT, at which time they are transcribed. Moreover, transcriptional activation of this class of RNA polymerase II genes is dependent on events prior to MBT that require protein synthesis during the early period of rapid cell division.

Unexpectedly, we observed that injection of excess DNA into cleaving embryos does not result in premature activation of endogenous genes. Furthermore, comparing normal and coenocytic embryos, we find that the patterns of newly made 4–8S RNAs at MBT differ both quantitatively and qualitatively and that DNA accumulation is reduced significantly in cleavage-arrested embryos (see also Takeichi et al. 1985). Despite this DNA deficiency in coenocytic embryos, RNA synthesis begins at the same time after fertilization as in normally cleaving embryos (cf. Newport and Kirschner 1982a). Thus, contrary to previous studies, we conclude that activation of transcription at MBT occurs at a fixed time, independent of the ratio of DNA to cytoplasm. However, DNA content does affect both the level of transcription (via gene dosage) and the point at which transcription of certain RNA polymerase III genes ceases (presumably via competition for limiting factors).

Results

Correct control of *X. laevis* embryonic U1 genes injected into cleaving embryos

To determine whether exogenous (injected) U1 snRNA genes were subject to the same transcriptional controls

as the endogenous U1 genes, one- or two-cell embryos were injected with a chimeric *X. laevis* embryonic U1 gene containing the 5'-flanking region of the xU1b1 gene but the coding region of the xU1b2 gene. Expression of this chimeric "xU1b2" gene in injected embryos should result in an increase in the ratio of newly synthesized xU1b2 to xU1b1 RNAs, which normally is ~1 : 4 in uninjected MBT embryos (Lund et al. 1987; cf. Fig. 1A). When 25 ng of this plasmid DNA was injected, very little xU1b2 RNA accumulated until MBT, at which point synthesis of xU1b2 RNA increased strikingly, as evidenced by the reversal in the ratio of the levels of xU1b2 to xU1b1 RNAs (Fig. 1A, cf. lanes 3–5 and 8–10). When even larger amounts of template DNA were injected (i.e., 50 ng/embryo, or twice the normal DNA content of an MBT embryo), a similar activation of the exogenous snRNA genes occurred at MBT (Fig. 1C, lanes 1–3).

While the level of xU1b2 RNA synthesis at the very early stages of MBT was increased slightly by the extra DNA, in no case was the timing of transcriptional activation of the endogenous snRNA genes altered significantly (Fig. 1A,C, and data not shown). Contrary to the conclusions of previous studies (Newport and Kirschner 1982a,b), these results indicate that the onset of embryonic transcription is independent of the ratio of DNA (or nucleus) to cytoplasm. They also demonstrate that exogenous U1 snRNA genes are subject to the same control(s) as the endogenous and injected rRNA or mRNA genes (Busby and Reeder 1983; Krieg and Melton 1985), transcribed by RNA polymerase I and II. However, transcription of injected plasmid DNA sequences by RNA

Figure 1. Expression of injected *X. laevis* U1 genes in cleaving embryos is developmentally controlled. (A) Exogenous xU1b genes are subject to normal developmental control. Cleaving embryos were injected at the one- to two-cell stage with either [α - 32 P]GTP alone (lanes 1–5) or [α - 32 P]GTP plus 25 ng/embryo of pXU1b (1/1/2) DNA (lanes 6–10) and incubated at 21°C. Total RNAs were prepared from pools of six to eight embryos at different times after fertilization (hours) and analyzed by electrophoresis in a 12% partially denaturing polyacrylamide gel. Each lane contains the RNAs of two embryos; the autoradiogram was exposed for 48 hr without intensifying screen. The major RNA polymerase II (U1–U5 snRNAs) and RNA polymerase III (tRNA, 5S and 7S RNAs) transcripts encoded by endogenous genes are indicated. (●) pBR322-encoded RNA polymerase III transcripts (cf. Newport and Kirschner 1982b). (B) Expression of injected U1 snRNA genes requires protein synthesis during the early cleavage stages. Untreated or DNA-injected embryos, labeled in parallel to those shown in A, were incubated in the presence of cycloheximide (100 μ g/ml), added ~4 hr prior to MBT. The accumulated 32 P-labeled RNAs were analyzed as in A. The 4S and larger mitochondrial transcripts (Mt.) made prior to MBT (see text and Discussion) are indicated by brackets. (C) Transcriptional activation at MBT is independent of ongoing protein and DNA synthesis. Embryos were injected with [α - 32 P]GTP plus 50 ng/embryo of pXU1b (1/1/2) DNA and incubated in the absence (lanes 1–3) or presence (lanes 4,5) of 100 μ g/ml of cycloheximide, added ~100 min prior to MBT. The accumulated 32 P-labeled RNAs were analyzed as in A.

polymerase III occurred prior to MBT (indicated by dots, e.g., Fig. 1A, lanes 6,7), as has been reported previously for injected tRNA and 5S rRNA genes (Newport and Kirschner 1982b; Brown and Schlissel 1985).

Protein synthesis requirement for transcriptional activation of snRNA genes at MBT

As shown in Figure 1B, transcription of exogenous xU1b genes, but not of the pBR322 vector sequences, was almost completely abolished when cycloheximide was added to the embryos several hours prior to MBT (i.e., ~1 hr after DNA injection) (lanes 4–7). Under these conditions, when the cell cycle is blocked and DNA replication is inhibited (Harland and Laskey 1980; Miake-Lye et al. 1983), the injected xU1b genes were replicated only to a limited extent (not shown), although they presumably were assembled into chromatin (Newport and Kirschner 1982b). However, the number of xU1b genes injected was in excess of the number of endogenous genes normally present at MBT, so this low level of transcription could not be accounted for by the lack of templates. Also, snRNA synthesis was not dependent on ongoing DNA replication, as activation of transcription from either injected or endogenous U1 genes was not prevented by addition of cycloheximide (or aphidicolin) to embryos shortly before MBT (Fig. 1C, lanes 4,5; Fig. 7, below, and data not shown). Thus, activation of snRNA transcription at MBT, unlike RNA polymerase III transcription, apparently requires the synthesis of one or more proteins in the early cleavage embryo.

Differential transcription of injected xU1b1 and xU1b2 genes

To determine whether the two exogenous xU1b genes were differentially transcribed at MBT, as are the chromosomal copies (e.g., Fig. 1A, lanes 3–5), embryos were injected with plasmid DNA carrying the full-length xU1b repeat unit, which contains one copy each of the xU1b1 and xU1b2 genes (Krol et al. 1985). As expected, transcription of both of the exogenous xU1b genes was activated at MBT (Fig. 2, cf. lanes 2 and 4). But clearly the synthesis of xU1b1 RNA exceeded that of xU1b2 RNA (lane 2), showing that expression of injected xU1b genes is correctly regulated with respect both to the timing of activation and to the preferential transcription of the xU1b1 gene versus the xU1b2 gene.

Expression of endogenous genes prior to MBT

A longer autoradiographic exposure of the gel shown in Figure 2 revealed two interesting findings about the RNAs synthesized in early-cleaving embryos (Fig. 2, right). First, the pattern of newly made RNAs at the onset of transcription (lane 8) differed significantly from that observed at later times (lane 5; see also Fig. 4, below). Second, small amounts of 4S RNA and several much larger transcripts were made prior to MBT (labeled

Figure 2. Exogenous xU1b1 and xU1b2 genes are transcribed differentially at MBT. Cleaving embryos were injected with either [α - 32 P]GTP plus 25 ng/embryo of pXU1b (111/222) DNA (lanes 1,2) or [α - 32 P]GTP alone (lanes 3–8). The accumulated 32 P-labeled RNAs present in pre-MBT (lanes 1,3,6,7), MBT (lanes 2,4,8), or early gastrula (lane 5) embryos were analyzed as in A. Lanes 7 and 8 represent a longer exposure (24 hr with screen) of lanes 3 and 4 (72 hr without screen), demonstrating the presence of newly made mitochondrial 4S RNAs prior to MBT and as early as 4 $\frac{3}{4}$ hr after fertilization (lane 6) (see also Fig. 1B).

Mt. in Fig. 2, lanes 6,7). Because these pre-MBT transcripts have mobilities identical to those of the RNAs synthesized in unfertilized eggs (in which transcription of nuclear genes is undetectable), they most likely represent mitochondrial RNA transcripts (Lund and Dahlberg 1987). This is consistent with the observation that the same set of RNAs was synthesized prior to MBT in cycloheximide-treated embryos (e.g., Fig. 1B, lane 1) in which the nuclear DNA content was greatly reduced (not shown) owing to the blockage of chromosomal DNA replication (see above).

Specificity of control of snRNA transcription in embryos

As a further test of the specificity of the embryo system, we compared the expression of a wild-type human U1 gene called HHH (Lund and Dahlberg 1984), with that of a chimeric human U1 gene, called HXH, in which the 5' proximal region of the promoter had been substituted with that of the frog xU1b2 gene (Fig. 3A). Consistent with our previous studies (Lund et al. 1987), this chimeric *Xenopus*–human U1 gene was transcribed much

Figure 3. Transcription of injected U1 snRNA genes in developing embryos is species specific. (A) Injected *X. laevis* U1 genes are transcribed much more efficiently than human U1 genes. Cleaving embryos were injected with either [α - 32 P]GTP alone (Cont.) or [α - 32 P]GTP plus 16–18 ng/embryo of pHHH (HHH) or pHXH DNA (HXH), and total 32 P-labeled nucleic acids were isolated at the indicated times after fertilization (hours) and analyzed for RNA as in Fig. 1. Differences in levels of endogenous RNA transcripts are the result of changes in the levels of genomic DNAs (see B). The line drawing shows the structures of the human (HHH) and the chimeric human–*Xenopus* (HXH) U1 genes. (B) Injection of exogenous DNA interferes with the replication of endogenous chromosomal DNA in cleaving embryos. Aliquots of the total 32 P-labeled nucleic acids used in A were treated with DNase-free RNase A and analyzed for DNA by electrophoresis in a 0.8% agarose gel. After staining with ethidium bromide (top), the gel was dried and subjected to autoradiography (bottom). The electrophoretic mobilities of mitochondrial DNA (Mito.), high-molecular-weight chromosomal DNA (Chrom.), and supercoiled (I) and relaxed (II) forms of the plasmid DNAs are indicated. Lanes M contain markers of unlabeled plasmid DNAs. Mitochondrial DNA is unlabeled because it is not replicated at this stage of *Xenopus* development.

more efficiently than was the wild-type human U1 gene (Fig. 3, cf. 10-hr lanes of HXH and HHH), demonstrating that *X. laevis* snRNA transcription factors of early embryos manifest the same species specificity as mature stage VI oocytes.

Reduction of chromosomal DNA accumulation in DNA-injected embryos

From the results presented above (Figs. 1A, 3A, and data not shown), it appeared that the level of endogenous 4–8S transcripts always was lower in DNA-injected embryos than in uninjected control embryos (e.g., Fig. 3A, cf. control and HHH or HXH). To determine whether this decrease reflected changes in the level of chromosomal DNA templates (i.e., gene dosage), we compared the DNA content of these embryos directly by agarose gel electrophoresis (Fig. 3B). Both ethidium bromide staining (top) and incorporation of 32 P into high-molecular-weight DNA (bottom) showed that chromosomal DNA replication was retarded in the DNA-injected embryos. However, because of the presence of exogenous plasmid DNA, the total amount of DNA in injected embryos was comparable to that of control embryos. Thus, injection of relatively large amounts of exogenous DNA (15–50 ng/embryo) interferes with nuclear DNA synthesis, resulting in lower numbers of endogenous genes at the time of MBT and, hence, in a decrease in the level of endogenous RNA synthesis.

Differences in transcription between cleaving and cleavage-arrested embryos at MBT

As shown in Figures 1 and 2, injection of exogenous DNA into dividing embryos did not lead to premature activation of transcription of endogenous genes. This result is contrary to previous studies that utilized cleavage-arrested (coenocytic) embryos (Newport and Kirschner 1982a,b). To determine whether cleaving and cleavage-arrested embryos differed with respect to transcriptional activation, we compared the synthesis of 4–8S RNAs in these two types of embryos. As shown in Figure 4, the pattern of small RNAs transcribed in the cleavage-arrested embryos at the MBT differed significantly from that of normal embryos. In particular, several RNAs (arrows) that were barely detectable in control embryos accumulated in large amounts in embryos whose cleavage had been arrested either by gentle centrifugation (stratified) or by cytochalasin B treatment. Moreover, the levels of newly made snRNA and tRNA transcripts were greatly reduced in the cleavage-arrested embryos (e.g., cf. lanes 4, 7, and 11). The onset of transcription, however, occurred at the same time as in normal embryos (cf. lanes 1 and 2 with lanes 8 and 9; see also Newport and Kirschner 1982a).

The RNAs that accumulated at high levels in the cleavage-arrested embryos were characterized further by RNase T1 fingerprinting (Fig. 5A and data not shown). Neither of the RNAs in the bands labeled 180 or 140 were related to *X. laevis* snRNAs of similar gel mobili-

Figure 4. Cleavage-arrested and cleaving embryos produce distinct patterns of newly synthesized 4–8S RNAs at MBT. Analysis of ^{32}P -labeled 4–8S RNAs synthesized in stratified (lanes 1–4), cytochalasin B-treated (lanes 5–7), or untreated control embryos (lanes 8–11) around the time of MBT. Cleaving and cleavage-arrested embryos were labeled by injection of [α - ^{32}P]GTP and incubated at 24°C (hence MBT occurred earlier than in Fig. 1); total RNAs were isolated at the indicated times (hours) and analyzed as in Fig. 1A. The RNAs of bands 180, 140, and 120 (the approximate length in nucleotides) were further characterized by RNase T1 fingerprinting (Fig. 5 and data not shown).

ties (cf. Fig. 4). Instead, the two bands contained mixtures of closely related but slightly heterogeneous RNAs, whose fingerprints resembled those predicted from the sequences of *X. laevis* OAX DNA (Ackerman 1983) or satellite I DNA (Lam and Carroll 1983; Meyerhof et al. 1983). Comparison of the fingerprints of the RNA in band 180 with that of authentic OAX RNA (Fig. 5C) transcribed by RNA polymerase III from a cloned repeat unit of satellite I DNA (Lam and Carroll 1983) (Fig. 5B) confirmed this assignment. The minor differences between the two fingerprints (indicated by arrows) are consistent with the sequence heterogeneities known to exist between different repeat units of satellite I DNA (cf. Lam and Carroll 1983; Meyerhof et al. 1983) and indicate that multiple OAX transcription units are expressed in the embryos. Thus, the predominant labeled 4.5–6S RNAs in cleavage-arrested embryos are RNA polymerase III transcripts of satellite I DNA rather than the normal complement of snRNAs.

DNA deficiency in cleavage-arrested embryos

Because control of RNA synthesis was demonstrated to be abnormal in coenocytic embryos (Fig. 4), we examined whether chromosomal DNA levels also were altered in such embryos. The DNA contents of stratified and normal embryos were compared directly by the use of aga-

rose gel electrophoresis and ethidium bromide staining. The results of such analyses (Fig. 6 and data not shown) demonstrated that cleavage-arrested embryos were deficient in DNA at least 1 hr (or two cell divisions) prior to MBT and that the level of DNA normally present at MBT (~24 ng/embryo; Newport and Kirschner 1982b) was not reached in coenocytic embryos until several hours after MBT.

Because the activation of global transcription of 4–8S

Figure 5. OAX RNAs encoded by satellite I DNA are the predominant transcripts in cleavage-arrested embryos. (A,C) Two-dimensional RNase T1 fingerprints of band 180 RNA (A) made in cleavage-arrested embryos or authentic OAX RNA (C) made in oocytes injected with pE190 DNA (cf. B). The first dimension (high-voltage electrophoresis at pH 3.5) was from right to left, and the second dimension (homochromatography) was from top to bottom. (5') The 5'-terminal nucleotides (pppGp and ppGp); arrows indicate oligonucleotides that are specific to embryo- or pE190-encoded OAX RNAs, respectively. These sequence heterogeneities between OAX RNAs in A and C are consistent with the variant forms of cloned OAX genes (Lam and Carroll 1983; Meyerhof et al. 1983). The OAX RNA fingerprints shown here differ from the one previously published (Wakefield et al. 1983), which apparently had been made using *X. laevis* 5.8S rRNA by mistake (E. Lund and J.E. Dahlberg, unpubl.). (B) Polyacrylamide gel electrophoresis of ^{32}P -labeled RNAs synthesized in stage-V to -VI oocytes that had been injected with either [α - ^{32}P]GTP alone (–) or [α - ^{32}P]GTP plus 1–2 ng of pE190 DNA per oocyte (+). Each lane contains the 4–8S RNAs of three oocytes; the autoradiograph was exposed for 15 hr without screen.

Figure 6. Accumulation of chromosomal DNA is reduced in cleavage-arrested embryos. Analysis of chromosomal DNA content of cleaving or cleavage-arrested (stratified) embryos at different times (hours) after fertilization. Total nucleic acids (one embryo equivalent per lane) were separated in 0.8% agarose gels and stained with ethidium bromide to allow for direct quantitation of the high-molecular-weight chromosomal DNA present in individual embryos. The uniform (faint staining) band of mitochondrial DNA seen below the band of genomic DNA serves as an internal control for DNA recovery. (▲) The time of MBT.

RNAs occurs at about the normal time in coenocytic embryos (Fig. 4; see also Newport and Kirschner 1982a) in spite of their deficiency in chromosomal DNA content, the timing of transcriptional activation at MBT must be independent of the ratio of nucleus (or DNA) to cytoplasm. Moreover, the abnormal pattern of newly synthesized RNAs seen in cleavage-arrested embryos can be attributed to two factors: (1) the lower copy number of genes for snRNAs and tRNAs, and (2) the prolonged synthesis of OAX RNA (see below).

Normal repression of satellite I DNA transcription in early embryos

A closer examination of the RNAs synthesized at the onset of transcription in normally cleaving embryos also indicated the presence of small amounts of OAX RNA (Fig. 4, lanes 8,9; Fig. 2, lane 8). However, very little of this unstable RNA remained at 7 hr (Fig. 4, lane 10), indicating that transcription of satellite I DNA occurs only transiently, early in MBT. As predicted, the synthesis of these early 5–6S transcripts (plus tRNA and 7S RNA) was inhibited by tagetitoxin, a specific inhibitor of RNA polymerase III transcription (Steinberg et al. 1990) but not by low concentrations of α -amanitin, which inhibits only RNA polymerase II transcription (data not shown).

In cleavage-arrested embryos, OAX RNA continued to accumulate for several hours after MBT (Fig. 4, lanes 3,6). This is consistent with previous pulse-labeling experiments (Newport and Kirschner 1982a) demonstrating that these transcripts are synthesized at a high rate for at least 2–3 hr after MBT. Thus, transcription of sat-

ellite I DNA appears to cease only when the level of DNA attained is comparable to that normally present at MBT (cf. Fig. 6). Unexpectedly, OAX synthesis also was inactivated when protein synthesis (and, hence, DNA replication) was inhibited by addition of cycloheximide shortly before MBT (Fig. 7, cf. lanes 1 and 2 with 5 and 6). Likewise, OAX RNA accumulation was significantly reduced when the total level of DNA was increased by injection of a relatively large amount of unrelated plasmid DNA (50 ng of pXIU1b DNA/embryo) into early embryos (lanes 3,4). Transcription of satellite I DNA, therefore, may be selectively repressed when the rate of chromosomal DNA replication is reduced artificially, either by cycloheximide treatment (Harland and Laskey 1980; Miake-Lye et al. 1983) or by increasing DNA levels through DNA injection.

Discussion

Control of transcriptional activation at MBT

The results presented here show that the timing of transcriptional activation at the MBT of *Xenopus* embryo-

Figure 7. Transcription of OAX RNA depends on ongoing protein synthesis. Analysis of ^{32}P -labeled RNAs made in cleavage-arrested (stratified) embryos that were either left untreated (lanes 1,2), injected with 50 ng/embryo of pXIU1b (1/1/2) DNA at ~3 hr after fertilization (DNA; lanes 3,4), or treated with cycloheximide (100 $\mu\text{g}/\text{ml}$ of medium) starting ~1 hr prior to MBT (Cyclo; lanes 5,6). Cleavage-arrested and control cleaving embryos (lane 7) were labeled by injection with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$, and the accumulated RNAs were analyzed at different times after fertilization (hours), as in Fig. 1. Arrowheads denote major OAX transcripts; (●) RNA polymerase III transcripts encoded by pBR322 vector sequences in the pXIU1b DNA.

genesis is independent of the ratio of nucleus to cytoplasm per se. For example, injection of a large amount of DNA did not promote premature expression of either exogenous or endogenous snRNA genes in cleaving embryos (Figs. 1 and 2). Moreover, the onset of transcription of 4–8S RNAs was not delayed in coenocytic embryos (Fig. 5; Newport and Kirschner 1982a), despite the greatly reduced level of chromosomal DNA present at MBT (Fig. 6). Likewise, activation of rRNA transcription occurs normally in such DNA-deficient embryos (Takeichi et al. 1985). Thus, we conclude that control of RNA synthesis is not mediated solely by DNA titration of repressor-like molecules. Instead, we favor a model in which initiation of transcription is triggered by events associated with the autonomous cell cycle oscillator, which operates (in the cytoplasm) independently of the nucleus during the early cleavage stages (Hara et al. 1980; Newport and Kirschner 1984; Kimelman et al. 1987; Dabauvalle et al. 1988). This model is also supported by the observations that both snRNA (Lund et al. 1987) and mRNA genes (Bendig and Williams 1984) injected into unfertilized eggs remain transcriptionally inactive until 6–8 hr after the eggs are parthenogenetically activated by the DNA injection, that is, until the time of MBT in developing embryos.

Requirement for early protein synthesis in transcriptional activation of snRNA genes

Protein synthesis is required early for the establishment of competence to transcribe injected snRNA genes, both in developing embryos (Fig. 1B) and in activated unfertilized eggs (Lund et al. 1987). In contrast, transcription of exogenous RNA polymerase III genes, such as tRNA (Newport and Kirschner 1982b) and 5S rRNA genes (Brown and Schlissel 1985), or sequences in the pBR322 vector DNA (Figs. 1 and 2) are unaffected by early inhibition of protein synthesis. Unfortunately, it is not possible to determine whether expression of endogenous genes is similarly affected because cycloheximide treatment indirectly blocks chromosomal DNA replication (Harland and Laskey 1980; Miake-Lye et al. 1983) and the consequent low gene copy number makes it is very difficult to detect the transcripts. The requirement for early protein synthesis thus may be specific to snRNA genes (and, perhaps, other RNA polymerase II genes), and could indicate a need to synthesize or modify transcription factors or a need to “activate” chromatin by several rounds of DNA replication. Similarly, early protein synthesis is required for zygotic transcription during *Drosophila* development (Edgar and Schubiger 1986), and an early period of DNA synthesis is required for the subsequent expression of certain mRNA genes in *Caenorhabditis elegans* embryos (Edgar and McGhee 1988).

Transcription prior to MBT in developing embryos

When translation is not inhibited until shortly before MBT, activation of both tRNA and snRNA transcription does occur (Figs. 1C and 7). While the lack of protein

synthesis at this later stage reduces the level of endogenous RNA synthesis, owing to the lower copy number of templates, it apparently has little or no effect on the timing of transcriptional activation (Fig. 1). Likewise, the low level of U1 transcription that can be detected during early inhibition of protein synthesis appears to be correctly timed (Fig. 1B). These results are inconsistent with the conclusion of Kimelman et al. (1987) that similar cycloheximide treatment leads to premature onset of tRNA synthesis. However, the source of the tRNA-sized molecules was not determined in that study, and, as demonstrated here, several size classes of mitochondrial RNA transcripts, including 4S RNAs, are synthesized prior to MBT both in the absence (Fig. 1B) and presence of protein synthesis (Fig. 2; see also Fig. 1 of Forbes et al. 1983a). Thus, 4S mitochondrial RNAs, rather than transcripts from prematurely activated nuclear tRNA genes, could account for the low level of tRNA-sized transcripts detected by Kimelman et al. (1987) in pre-MBT embryos.

Aberrant nucleic acid biosynthesis in cleavage-arrested embryos

The disparity between our results and those of previous studies is principally due to the types of assays used to examine DNA and RNA synthesis. We compared directly the level of accumulated DNA in cleaving and coenocytic embryos at various times after fertilization using DNA stains (Figs. 3B and 6 and data not shown) and did not rely on incorporation of labeled nucleotide precursors of unknown specific activity (Newport and Kirschner 1982a). Furthermore, we characterized the accumulated RNAs by RNase T1 fingerprinting, in addition to polyacrylamide gel electrophoresis (Fig. 5 and data not shown).

DNA accumulation in cleavage-arrested embryos was clearly retarded (Fig. 6; see also Takeichi et al. 1985). Perhaps this deficiency is related to the generation of large polyploid nuclei during the syncytial nuclear divisions in these cells (Newport and Kirschner 1982a, 1984). The resulting low level of DNA at MBT has two striking effects on accumulation of RNA (Fig. 4). First, the reduced copy numbers of templates for snRNA and tRNA cause abnormally low levels of these endogenous RNAs to accumulate. Second, most of the newly synthesized 4.5–6S RNAs in coenocytic embryos are not normal cellular RNAs, but are OAX RNAs—a collection of RNA polymerase III transcripts encoded by the highly repetitive satellite I DNA sequences (Fig. 5; Ackerman 1983; Lam and Carroll 1983; Wakefield et al. 1983). This aberrant expression of satellite I DNA might stem from lack of competition for transcription factors by other RNA polymerase III genes (Andrews et al. 1984) or from an extended period of relatively short cell cycles that could persist in coenocytic embryos owing to the abnormally low DNA content (cf. Newport et al. 1985). Thus, interpretations of results obtained using cleavage-arrested embryos are complicated by a variety of artifacts that obscure the normal events of transcriptional activation of tRNA and snRNA genes.

Expression of satellite I DNA during early embryogenesis

In cleaving embryos, synthesis of OAX RNA occurs only transiently, at the onset of zygotic transcription, and ceases when the cell cycle and DNA synthesis slow down after MBT (Fig. 4; see also Wolffe 1989; Andrews et al. 1991; Cohen and Reynolds 1991). Hence, the strong curtailment of OAX transcription following inhibition of protein synthesis (Fig. 7) might be the result of inactivation of the OAX genes when rapid DNA replication is prevented (cf. Wolffe and Brown 1988).

Previously, the synthesis of OAX RNA has been observed only in oocytes injected with somatic cell nuclei (Wakefield et al. 1983) and in uninjected oocytes (Ackerman 1983; Jämrich et al. 1983; Lund and Dahlberg 1989) but not in normal somatic cells (Lam and Carroll 1983). Comparison of OAX and tRNA gene sequences suggests that an ancestral OAX gene could have arisen by fusion of two tRNA genes (D. Carroll, pers. comm.). Because OAX genes are much less abundant in other strains of *Xenopus* (Lam and Carroll 1983), it seems unlikely that OAX RNA per se is essential for normal *X. laevis* development; however, the act of OAX transcription may still be important for control of RNA polymerase III activity.

Transcriptional control of injected U1 genes in cleaving embryos

Cleaving embryos injected at the one- to two-cell stage with cloned snRNA genes represents a promising experimental system for the study of developmental control of snRNA transcription. The benefits of this system contrast with those of cleavage-arrested embryos (see above) or stage-VI oocytes. As we have shown earlier (Lund et al. 1987), transcription of injected snRNA genes in oocytes is both efficient and accurate but essentially uncontrolled (except by competition for transcription factors that become limiting when DNA templates are in excess).

After injection into cleaving embryos, *X. laevis* U1 RNA genes, like genes encoding mRNA (e.g., Krieg and Melton 1985) and rRNA (Busby and Reeder 1983), are subject to the same developmental controls as the endogenous genes, remaining quiescent until MBT (Fig. 1). This contrasts with the behavior of injected 5S rRNA genes (Brown and Schlissel 1985) and tRNA genes (Newport and Kirschner 1982b), which, unlike the chromosomal genes, are transcribed prior to MBT. Upon activation, exogenous xU1b1 and xU1b2 genes are transcribed with the same relative efficiency as their endogenous counterparts (Fig. 2). However, human U1 genes are transcribed very poorly at MBT (Fig. 3), presumably because these heterologous genes lack one or more specific transcription signals (Dahlberg and Lund 1988) required for efficient expression in *X. laevis* cells (Lund et al. 1987). This differential expression of the two types of U1 genes should be useful in the identification of *cis*-acting control elements and the isolation of specific snRNA transcription factors.

Materials and methods*Plasmids*

The plasmids used were pXIU1b (1/1/1 : 2/2/2), which contains one copy of the full-length repeat of *X. laevis* embryonic U1 genes (Krol et al. 1985; Lund et al. 1987); pXIU1b (1/1/2), which contains a chimeric *X. laevis* U1 gene with the xU1b1 promoter region fused to the xU1b2-coding region (Lund et al. 1987); pHU1-1 (dl-231) (here referred to as pHHH), which contains a human U1 gene with 231 bp of 5'-flanking region sequences (Skuzeski et al. 1984); and pHHH, which contains the enhancer and coding region of the human U1 gene (as in pHHH), but the PSE region of the xU1b2 gene. pHHH was constructed by substitution of the sequences between positions -203 and -4 of the human U1 gene with the comparable sequences (positions -219 to -4) of the xU1b2 gene (M. Schimerlik and E. Lund, unpubl.).

Preparation and injection of embryos

Adult female and male *X. laevis* were purchased from Xenopus I (Ann Arbor, MI), kept at 18°C on a 12 hr/12 hr day/night cycle and fed beef liver two to three times a week. To induce egg laying, frogs were preinjected with 100 units of pregnant mare serum gonadotropin (Sigma), followed 3–7 days later by 600–800 units of human chorionic gonadotropin (Sigma) (Newport and Kirschner 1982a). Fifteen to eighteen hours thereafter eggs were collected over a 10- to 30-min period into MMR (0.1 M NaCl, 2 mM KCl, 1 mM MgSO₄, 2.0 mM CaCl₂, 5.0 mM HEPES, 0.1 mM EDTA, pH 7.8) containing 100 µg/ml of penicillin and 50 µl/ml of streptomycin. To generate synchronized embryos, the eggs were then fertilized (in H₂O) by rubbing with a bisected testis (*X. laevis*) and subsequently dejellied by treatment with 2% cysteine-HCl (adjusted to pH 7.8) as detailed by Newport and Kirschner (1982a). Cleavage-arrested embryos were obtained either by centrifugation onto a cushion of 50% Ficoll (type 400, Sigma) in MMR for 10 min at 500g (Newport and Kirschner 1982b) or by incubation with cytochalasin B (Sigma) at 5 µl/ml in MMR, 5% Ficoll (Newport and Kirschner 1982a).

Fertilized, dejellied embryos (kept at 20–24°C in MMR, 5% Ficoll) were injected at the one- to two-cell stage with 20–40 nl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA at pH 7.6) containing 1.0 µCi of [α -³²P]GTP (400 Ci/mmol, Amersham) plus 15–50 ng of U1 plasmid DNA (as specified in the figure legends). Control embryos received only [α -³²P]GTP. In some cases, injected embryos were transferred into 1/10× MMR, 5% Ficoll at 4–5 hr after fertilization. To block protein synthesis, cycloheximide (Sigma) was added to the medium at 100 µl/ml. To inhibit transcription by RNA polymerase II or III, two- to four-cell embryos were injected with 40–50 nl of α -amanitin or tagetitoxin solutions to give final intracellular concentrations of 2 µg/ml or 30 µM, respectively.

Injection of oocytes

To produce authentic ³²P-labeled OAX RNA, stage-V to -VI oocytes were coinjected with 0.5 µCi of [α -³²P]GTP and 1–2 ng of pE190 plasmid DNA (Lam and Carroll 1983), which contains one copy of the 750-bp *Hind*III repeat unit of satellite I DNA. After incubation at 18°C for 16–18 hr, total RNA was isolated (Lund et al. 1987) and analyzed as described below.

Analyses of RNA and DNA synthesis

To monitor RNA synthesis, ³²P-labeled embryos were collected (three to eight embryos per time point) and homogenized in

proteinase K buffer [0.1 ml/embryo: 50 mM Tris-HCl, 10 mM EDTA, 0.8 µg/ml of Proteinase K (Boehringer Mannheim), 1% SDS at pH 7.6]. After incubation at 37°C for 2–4 hr, total nucleic acids were prepared by extraction with phenol-chloroform-isoamylalcohol (24 : 24 : 1) and precipitation with ethanol. The ³²P-labeled transcripts were analyzed by electrophoresis in partially denaturing gels containing 12% (30 : 0.8) polyacrylamide, 7 M urea, and 1× TEB (85 mM Tris-borate, 2 mM EDTA at pH 8.3), as described previously (Krol et al. 1985; Lund et al. 1987). Autoradiography of the wet gels was for 20–96 hr without, or for 24–48 hr with, intensifying screen as indicated in the figure legends.

For further analyses, individual RNA species were eluted from the gels, digested with RNase T1, and subjected to two-dimensional fingerprinting (Barrell 1971), using homomix C and polyethyleneimine (PEI) thin-layer plates for the second dimension (Lund et al. 1984, 1987).

To follow DNA synthesis, total nucleic acids were prepared from unlabeled or ³²P-labeled embryos, and the accumulated DNA molecules were analyzed by electrophoresis in 0.8% agarose gels. After electrophoresis, the gels were stained with ethidium bromide, and DNA was quantified by visualization under UV light. In the case of ³²P-labeled samples, the gels were then dried and subjected to autoradiography for 12 hr without intensifying screens.

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