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In vitro synthesis of vertebrate U1 snRNA

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We have developed a DNA-dependent *in vitro* transcription system for vertebrate snRNA genes. By isolating the nuclei (germinal vesicles, GVs) of *Xenopus laevis* oocytes under oil to maintain the *in vivo* composition of their internal milieu, we are able to prepare nuclei that retain their ability to synthesize snRNAs efficiently. Homogenates of these GVs synthesize correctly initiated and terminated U1 snRNA using exogenous *X.laevis* U1 genes as templates. The templates may be either injected into the nucleus prior to its isolation or added to the nuclear homogenate.

Key words: in vitro snRNA synthesis/germinal vesicles/U1 snRNA transcription/isolated nucleus

Introduction

The synthesis of vertebrate snRNAs is an extremely efficient process *in vivo* with RNA polymerase II initiating transcription about 20–25 times per minute on each snRNA gene (reviewed by Dahlberg and Lund, 1988). In contrast, in isolated nuclei of mammalian cells, run-off snRNA transcripts are the predominant products and newly initiated snRNAs are barely detectable (Kunkel and Pederson, 1985; Lobo and Marzluff, 1987). Furthermore, the commonly used RNA polymerase II *in vitro* transcription systems derived from mammalian cells (Manley *et al.*, 1979; Dignam *et al.*, 1983) are incapable of synthesizing correctly initiated U1 or U2 snRNA transcripts (Murphy *et al.*, 1982; Westin *et al.*, 1984; our unpublished results).

Recently, a DNA-dependent *in vitro* transcription system was developed for sea urchin U1 RNA; this nuclear extract, derived from sea urchin blastulae, can use only homologous, invertebrate, snRNA genes as templates (Morris *et al.*, 1986). Conversely, neither sea urchin (Strub and Birnstiel, 1986; Birnstiel and Schaufele, 1988) nor *Drosophila* snRNA genes can be transcribed in vertebrate cells such as *Xenopus* oocytes (Saba *et al.*, 1986). This specificity of snRNA transcription presumably reflects significant functional differences between the promoters of invertebrate and vertebrate snRNA genes (Dahlberg and Lund, 1988). Thus, to date there has been no report of an *in vitro* transcription system capable of transcribing exogenous vertebrate snRNA genes.

The lack of *in vitro* assays led us and others to use microinjected *Xenopus laevis* oocytes to analyze vertebrate snRNA gene transcription (e.g. Murphy *et al.*, 1982; Mattaj and Zeller, 1983; Westin *et al.*, 1984; Ares *et al.*, 1985; Hoffmann *et al.*, 1986). This *in vivo* transcription system allowed the definition of regions of the snRNA promoter needed for efficient transcription (Skuzeski *et al.*, 1984; Mattaj *et al.*, 1985; Bark *et al.*, 1986; Murphy *et al.*, 1987; see also Dahlberg and Lund, 1988 and references therein) and established requirements for snRNA 3′ end formation (Yuo *et al.*, 1985; Neuman de Vegvar *et al.*, 1986; Ciliberto *et al.*, 1986). The oocyte's large stockpiles of both RNA polymerase II (Roeder, 1974) and snRNA-specific transcription factors allows for a remarkably high rate of transcription of injected, exogenous snRNA genes (Lund *et al.*, 1987).

We have now taken advantage of the efficiency and ease of manipulation of *X.laevis* oocyte nuclei, or germinal vesicles (GVs), to develop an *in vitro* transcription system for vertebrate snRNA genes. As reported here, manually isolated GVs maintain their high capacity and precision for snRNA synthesis *in vitro*, provided they are isolated under oil—a condition which prevents changes in the internal nuclear composition (Paine, 1987; Paine *et al.*, in preparation). Moreover, we demonstrate that *X.laevis* U1 RNA genes are accurately transcribed when added *in vitro* to a homogenate of such 'native' GVs. This report is the first demonstration of DNA-dependent *in vitro* synthesis of vertebrate U1 snRNA.

Results

In vitro transcription of pre-injected snRNA genes in isolated GVs

We previously showed (Lund *et al.*, 1987) that the rate of transcription of homologous U1 snRNA genes (by RNA polymerase II) micro-injected into *X.laevis* stage VI oocytes is comparable to that of genes transcribed by RNA polymerase III, such as 5S rRNA genes or tRNA genes (Gurdon and Brown, 1978; Gurdon and Melton, 1981). However, when GVs were pre-injected *in vivo* with *X.laevis* U1 genes and subsequently isolated in an aqueous medium under conditions that preserve high levels of RNA polymerase III activity (Birkenmeier *et al.*, 1978) no snRNA synthesis was detected (Figure 1A). As an internal control to demonstrate that the isolated nuclei had been correctly injected, a somatic 5S rRNA maxi-gene (Wormington *et al.*, 1981), co-injected with the embryonic U1 genes (Lund *et al.*, 1984; 1987), continued to be actively transcribed.

Negative results were also obtained using a variety of aqueous media (cf. Materials and methods), indicating that GVs isolated by standard methods lose their ability to transcribe snRNA genes efficiently. This is also the case with aqueously isolated nuclei from cultured mammalian cells (Kunkel and Pederson, 1985; Lobo and Marzluff, 1987; our unpublished results).

To prevent the loss of snRNA gene transcription activity, we sought isolation conditions more likely to preserve the native state of the oocyte nucleus. Recently it has been demonstrated that while GVs isolated in aqueous buffer lose

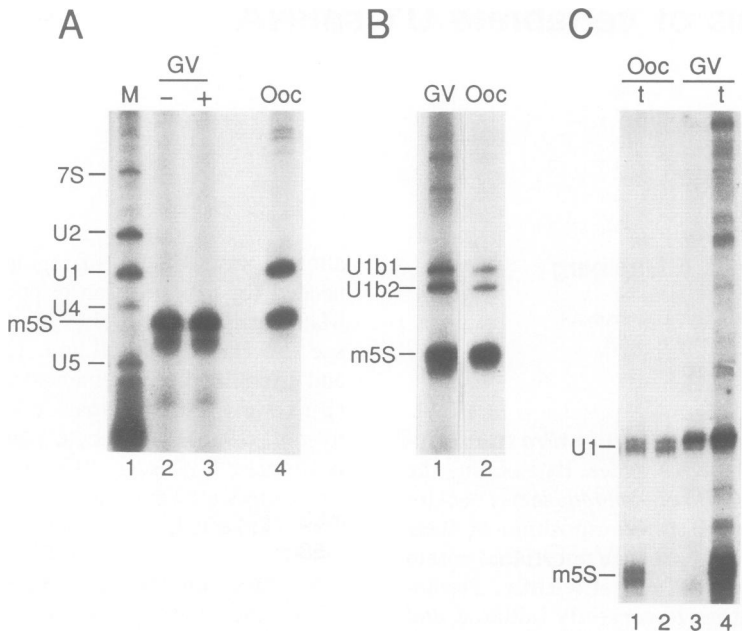

Fig. 1. *In vitro* synthesis of U1 RNA in isolated GVs. (**A**) Analysis of the newly synthesized RNAs made by pre-injected GVs (GV) isolated in I-buffer, in the absence of (−) (**lane 2**) or presence (+) (**lane 3**) of 2 μg/ml α-amanitin. Electrophoresis was in a denaturing 10% (38.9:1.1) polyacrylamide gel and the markers were *in vivo*-labeled 4−8S RNAs made by *X.laevis* blastula embryos (M) (**lane 1**) or by pre-injected intact oocytes (Ooc) (**lane 4**). (**B**) Analysis of the RNAs made by pre-injected GVs that were isolated and incubated under oil (GV) (**lane 1**) or by pre-injected intact oocytes that were pulse-labeled for 2 h (Ooc) (**lane 2**). Total RNAs equivalent to 11 GVs or three oocytes, respectively, were electrophoresed in a partially denaturing 12% (38.9:1.1) polyacrylamide gel, which separates the embryonic U1b1 and U1b2 RNAs. (**C**) Analysis of hybrid-selected U1 RNA transcripts (**lanes 2** and **3**) and total RNAs (t) (**lanes 1** and **4**) made by pre-injected oil-isolated GVs (**lanes 3** and **4**) or by intact oocytes (**lanes 1** and **2**). Electrophoresis was in a 8% (30:1) sequencing gel which separates the longer 3′ extended pre-U1 RNAs from the mature U1 RNA (compare **lanes 2** and **3**). *X.laevis* stage V−VI oocytes were pre-injected into the nucleus with a mixture of 4 ng of U1 DNA pX1U1b and 0.1 ng of maxi-5S (m5S) DNA (pXbs+20) and GVs were isolated by manual dissection (cf. Materials and methods). RNA synthesis in isolated GVs or intact oocytes was monitored by incorporation of [α-^{32}P]GTP (added after GV isolation). The autoradiograms were exposed for 48 (**A**), 15 (**B**) and 90 h (**C**) without intensifying screens.

more than 50% of their protein content within 5−10 min after dissection (Paine *et al.*, 1983), GVs isolated under oil retain many *in vivo* characteristics, including transcriptional activity; presumably this is because they do not experience diffusive loss of proteins and small solutes (Paine, 1987; Paine *et al.*, in preparation).

We found that GVs isolated by the oil method from oocytes pre-injected with the U1 genes do indeed actively synthesize U1 RNA (Figure 1B, lane 1). Analysis of hybrid-selected U1 transcripts (Figure 1C) confirmed that most of the newly synthesized U1 transcripts are the size of pre-U1 RNAs which have unprocessed 3′ extended ends (Eliceiri and Sayavedra, 1976). This lack of 3′ end trimming was expected for U1 RNAs made in the absence of cytoplasm (Eliceiri, 1980; Madore *et al.*, 1984; H.Neuman de Vegvar and J.E.Dahlberg, in preparation). The two bands of xU1b RNA in Figure 1B represent transcripts of the two different xU1b genes in the template (Krol *et al.*, 1985; Lund *et al.*, 1987); the fidelity of the GV system is reflected in the fact that the ratio of U1b1 and U1b2 transcripts was the same as in intact oocytes (Figure 1B, lane 2).

Digestion of the gel-purified U1 RNAs with RNase T2 (Figure 2A) demonstrated that these pre-U1 RNAs contained a radiolabeled 5′ cap-structure, indicating that most, if not all, of the *in vitro* made U1 RNAs represented *de novo* initiated transcripts rather than run-off products. However, their cap-structure differed from the $m_3^{2,2,7}$ G-cap of mature U1 RNAs made in intact oocytes (compare upper and lower panels). Direct analysis of the 5′ terminal nucleotides by digestion with tobacco acid pyrophosphatase (TAP) (Figure 2B) confirmed that U1 RNA made in isolated GVs contained an m_1^7G-cap, like that present on the nascent (nuclear) U1 RNA made *in vivo* (Skuzeski *et al.*, 1984; Mattaj, 1986; E.Lund, unpublished results).

We conclude that GVs which are isolated under conditions designed to keep them intact constitute a highly efficient and accurate *in vitro* system for U1 synthesis.

U1 RNA synthesis in disrupted pre-injected GVs

To determine if *in vitro* snRNA synthesis requires undisturbed nuclear architecture and/or maintenance of the *in vivo* ionic concentrations of GVs, GVs isolated under oil from pre-injected oocytes were incubated with or without manual disruption and in the absence or presence of an ~14-fold excess volume of transcription buffer. Neither disruption nor dilution alone abolished snRNA transcription (Figure 3A), but each treatment resulted in a significant decrease in the level of transcription of U1 RNA relative to that of maxi-5S RNA (e.g. cf. lanes 1 and 3, and 2 and 4). It is unclear whether this alteration in the ratio of transcripts resulted only from an increase in 5S RNA synthesis (see Discussion) or also from a decrease in U1 RNA synthesis, since the absolute amounts of the two types of transcripts made under the different conditions could not be compared directly (due to variations in the specific activities of the [^{32}P]GTP precursor).

When the oil-isolated GVs were disrupted in transcription buffer, RNA polymerase III transcription of vector

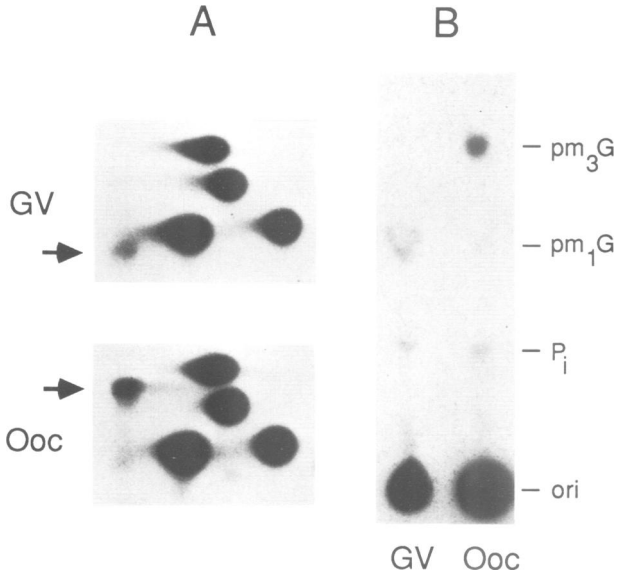

Fig. 2. Analyses of the 5′ cap-structure. U1 RNAs synthesized *in vitro* by oil-isolated GVs (GV) or *in vivo* by intact oocytes (Ooc) were gel-purified as in Figure 1B, and treated with RNase T2 (**A**) or tobacco acid pyrophosphatase (**B**) and the digests were analyzed by thin-layer chromatography. (**A**) arrows indicate the positions of the unique RNase T2-resistant cap-structures among the four common 3′ ribonucleoside monophosphates; (**B**) the migration positions of P_i, $pm_1{}^7G$ and $pm_3{}^{2,2,7}G$ are indicated. The autoradiograms were exposed for 6 (**A**) and 14 (**B**) days with intensifying screens.

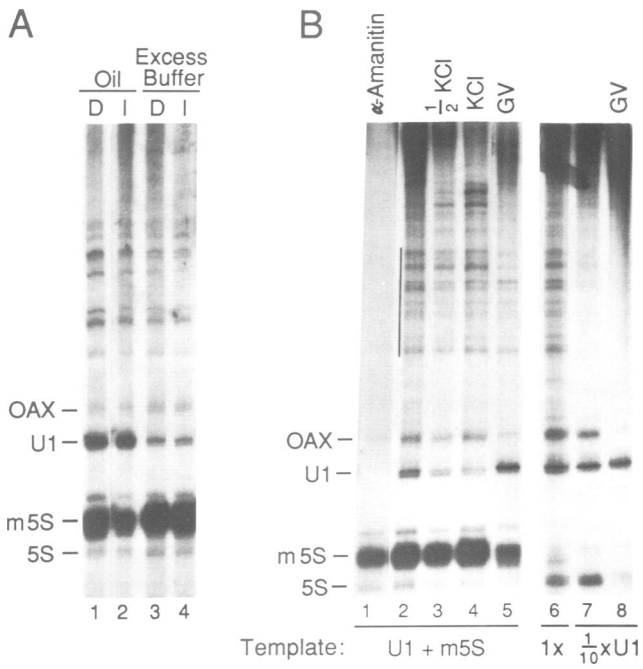

Fig. 3. *In vitro* synthesis of U1 RNA transcripts in homogenates of pre-injected GVs. (**A**) Comparison of the RNAs made *in vitro* by the equivalent of five pre-injected GVs that were either kept intact (I) or disrupted (D) in the absence (Oil) or presence of an ~14-fold excess of transcription buffer (relative to the GV-volume). (**B**) Analysis of the RNAs made by the equivalent of three intact GVs (**lanes 5** and **8**) or three homogenized GVs (**lanes 1–4, 6** and **7**), which had been pre-injected either with a mixture of 4 ng of U1 DNA and 0.1 ng of maxi-5S DNA (U1+m5S), or with 4 ng (1×) or 0.4 ng (1/10 × U1) of U1 DNA alone. The GV-homogenates contained a 5-fold excess of standard GV transcription buffer either without (**lanes 2, 6** and **7**) or with (**lane 1**) 2 μg/ml of α-amanitin, or modified transcription buffer with 50 mM each of KCl and KOAc (1/2 KCl) (**lane 3**) or 100 mM KCl (KCl) (**lane 4**). Electrophoresis was in 10% (38.9:1.1) sequencing gels, and the autoradiograms were exposed for 20 (**A**) and 30 h (**B**) without screens. The mobilities of endogenous *X.laevis* 5S and OAX RNAs are indicated.

sequences in the U1 plasmid DNA (i.e. the 6–8S RNAs indicated by the vertical line in Figure 3B, lane 2) increased in parallel with transcription of the injected maxi-5S DNA and the endogenous 5S and OAX genes (e.g. compare lanes 2 and 5). This background transcription could be eliminated almost completely without a comparable loss of U1 synthesis by decreasing the amount of pre-injected U1 template DNA (compare lanes 6 and 7). As expected, U1 synthesis was abolished in the presence of low levels of α-amanitan (lane 1), confirming that transcription was catalyzed by an RNA polymerase II like activity. Although a slight (2–3-fold) reduction in the level of U1 synthesis resulted from substitution of potassium chloride for potassium acetate in the transcription buffer (lanes 2–4), this vertebrate U1 transcription is much less sensitive to inhibition by chloride ions than is RNA polymerase II transcription in aqueous extracts of yeast nuclei (Lue and Kornberg, 1987).

We conclude that oil-isolated *X.laevis* GVs utilizing preformed U1 snRNA transcription complexes retain their capacity for efficient and accurate U1 synthesis, and that this activity is stable to physical disruption of the nuclei in excess transcription buffer.

Transcription of exogenously added U1 genes in GV homogenates

We tested homogenized oil-isolated GVs for faithful transcription of U1 genes that were added after nuclear isolation, rather than by injection prior to GV isolation. In the absence of added DNA, the homogenized GVs, like intact GVs, synthesize only small amounts of endogenous 5S and OAX RNAs (Figure 4A, lane 2). Introduction of DNA containing only the *X.laevis* xU1b1 gene resulted in a complex mixture of transcripts (lanes 3–5), most, but not all, of which were probably synthesized by RNA polymerase III (lanes 7 and 8). Hybrid-selection of these *in vitro* made transcripts revealed small amounts of U1-sized RNAs in addition to several other longer U1 transcripts (Figure 4B, lanes 2 and 3) that hybridized specifically to the template strand of the U1 coding region sequences (cf. lanes 3 and 5). Likewise, precipitation with an m^7G-cap-specific antibody (Munns *et al.*, 1982) demonstrated the synthesis of U1-sized transcripts containing m^7G-caps (Figure 4C, lane 3).

The synthesis of U1-sized RNAs in GV homogenates was inhibited by low levels of α-amanitin (cf. lanes 3 and 4 in Figures 4B and C) and was dependent on the addition of template DNA (e.g. Figure 4C, lane 5), indicating that the transcripts were pre-U1 RNAs transcribed by RNA polymerase II and encoded by the exogenously added U1 gene templates. (The indicated transcripts seen both in the absence and presence of α-amanitin in Figure 4C resulted from non-specific binding of the abundant RNA polymerase III transcripts to the immunoadsorbent; cf. legend to Figure 4). As expected, the GV homogenate transcribed RNA polymerase III genes like the *Xenopus* maxi-5S or OAX genes (Figure 4A, lane 1 and data not shown) or a mouse U6 snRNA gene (lanes 7 and 8) very accurately and efficiently (cf. legend to Figure 4A).

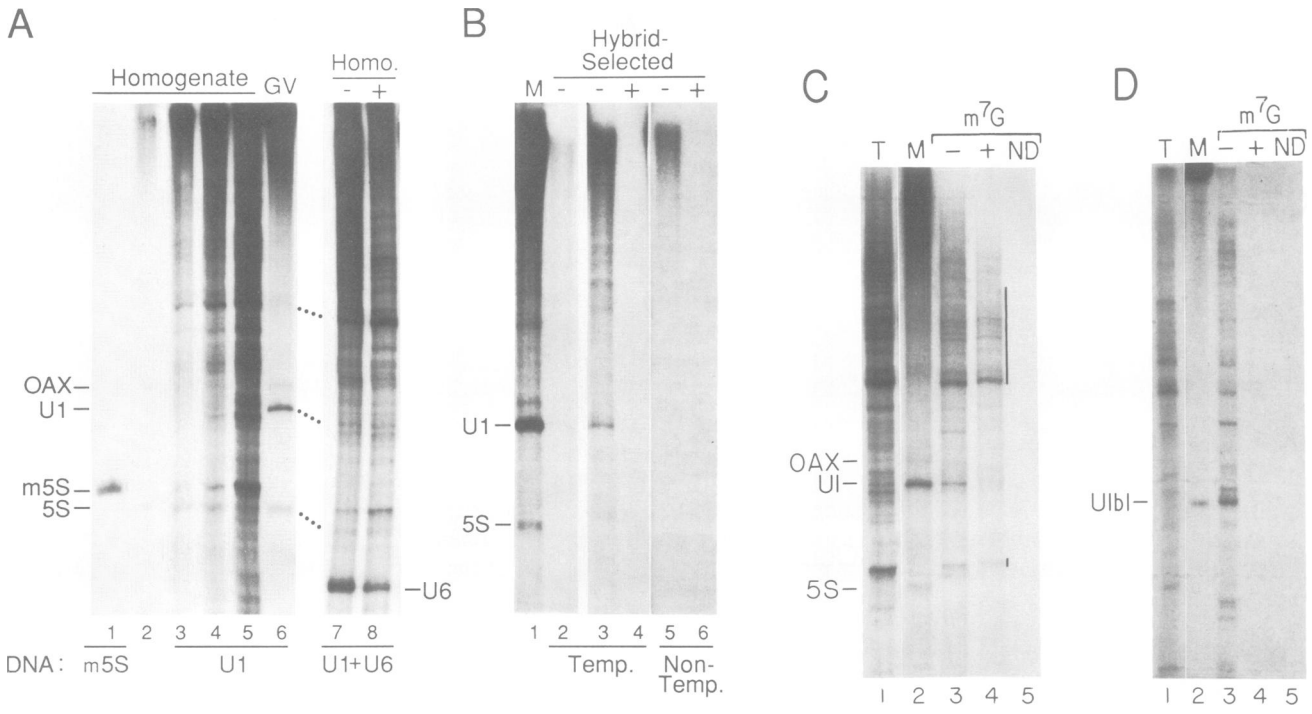

Fig. 4. DNA-dependent *in vitro* synthesis of U1 RNA in GV homogenates. (**A**) Analyses of the transcripts made by GV homogenates after *in vitro* addition of 0.7 ng of maxi-5S DNA (**lane 1**), no DNA (**lane 2**), 9 ng (**lane 3**), 18 ng (**lane 4**) or 45 ng (**lane 5**) of U1 DNA (pX1U1b1) or a mixture of 9 ng of U1 DNA and 0.9 ng U6 DNA (pmU6) (**lanes 7** and **8**) per GV-equivalent; transcription was in the absence (−) (**lanes 1−7**) or presence (+) (**lane 8**) of 2 mg/ml α-amanitin. A marker of U1 RNA made by homogenized, pre-injected GVs (GV) is shown in **lane 6**. Total RNAs made by the equivalent of three GVs were electrophoresed as in Figure 3, except the samples in **lanes 1−6** and **lanes 7** and **8** were run in separate gels. The autoradiograms were exposed for 0.5 (**lane 1**) or 40 h (**lanes 2−8**) without screens. (**B**) Analysis of the transcripts prepared by selective hybridization to single-stranded DNA probes containing either the template (Temp.) or non-template (Non-Temp.) of a human U1 gene coding region. The selected RNAs made by the equivalent of 12 GVs [as in (A), lane 3] in the absence (−) (**lanes 2, 3** and **5**) or presence (+) (**lanes 4** and **6**) of 2 μg/ml α-amanitin; **lanes 2** and **3** are the same sample. The U1 marker (**lane 1**) corresponds to the sample in lane 6 of (A); the autoradiograms were exposed for 16 h (**lanes 1** and **2**) or 4 days (**lanes 3−6**) with intensifying screens. (**C**) Analysis of the transcripts prepared by precipitation with an m^7-G-cap-specific antibody using total RNA made by the equivalent of 10 GVs in the absence (−) (**lane 3**) or presence (+) (**lane 4**) of 2 μg/ml α-amanitin, or without added U1 template DNA (ND) (**lane 5**). RNAs equivalent to the total in 0.5 GVs (T) (**lane 1**) and the U1 marker (M) (**lane 2**) are comparable to the samples in lanes 5 and 6 of (**A**), respectively. The vertical lines next to lane 4 indicate the background of non-specifically bound RNA polymerase III transcripts in this particular experiment (compare with lane 4 of [**D**]). The autoradiograms were exposed for 16 h (**lane 1**) or 12 days (**lanes 2−5**) without screens. (**D**) Analysis of the m^7G-antibody precipitable RNAs [as in (**C**)] using a partially denaturing 12% (38.9:1.1) polyacrylamide gel like that of Figure 1B. RNAs equivalent to the total in 0.2 GVs (**lane 1**) and the precipitates from eight GVs (**lanes 3−5**) were analyzed; the marker (M) (**lane 2**) was *X.laevis* U1b1 RNA made *in vivo* by injected oocytes. The autoradiograms were exposed for 25 h without (**lane 1**) and with (**lanes 2−6**) intensifying screens.

To ensure that the U1-sized transcripts were in fact authentic xU1b1 RNAs (and not fortuitous RNA polymerase II transcripts containing U1 sequences), both the antibody-precipitated and the hybrid-selected RNAs were electrophoresed under partially denaturing conditions in a gel system that separates according to U1 RNA structure (Figure 4D and data not shown). Again, the 'α-amanitin-sensitive' U1-sized RNA (lanes 3 and 4) co-migrated with the marker of *X.laevis* xU1b1 RNA (lane 2). Thus, we conclude that the GV homogenate carries out DNA-dependent *in vitro* synthesis of correctly initiated and terminated *Xenopus* U1 RNA.

Discussion

Previously, we and others (Murphy *et al.*, 1982; Westin *et al.*, 1984; S.Gunderson, personal communication) have found that aqueous extracts of mammalian nuclei or whole cells are inactive for transcription of vertebrate snRNA genes. In this paper, we have demonstrated for the first time the synthesis of vertebrate U1 snRNA in a DNA-dependent *in vitro* system. Two characteristics of the present *in vitro* system contribute to this success: (i) the use of *Xenopus* oocytes as the starting material, and (ii) the use of oil as the isolation medium for the oocyte nuclei.

Because mature oocytes of *X.laevis* contain a large excess of snRNA-specific transcription factors (in addition to RNA polymerase II), the capacity of a single oocyte for snRNA synthesis is equivalent to that of $1-2 \times 10^6$ somatic cells. Hence, exogenous (i.e. micro-injected) vertebrate snRNA genes are transcribed at an exceptionally high rate (Figure 1; Lund *et al.*, 1987). Moreover, transcription of endogenous snRNA genes is negligible due to the low amount of *Xenopus* chromosomal DNA in the GV (i.e. 12 pg versus 12 μg/2×10^6 somatic cells).

The use of the oil isolation procedure to prepare GVs, unlike standard aqueous methods, allows the GVs to retain their high capacity for *de novo* snRNA synthesis (Figures 1 and 2). Presumably, this is because the oil prevents the diffusive loss of nuclear metabolites, cofactors, and proteins that inevitably occurs when nuclei are isolated in aqueous buffers (Paine *et al.*, 1983; Lue and Kornberg, 1987). Using oil-isolated GVs, we are able to demonstrate *in vitro* transcription of exogenously added U1 genes which exceeded

by several orders of magnitude the levels reported for transcription of endogenous U1 genes in aqueously isolated mammalian cell nuclei (Kunkel and Pederson, 1985; Lobo and Marzluff, 1987). [This estimate is based on the calculation that a single GV injected with 1 ng of *X.laevis* U1 DNA ($\sim 1.5 \times 10^8$ U1 genes) is equivalent to $\sim 2 \times 10^6$ mouse cell nuclei, each of which contains 50–60 transcriptionally active U1 genes (Dahlberg and Lund, 1988)]. Moreover, *de novo*-initiated pre-U1 RNAs are the predominant transcripts in oil-isolated GVs (Figures 1B and 2), whereas these RNAs comprise only a very small fraction of the total labeled U1 transcripts in isolated mouse cell nuclei (cf. Figure 3 of Lobo and Marzluff, 1987).

Transcription of *X.laevis* U1 genes injected into oocyte nuclei appears to be about as efficient in oil-isolated intact GVs as in whole oocytes (Figure 1). Furthermore, homogenization of pre-injected GVs had little effect on overall efficiency or fidelity (Figure 3), demonstrating that strict integrity of the nuclear membrane (and presumably intranuclear structure) is not essential for snRNA synthesis. This continued high level of transcription of pre-injected snRNA genes in oocyte nuclear homogenates should allow the isolation of active transcription complexes.

Although the efficiency of transcription of U1 templates added to homogenates *in vitro* is relatively low, correctly initiated and terminated *X.laevis* pre-U1 RNAs are synthesized (Figure 4). It is at present unclear if the lower rate of synthesis of U1-sized RNA (compared to that using genes pre-injected into the intact oocyte) reflects inefficient transcription initiation at the snRNA promoter or the lack of correct 3′ end formation (or a combination of both).

Analysis of the 5′ cap of the *in vitro* synthesized U1 transcripts showed that synthesis was initiated *in vitro*, after the addition of [α-^{32}P]GTP (Figure 2). Maturation of pre-U1 RNA, including hypermodification of the 5′ cap and trimming of the 3′ end of pre-U1 RNAs occur in the cytoplasm (Mattaj, 1986; Madore *et al.*, 1984; H.Neuman de Vegvar and J.E.Dahlberg, in preparation). Because the cytoplasm is virtually all removed during GV isolation under oil, it is not surprising that the accumulated U1 transcripts have m_1^7G-caps (rather than $m_3^{2,2,7}$G-caps) and are slightly longer at their 3′ ends. We have not yet examined whether the pre-U1 RNAs made *in vitro* can be exported from the GVs, as has been reported in the case of snRNA synthesis in aqueously isolated nuclei of mouse cells (Lobo and Marzluff, 1987).

We have found that transcription of pre-injected U1 genes in GV homogenates is relatively insensitive to 2-fold variations in either K^+ or Mg^{2+} ions (data not shown). But it remains to be determined whether the conditions used here are optimal for the DNA-dependent U1 synthesis in the homogenate. We note that unlike RNA polymerase II transcription in aqueous extracts of *Saccharomyces cerevisiae* nuclei (Lue and Kornberg, 1987), snRNA transcription in oil-isolated GV homogenates is not greatly inhibited by chloride ions (Figure 3B).

Consistent with previous studies (e.g. Birkenmeier *et al.*, 1978; Wormington *et al.*, 1981; Peck *et al.*, 1987), we find that *Xenopus* 5S rRNA genes are expressed very efficiently in both intact and homogenized oil-isolated GVs. However, both the average length of the maxi-5S transcripts and the levels of transcription are increased significantly in homogenates as compared to intact GVs (cf. lanes 1 or 2 with lane 5 of Figure 3B). This result raises the intriguing possibility that homogenization might release or activate a factor, such as the La-antigen, which acts as a termination factor and is needed for efficient transcription by RNA polymerase III *in vitro* (Gottlieb and Steitz, 1987, 1988).

It is unclear what limits transcription in the GV homogenate. A mouse U6 gene added to the homogenate is transcribed much more efficiently than are the *X.laevis* U1 genes (Figure 4A). Although the U6 gene is transcribed by RNA polymerase III, its promoter also contains transcription signals normally present in the RNA polymerase II U1(-U5) snRNA genes (Das *et al.*, 1987; Krol *et al.*, 1987; reviewed in Dahlberg and Lund, 1988). In particular, the proximal snRNA promoter element (PSE or 'snRNA TATA-box') is required for U6 RNA synthesis (Carbon *et al.*, 1987; Kunkel and Pederson, 1988; Das *et al.*, 1988). Thus, the snRNA-specific transcription factor(s) that interacts with the PSE (Gunderson *et al.*, 1988) is unlikely to be the limiting component(s) for U1 RNA synthesis *in vitro*. The availability of the *in vitro* system described here gives us the opportunity to define just what factors participate in snRNA synthesis and how they function.

Materials and methods

DNA templates

The U1 templates were *X.laevis* embryonic U1 genes (Lund *et al.*, 1984) cloned in pBR322; they contained either a full length repeat with one copy of each of the xU1b1 and xU1b2 genes [pX1U1b (111/222)] or only the xU1b1 gene (pX1U1b1) (Krol *et al.*, 1985; Lund *et al.*, 1987). The *Xenopus borealis* maxi-5S template, pXbs+20 (Wormington *et al*, 1981), was kindly provided by D.D.Brown. The mouse U6 template, subclone −315/+287 (Das *et al.*, 1988) of pmU6−52 (Oshima *et al.*, 1981), was a gift from R.Reddy.

Oocyte preparation and injection

The maintenance of *X.laevis* female frogs, the preparation of oocytes and conditions of injection were as previously described (Krol *et al.*, 1985; Lund *et al.*, 1987). Oocytes were injected into the nucleus with 0.1–4.0 ng of circular plasmid DNAs. Prior to GV isolation, injected oocytes were incubated at 18°C for 2–4 h to allow for chromatin assembly. For preparation of *in vivo* made ^{32}P-labeled U1 RNAs, oocytes were injected with 0.5–1.0 μCi of [α-^{32}P]GTP into the cytoplasm and incubation was continued for 2–15 h.

Isolation of germinal vesicles in aqueous buffer

Oocyte nuclei [germinal vesicles (GVs)] were isolated from pre-injected oocytes by manual dissection (Feldherr and Richmond, 1978) into ice-cold I buffer, which is isolation medium [20 mM Tris–HCl, pH 7.5, 75 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, 2% PVP-360 (Clark and Merriam, 1977)] supplemented with 5 mM $MgCl_2$ and 0.1 mM EDTA. Initial experiments showed that omission of the priming step (Birkenmeier *et al.*, 1978) or isolation into J-buffer (10 mM Hepes, pH 7.4, 70 mM NH_4Cl, 7 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM DTT, 10% glycerol) with or without 1–2% PVP-360 (Birkenmeier *et al.*, 1978; Peck *et al.*, 1987) or a buffer based on the intra-cellular medium of oocytes (102 mM KCl, 11.1 mM NaCl, 7.2 mM K_2HPO_4, 4.8 mM KH_2PO_4, pH 7.0) (Feldherr and Richmond, 1978; Paine *et al.*, 1983) did not result in increased activity of the isolated GVs for snRNA transcription (data not shown).

Isolation of GVs under mineral oil

Oil-isolated GVs were prepared in a similar manner except that dissection was performed at room temperature (20–22°C) under mineral oil [American Standard, White oil no. 31-USP (heavy)] that had been pre-saturated with intracellular medium (see above). To remove excess oocyte incubation medium, oocytes were first blotted briefly on Whatman 3 MM paper, then submerged completely under oil and cut open with a 22 gauge hypodermic needle. Such oil-isolated GVs remain transcriptionally active for >4 h after isolation (Paine,P.L., Miller,P.S., Johnson,M.E., Lau,Y.-G., Tluzek, L.J.M. and Horowitz,S.B., in preparation; our unpublished results), thus permitting the collection of at least 100–150 GVs for use in the same experiment.

In vitro RNA synthesis in GV preparations

For *in vitro* synthesis of U1 RNA, intact (or disrupted) pre-injected GVs (kept under oil) were labeled by fusion with 0.25–0.5 μCi of [α-^{32}P]GTP per GV (or GV-equivalent) in a 5–10 nl microdroplet of TE (10 mM Tris–HCl, pH 7.6, 0.1 mM EDTA) formed by extrusion from a blunt-end micropipette (internal diameter 10–20 μm). Incubation was for 60–120 min at 18–22°C under oil. Isolation and incubation of GVs at elevated temperatures (>25–27°C) inactivates snRNA synthesis.

For monitoring transcription in homogenized pre-injected GVs, groups of 10 intact GVs were transferred (under oil) into droplets of 5 μl (~12–14 GV volumes) of transcription buffer supplemented with 0.5 mM each of ATP, CTP and UTP, 20 μM of GTP and 5 μCi of [α-^{32}P]GTP. Transcription buffer is 20 mM Hepes, pH 7.4, 100 mM KOAc, 5 mM $MgSO_4$, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EGTA, 0.2 mM EDTA, 4 mM ATP, 1.5% PVP-360, 1 mM DTT, 1 mM PMSF, 20 mM creatine phosphate, 80 μg/ml creatine phosphokinase and 8–10% glycerol. Other additions or substitutions are indicated in the figure legends. Homogenization was accomplished by repeatedly drawing the suspension in and out of a fine-tipped micropipet (drawn-out capillary tubing) and incubation was for 60–120 min at 18–22°C under oil.

For DNA-dependent transcription in GV homogenates, uninjected oil-isolated GVs were homogenized as above in ~14 vol of transcription buffer containing circular template DNAs at 0.5–50 ng of DNA/GV equivalent, as specified in the legend to Figure 4. After pre-incubation of the homogenates for 30–60 min, unlabeled ribonucleoside triphosphates and [α-^{32}P]GTP were added to the same final concentrations as above by fusion with a droplet 1/5 to 1/10 the volume of the homogenate, and incubation was continued under oil for an additional 60–90 min.

Analyses of RNA transcripts

RNA synthesis *in vitro* was terminated by transfer of the isolated GVs or GV homogenates from the oil into 100 μl of proteinase K buffer (Krol *et al.*, 1985) per 10–25 GVs. After digestion for 1–2 h at 37°C, total RNAs were isolated by phenol extraction and ethanol precipitation and were analyzed by polyacrylamide gel electrophoresis either directly or after preparative hybridization to filter-bound human U1 DNA as described elsewhere (Murphy *et al.*, 1982). All gels contained TEB buffer (90 mM Tris-borate, pH 8.3, 2.3 mM EDTA) and were run at 40–50 V/cm for 3–5 h for 8–10% sequencing gels or at 12–15 V/cm for 16–18 h for 12% partially denaturing gels.

For determination of the 5′ cap-structures, ^{32}P-labeled U1 RNAs were eluted from the gels and digested with RNase T2 or tobacco acid pyrophosphatase and the digests were analyzed by one- or two-dimensional thin layer chromatography as described previously (Skuzeski *et al.*, 1984).

For precipitation with the m_1′G-cap-specific antibody (Munns *et al.*, 1982), total RNAs (equivalent to 10 GVs) were incubated with 10–25 μl of covalently-linked anti-cap antibody (generously supplied by T.Munns, Washington University, St Louis, MO) in 40–200 μl of TBS (10 mM Tris–HCl, pH 8.0, 140 mM NaCl) containing 0.05% Tween-20. After incubation for 1–3 h at 4°C, the agarose-beads were washed with 3 × 1 ml TBS, 0.05% Tween-20 and the bound RNAs were eluted by resuspension in TE containing 1% SDS and phenol extraction. The eluted RNAs were recovered by ethanol precipitation and analyzed by polyacrylamide gel electrophoresis.

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