Molecular Biology of the Cell
Vol. 3, 249–261, March 1992

Transcription on Lampbrush Chromosome Loops in the Absence of U2 snRNA

Alexander Tsvetkov,*† Michael Jantsch,* Zheng'an Wu,*‡ Christine Murphy,* and Joseph G. Gall*

*Department of Embryology, Carnegie Institution, Baltimore, Maryland 21210; †Institute of Cytology, St. Petersburg, USSR; and ‡Institute of Developmental Biology, Academia Sinica, Beijing, People's Republic of China

Submitted November 1, 1991; Accepted December 30, 1991

The five small nuclear RNAs (snRNAs) involved in splicing occur on the loops of amphibian lampbrush chromosomes and in hundreds to thousands of extrachromosomal granules called B snurposomes. To assess the role of these snRNAs during transcription and to explore possible relationships between the loops and B snurposomes, we injected single-stranded antisense oligodeoxynucleotides (oligos) against U1 and U2 snRNA into toad and newt oocytes. As shown before, antisense U1 and U2 oligos caused truncation of U1 and complete destruction of U2 snRNAs, respectively. However, injection of *any* oligo, regardless of sequence, brought on dramatic cytological changes, including shortening of the chromosomes and retraction of the lateral loops, with concomitant shutdown of polymerase II transcription, as well as disappearance of some or all of the B snurposomes. When injected oocytes were incubated for 12 h or longer in physiological saline, these changes were reversible; that is, the chromosomes lengthened, transcription (detected by ^{3}H-UTP incorporation) resumed on newly extended lateral loops, and B snurposomes reappeared. In situ hybridization showed that loops and B snurposomes had negligible amounts of U2 snRNA after recovery from injection of the anti-U2 oligo, whereas these structures had normal levels of U2 snRNA after recovery from a control oligo. Thus, the morphological integrity of B snurposomes and lampbrush chromosome loops is not dependent on the presence of U2 snRNA. Because transcription occurs in the absence of U2 snRNA, we conclude that splicing is not required for transcription on lampbrush chromosome loops.

INTRODUCTION

The lampbrush chromosomes of amphibian oocytes provide a useful system in which to study transcription and processing of pre-mRNA. These chromosomes consist of a central axis of inactive chromatin, the chromomeres, from which loops of transcriptionally active chromatin project laterally. Each loop contains one or more transcription units, which are recognizable in the light microscope by their "thin-to-thick" morphology. Transcription begins at the thin end of a transcription unit, where the nascent transcripts are short, and proceeds toward the thick end, where they are longer. Thus, the asymmetrical structure of a transcription unit reflects the direction of transcription, the greater mass of pre-mRNA and associated protein being at the downstream end of the unit. These general features of lampbrush chromosome organization are reviewed in Callan's monograph (Callan, 1986).

The proteins associated with the nascent transcripts have been studied extensively by immunofluorescence microscopy (Scott and Sommerville, 1974; Sommerville *et al.*, 1978; Martin and Okamura, 1981; Lacroix *et al.*, 1985; Moreau *et al.*, 1986; Leser and Martin, 1987; Roth and Gall, 1987; Piñol-Roma *et al.*, 1989; Angelier *et al.*, 1990; Wu *et al.*, 1991). Most transcription units stain with antibodies against the major heterogenous nuclear RNPs (hnRNPs), such as the A, B, and L proteins, although a few landmark loops remain conspicuously unstained. Likewise, most loops stain with antibodies against snRNP proteins and the essential splicing factor SC35 (Fu and Maniatis, 1990). All of the small nuclear RNAs (snRNAs)[1] involved in pre-mRNA splicing (U1, U2, U4, U5, and U6) are demonstrable on loops by in situ hybridization (Wu *et al.*, 1991). These localization

[1] Abbreviations used: GV, germinal vesicle; hn, heterogenous nuclear; oligo, oligodeoxynucleotide; sn, small nuclear.

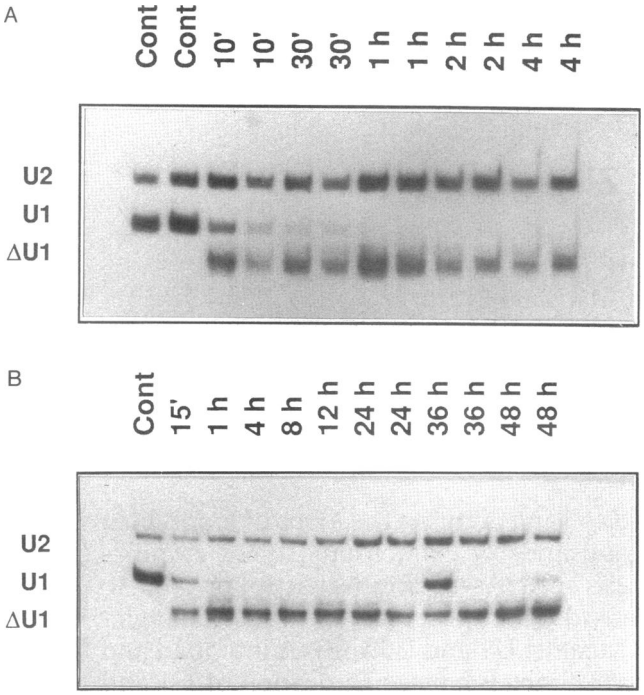


Figure 1. (A) Northern blot of RNA from GVs of *Notophthalmus* (2 GVs per lane). The first two lanes are from control uninjected oocytes, the rest from oocytes injected with the U1a oligo 10 min to 4 h previously. Roughly half the U1 snRNA is converted to a truncated form (Δ U1) in 10 min. U2 snRNA is unaffected. (B) Similar Northern blot of RNA from three GVs, with the time extended to show that the truncated U1 snRNA is stable for up to 48 h. Lane 9 shows incompletely degraded U1 snRNA, presumably due to poor injection of the U1a oligo into one of the oocytes.

studies show clearly that many components involved in the packaging and processing of pre-mRNA associate with the nascent chains while transcription is still in progress. It is not known, however, whether splicing itself takes place before release of the nascent transcripts.

Small nuclear RNPs (snRNPs) also exist in hundreds to thousands of nucleoplasmic granules, which we designate snurposomes (Gall and Callan, 1989; Wu *et al.*, 1991). Three types are distinguishable: the A snurposome, which contains only U1 snRNA and U1-associated proteins; the B snurposome, which contains the five splicing snRNAs, associated snRNP proteins, and other essential splicing factors such as SC35; and the C snurposome, whose composition is still unclear. The relationship between the snRNPs on the chromosome loops and those in the A, B, and C snurposomes is unknown.

The experiments reported here were undertaken to determine what effect the alteration or removal of splicing snRNAs might have on the chromosome loops and snurposomes. Earlier studies on *Xenopus* oocytes by Pan and Prives (1988, 1989) and Prives and Foukal (1991) showed that injection of single-stranded oligodeoxynucleotides (oligos) complementary to parts of U1 or U2 caused truncation (U1) or destruction (U2) of the snRNA and inhibited splicing of SV40 transcripts. A control oligo had no effect on the snRNAs or on splicing. Similar experiments by Hamm *et al.* (1989) examined the role of U1 and U2 in the formation of splicing complexes in *Xenopus* oocyte nuclei.

For most of our experiments, we used the three oligos described by Pan and Prives. We examined the morphology of the germinal vesicle (GV) contents at various times after injection and carried out in situ hybridization studies to determine the distribution of the snRNAs involved in splicing. We found that each of the three oligos, as well as several other unrelated oligos, had dramatic nonspecific effects. Each caused the loops to disappear and the chromosomes to shorten to a fraction of their original length, with concomitant shutdown of pre-mRNA synthesis. In many cases, the B snurposomes were reduced in number or disappeared entirely. Surprisingly, these morphological effects were completely reversible, and transcription resumed on newly extended chromosome loops when injected oocytes were held for 12–24 h in physiological saline. By comparing oocytes that had been injected with a control oligo to those injected with an oligo against U2 snRNA, it was possible to examine the recovery of the chromosomes and snurposomes in the presence or absence of U2 snRNA.

MATERIALS AND METHODS

Animals

Female newts *Notophthalmus viridescens* were obtained from Lee's Newt Farm (Oak Ridge, TN), and female toads *Xenopus laevis* were obtained from Xenopus I (Ann Arbor, MI).

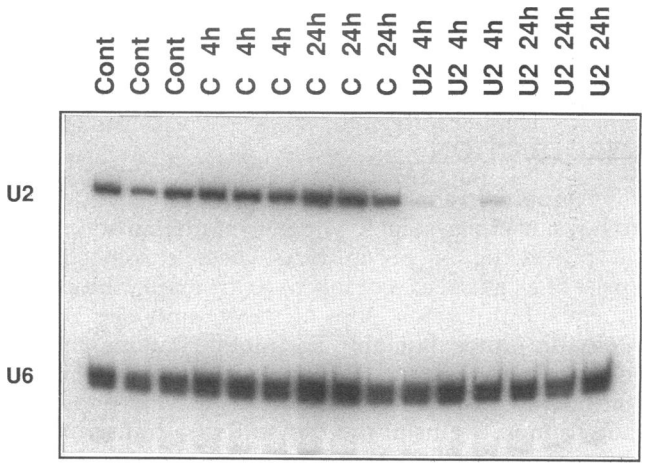


Figure 2. Northern blot of RNA from single GVs of *Notophthalmus* at various times after injection. The first three lanes are from control uninjected oocytes, the next six from oocytes injected with the C oligo, and the last six from oocytes injected with the U2b oligo. GVs from oocytes injected 24 h previously with the U2b oligo have control levels of U6 but essentially no U2 snRNA (<2% of the control by quantitation of the autoradiogram). The C oligo has no effect on the amount of U2 or U6 snRNAs.

Oocyte Injections

A sample of ovary was removed from an anesthetized animal and stored at 18–22°C in OR2 saline (Wallace *et al.*, 1973). Individual oocytes with their surrounding follicle cells were separated from the ovary wall with jeweler's forceps. To ensure the presence of active lampbrush chromosomes, *Xenopus* oocytes in the range of 0.9–1.1 mm diameter were chosen (roughly Dumont stage IV); for *Notophthalmus*, size was less critical, although in most cases oocytes of 1.1–1.5 mm diameter were used. For long-term experiments, oocytes must be handled gently to prevent degeneration, especially those of *Notophthalmus*. One of the first external signs of trouble is redistribution of pigment in the animal hemisphere, resulting in a stippled or mottled appearance. Because such damage may become evident within 2–4 h, we usually held oocytes this long before injection. Only oocytes with uniform pigmentation were injected.

Injections were performed with a glass needle using air pressure from a 50-ml plastic syringe (Kay, 1991). Each oocyte was injected with roughly 20–50 nl of a solution containing an oligo at 0.25–2.0 ng/nl. Injections were made into the cytoplasm to avoid cytological damage to the nucleus. After injection, oocytes were stored at 18–22°C in OR2.

Oligos

Single-stranded oligos were synthesized on an Applied Biosystems (Foster City, CA) 380B automated DNA synthesizer, purified by reversed-phase high-performance liquid chromatography, and dissolved at a concentration of 1 mg/ml in H_2O. Most experiments involved the three oligos described by Pan and Prives (1988). U1a is complementary to nucleotides 1–20 of *Xenopus* U1 snRNA: 5′ CTCCCCTGCCAGGTAAGTAT 3′. U2b is complementary to nucleotides 28–42 of *Xenopus* U2 snRNA: 5′ CAGATACTACACTTG 3′. C is unrelated to any snRNA: 5′TCCGGTACCACGACG 3′.

Northern Blots

snRNA from *Xenopus* and *Notophthalmus* GVs was analyzed by Northern blots. Individual GVs, or groups of 2–3 GVs, were homogenized in 10 mM tris(hydroxymethyl)aminomethane, pH 8.0, 1 mM EDTA, 0.2% sodium dodecyl sulfate. Total RNA was extracted with phenol, phenol/chloroform 1:1, and chloroform, precipitated with 0.3 M Na acetate in 70% ethanol with 5 μg of glycogen as carrier, and electrophoresed on 8 M urea, 10% acrylamide gels. RNA was transferred to nylon GeneScreen filters (New England Nuclear, Boston, MA) by electroblotting (Bittner *et al.*, 1980) and was hybridized with ^{32}P-labeled antisense probes against various snRNAs. Because of the large amount of snRNA in a GV, autoradiographic exposures of a few hours were adequate.

GV Spreads

Cytological preparations were made by isolating the GV in an isotonic Ca^{++}-free solution and then allowing the normally gelled contents to disperse in a more dilute saline. The conditions for *Xenopus* GVs are described in Gall *et al.* (1991) and those for *Notophthalmus* in Wu *et al.* (1991).

In Situ Hybridization With ^{3}H-Labeled Probes

The protocol followed in these experiments is given in Wu *et al.* (1991). Initially, the probe for U2 snRNA was a T3 antisense transcript copied from a human U2 clone, as described in that paper. In more recent experiments, we subcloned a 210 bp fragment of the *X. laevis* U2 snRNA gene (Hamm *et al.*, 1989) into pBluescript KS(+). From this we transcribed a T7 antisense RNA that contained the complement to the first 127 nt of the U2 snRNA and 83 nt of upstream sequences. This shorter probe gave less nonspecific binding to nucleoli. Probes were used at 1–2 × 10^5 cpm/μl.

RESULTS

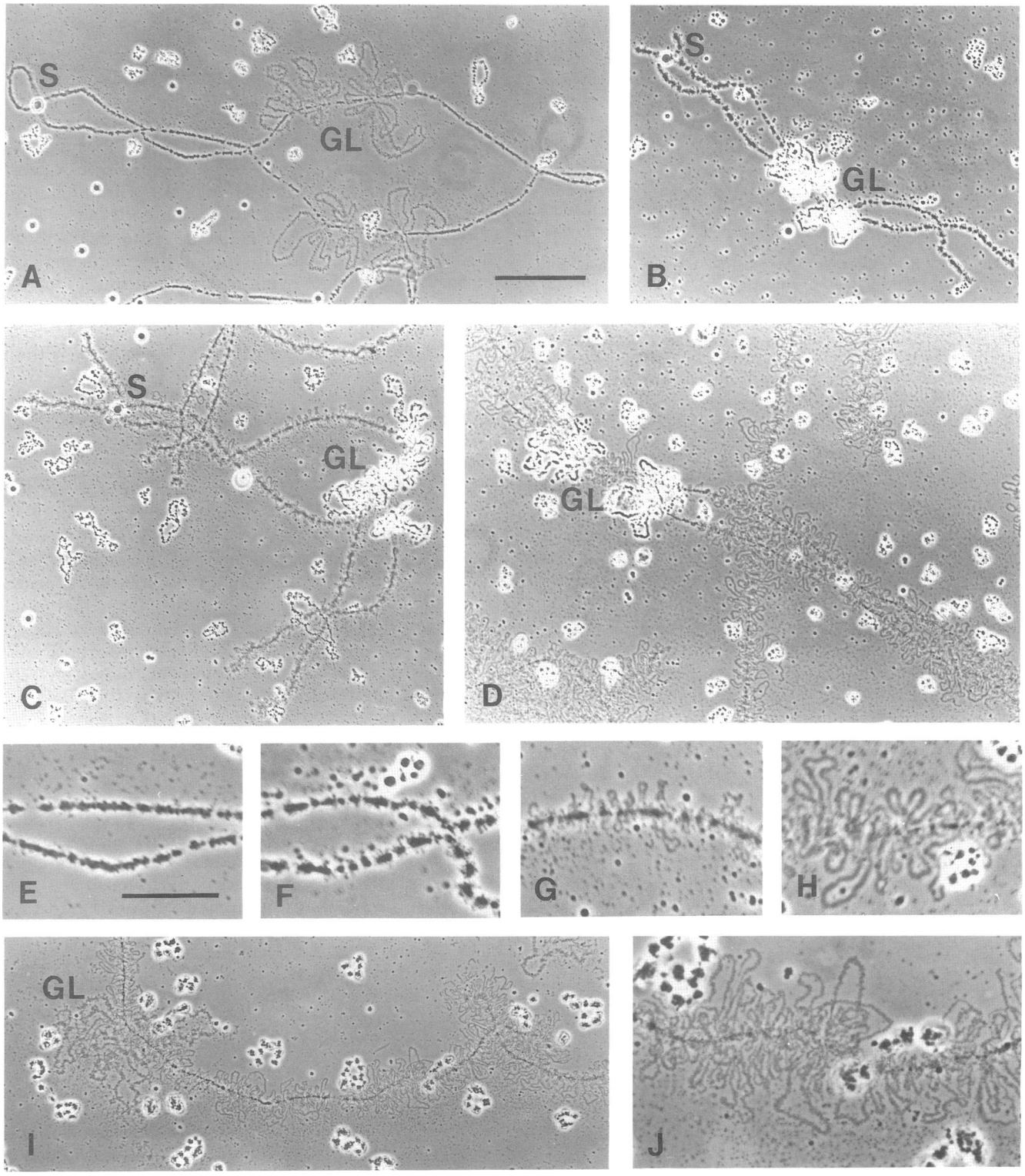
Specific Effects of Injected Antisense Oligos

In earlier studies on amphibian oocytes, antisense oligos against U snRNAs were usually injected directly into the GV. Because we wanted to minimize physical damage to the nuclear contents, we injected into the cytoplasm. Preliminary experiments showed that the oligos equilibrated across the nuclear envelope within minutes and effectively targeted the appropriate snRNA (see also Chin *et al.*, 1990). In typical experiments, ~20–50 nl of solution was injected into the animal hemisphere of an oocyte. The oligos were used at concentrations of ~0.25–2.0 ng/nl in distilled H_2O. As shown by Pan and Prives (1988, 1989) and Prives and Foukal (1991), the U1a oligo caused truncation of U1 snRNA within minutes; thereafter, the shortened U1 molecule remained stable for ≥48 h (Figure 1, A and B). The U2b oligo caused a similar rapid truncation of U2 snRNA, but in this case the product was unstable and disappeared from the GV after a few hours (Fig. 2). By extending the autoradiographic exposure, it was usually possible to detect a small amount of intact U2 snRNA in the GV. Quantitation of the autoradiographs showed that <1–2% of the original amount of U2 snRNA remained after 24 h. U1a and U2b oligos were specific for the snRNAs to which they were complementary; neither affected other snRNAs (Figures 1 and 2). The C oligo had no effect on any of the five splicing snRNAs (Figure 2 for U6). Because the injected oligos have a half-life of about 10 min in the oocyte, the GVs are effectively free of the oligo after 1–2 h (Cazenave *et al.*, 1987; Pan and Prives, 1988).

Nonspecific Effects of Injected Oligos

To assess what effect the truncation of U1 or removal of U2 snRNA might have on the lampbrush chromosomes and snurposomes, we made spread preparations of GV contents at various times after injection of the oligos. We found that injection of *any* oligo into the oocyte—U1a, U2b, C, or three other unrelated oligos of 15, 18, and 24 nucleotides—had immediate and dramatic cytological effects, beginning within the first few minutes after injection. The most conspicuous of these was retraction of the lampbrush loops, accompanied by overall shortening of the chromosomes to a fraction of their original length (Figure 3, A, B, E, and F). We emphasize that these effects were unrelated to the sequence of the oligo. However, they were not the result of the injection procedure itself because GVs from oocytes injected with H_2O retained their normal morphology. Similar cytological effects occur when oocytes are incubated in inhibitors of RNA synthesis, such as actinomycin or α-amanitin or when isolated GVs are treated with RNase or millimolar concentrations of Ca^{++} (see discussion in Callan, 1986).

In addition to the striking changes in the chromosomes, the number of B snurposomes was often re-


S
GL
A
S
GL
B
S
GL
C
GL
D
E
F
G
H
GL
I
J

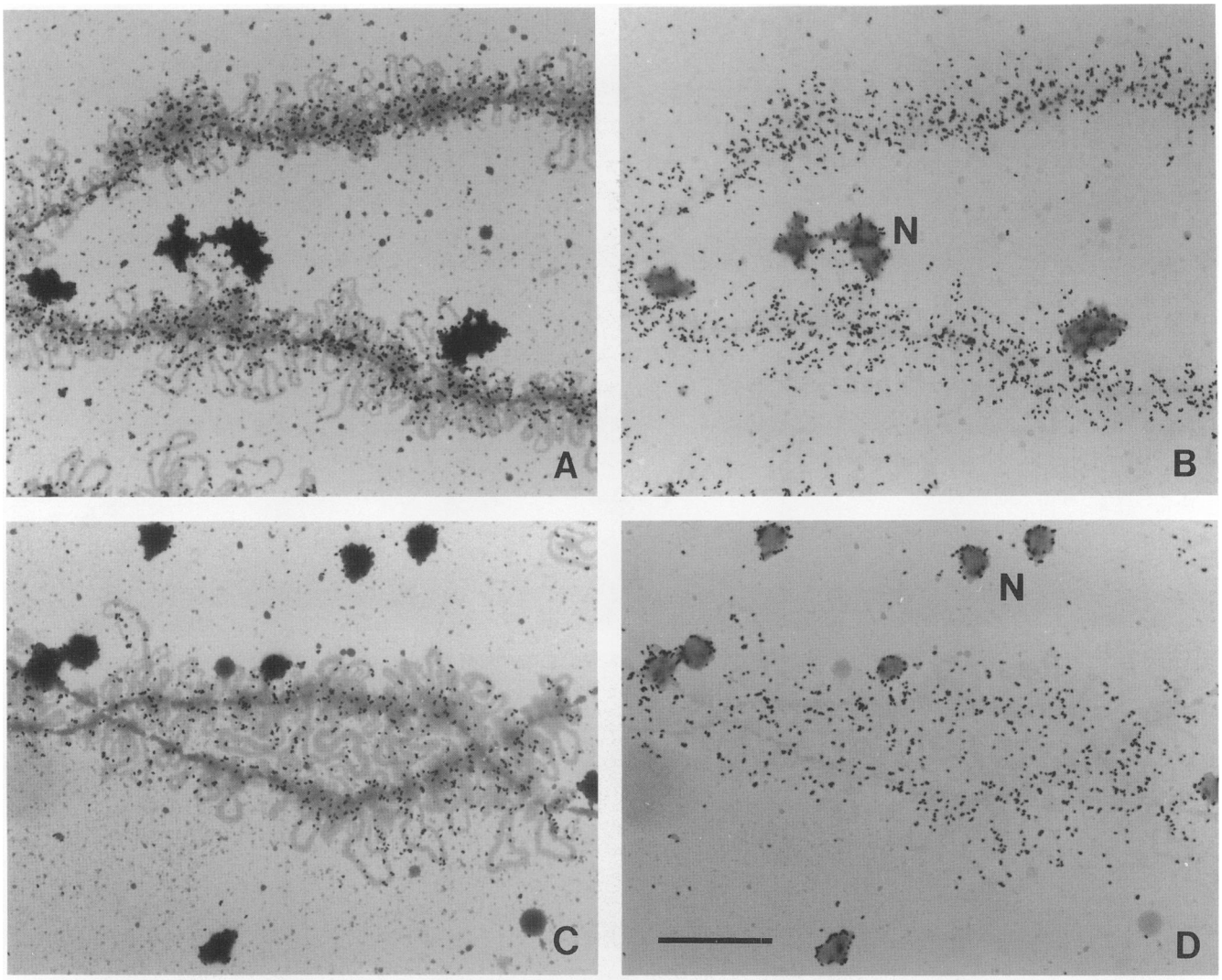
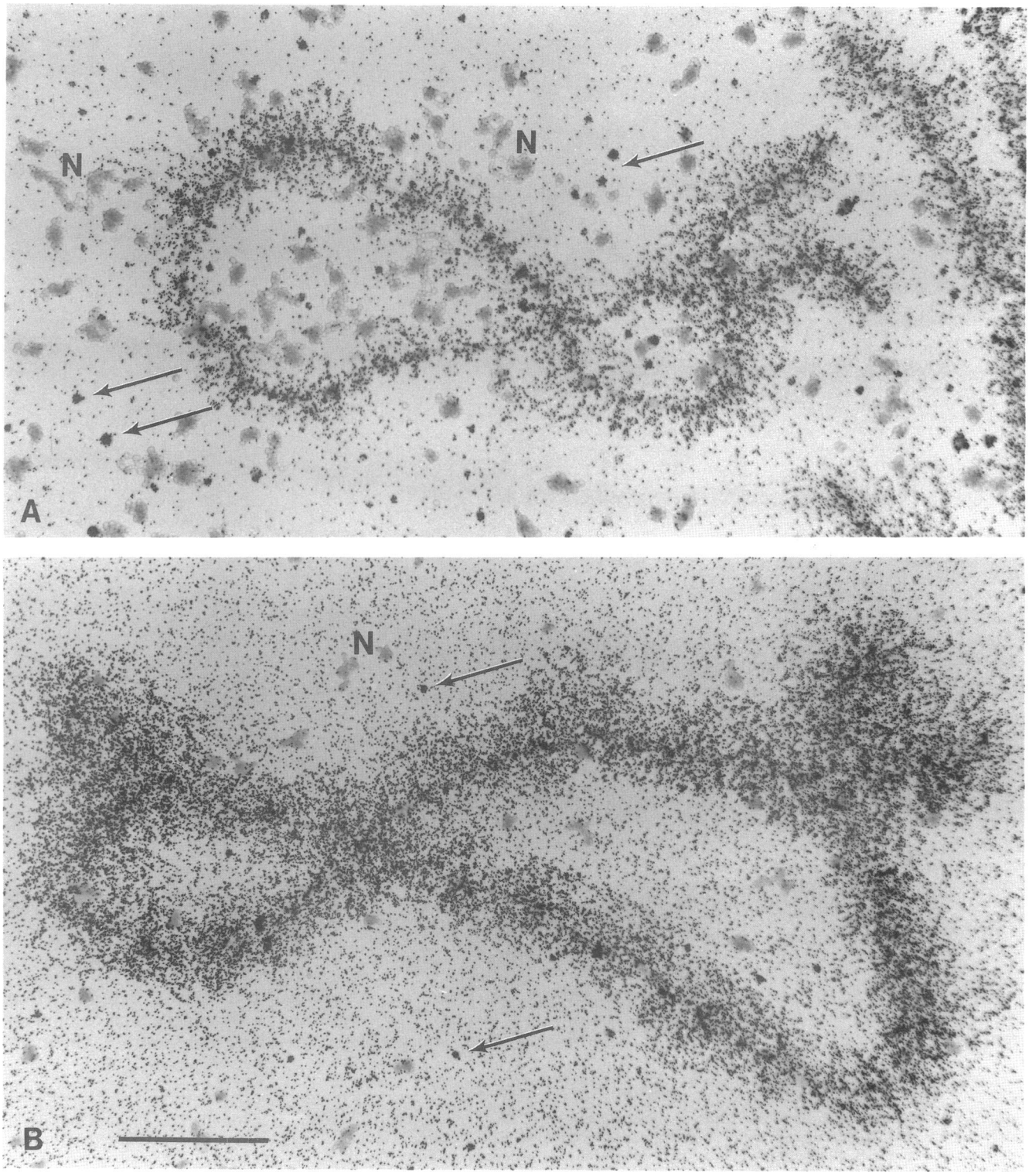


Figure 4. (A) Autoradiograph of spread GV contents from a *Notophthalmus* oocyte injected 4 h previously with ^{3}H-UTP. Photographed at 500 nm to show the chromosome loops stained with Coomassie blue. (B) The same area photographed at 450 nm to suppress the stain and accentuate the silver grains over the chromosomes and nucleoli (N). (C) An oocyte was injected with the U2b oligo, allowed to recover 20 h, and then injected with ^{3}H-UTP. Four hours later a GV spread was made and autoradiographed. Photographed at 500 nm to accentuate the chromosome loops. (D) The same area photographed at 450 nm to show silver grains above the chromosomes and nucleoli. Bar, 20 μm.

duced. In control *Xenopus* oocytes of the size used in these experiments, ~1.0 mm diameter, B snurposomes are invariably abundant, both free and on the surface of C snurposomes. It was thus easy to determine that in many cases injection of an oligo either eliminated the B snurposomes or reduced their number. The mag-

Figure 3. (A) Lampbrush chromosome 2 from *Notophthalmus* isolated 1 h after the oocyte had been injected with the U1a oligo. Of the several hundred loops that would normally be on this chromosome, only the giant loops (GL) remain. The chromosome has contracted to roughly half its normal length. S, attached sphere locus. A portion of the chromosome axis is enlarged in E. (B) Chromosome 2 from an oocyte injected 7 h previously with the U1a oligo. Overall contraction of the chromosome even more extreme than in A. The giant loops appear as a refractile mass near the center of the chromosome. An enlargement of the axis is shown in F. (C) Chromosome 2 from an oocyte injected 11 h earlier with the U1a oligo. The loops have begun to return, and the overall length of the chromosome has increased. The giant loops remain contracted. Enlargement in G. (D) The right end of chromosome 2 from an oocyte injected 21 h earlier with the U1a oligo. Both the overall chromosome length and the extent of loop development appear normal. The contracted giant loops indicate that this chromosome is derived from a recovered oocyte, not an accidentally uninjected one. Enlargement in H. (E, F, G, and H) Enlargements from A, B, C, and D, respectively, to show the state of the loops and chromomere axis at 1, 7, 11, and 21 h after injection of the U1a oligo. (I) The right end of chromosome 2 from a control uninjected oocyte. The giant loops are in their normal extended condition. (J) An enlargement from I to show normal loops; compare with the similar loops in H from a recovered oocyte. Bar in A, 50 μm for A, B, C, D, and I; bar in E, 20 μm for E, F, G, H, and J.


N
N
A
N
B

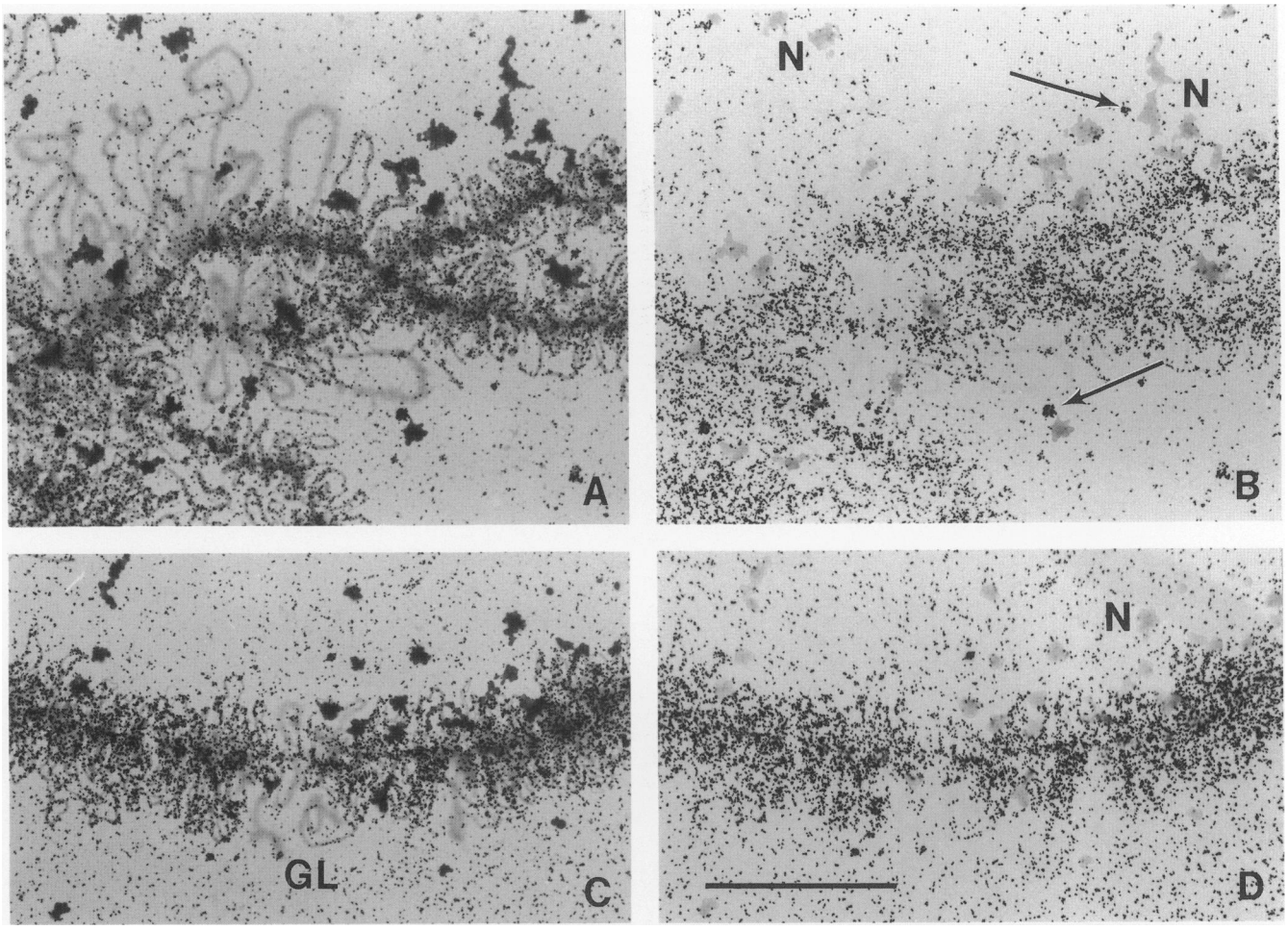


Figure 6. (A) Autoradiograph of a portion of chromosome 2 from a control uninjected oocyte of *Notophthalmus* after in situ hybridization with an antisense probe against U2 snRNA. The giant loops characteristic of this chromosome are among the very few loops in the entire genome that fail to label with the U2 probe. Photographed at 500 nm to accentuate the Coomassie blue stain in the loops and nucleoli. 14-d exposure. (B) Same area photographed at 470 nm to suppress the stain. The giant loops are invisible because they are not outlined by silver grains, as are essentially all other loops on the chromosome. B snurposomes (arrows) are intensely labeled. With the stain suppressed, one can see that the nucleoli (N) are at background level. (C) Autoradiograph of the giant loop (GL) region of chromosome 2 from an oocyte injected 24 h previously with the C oligo. The labeling pattern is essentially like that in A and B. The unlabeled loops near the middle are the giant loops, which have failed to re-extend. Photographed at 500 nm to accentuate the Coomassie blue stain. 14-d exposure. (D) Same region as in C, photographed at 450 nm to suppress the stain. Note that the giant loops and nucleoli are unlabeled. Bar, 50 μm.

nitude of this effect was more difficult to assess in *Notophthalmus* because of naturally occurring variability in the number of Bs from oocyte to oocyte. In many cases, however, injected *Notophthalmus* GVs had no B snurposomes detectable either by direct phase contrast observation or by immunofluorescent staining with specific antibodies. In both *Xenopus* and *Notophthalmus*, the morphology and staining of C snurposomes were unaffected by the oligos, except for the loss of B snurposomes usually found on their surface.

Recovery of Injected Oocytes

When injected oocytes were held in physiological saline (OR2) at 18–20°C, the nuclear contents gradually re-

Figure 5. (A) Autoradiograph of spread GV contents from a control uninjected *Notophthalmus* oocyte after in situ hybridization with an antisense probe against U2 snRNA. One complete lampbrush chromosome is shown (No. 10 or No. 11). Silver grains above the loops are evident, although the loops themselves are not visible in this photograph, which was taken at 470 nm to suppress the Coomassie blue stain. B snurposomes (arrows) are black due to overlying silver grains. Nucleoli (N) are at background level. 14-d exposure. (B) Autoradiograph of chromosome 10 or 11 from a *Notophthalmus* oocyte that had been injected 24 h previously with the C oligo. Photographed at 470 nm to suppress the stain and accentuate the silver grains. The labeling pattern is essentially like that of the control in A. As is often true of recovered chromosomes, the loops are unusually well developed and heavily labeled. 14-d exposure. Bar, 50 μm.

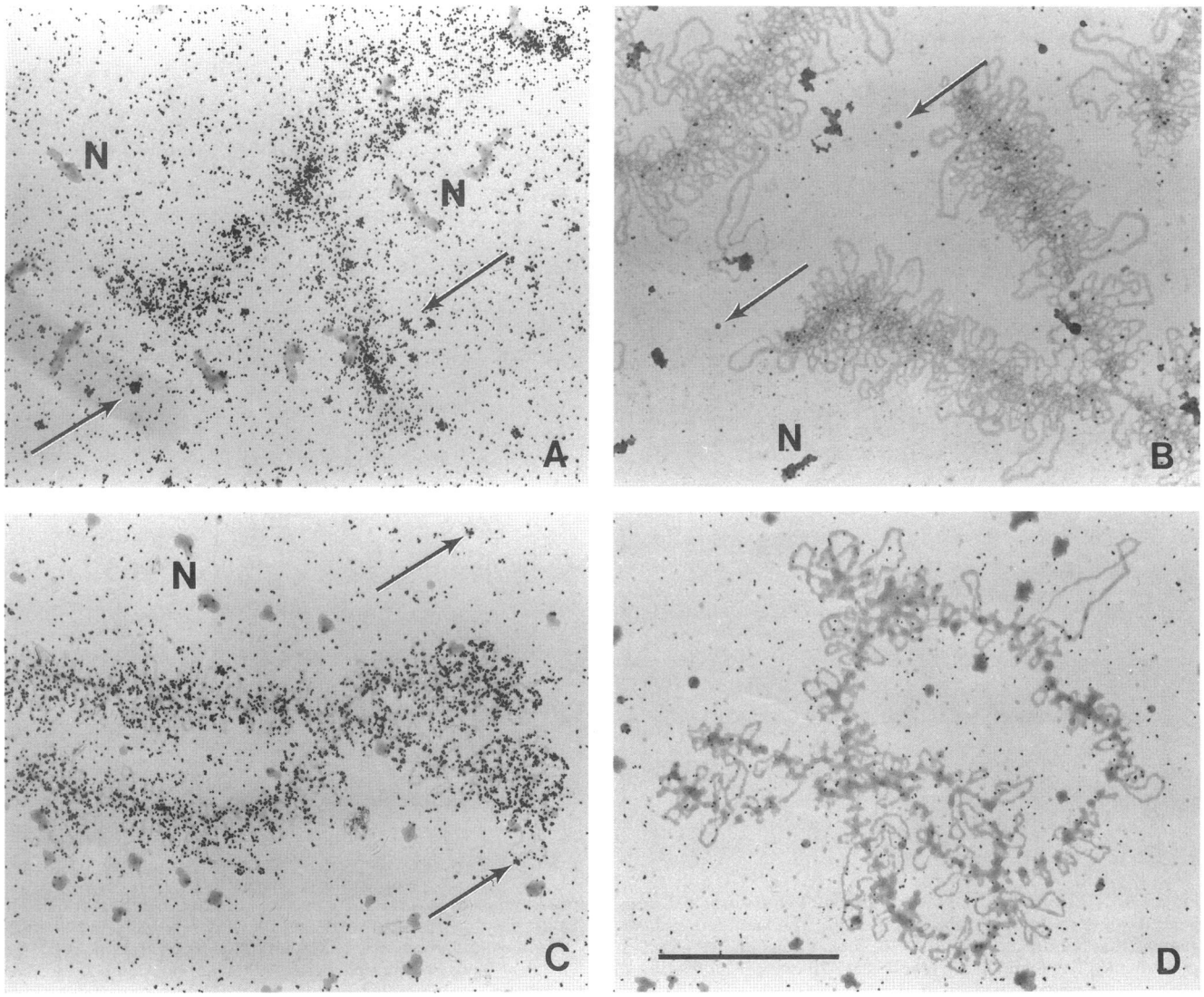


Figure 7. (A) Autoradiograph of spread GV contents from an uninjected control oocyte of *Notophthalmus* hybridized in situ with an antisense probe against U2 snRNA. Photographed at 458 nm to suppress the Coomassie blue stain. Silver grains occur over the chromosomes and the B snurposomes (arrows); nucleoli (N) are negative. 6-d exposure. (B) Autoradiograph of spread GV contents from an oocyte injected 24 h previously with the U2b oligo hybridized as in A. Nothing in the preparation is labeled above background level, although the stain obscures this fact in the snurposomes (arrows) and nucleoli. Photographed at 500 nm. 6-d exposure. (C) Autoradiograph of GV contents from an oocyte of *Notophthalmus* injected at 0 h and again at 24 h with the C oligo; preparation made at 48 h. In situ hybridization with an antisense probe against U2 snRNA. Label occurs above the chromosomes and B snurposomes (arrows) but not above the nucleoli. Photographed at 470 nm to suppress the Coomassie blue stain. 14-d exposure. (D) Autoradiograph of GV contents from an oocyte injected twice with the U2b oligo. This GV and the control GV shown in C were centrifuged onto the same slide and processed through all steps of hybridization and autoradiography together. No structures are labeled above background level. Photographed at 480 nm. 14-d exposure. Bar, 50 μm.

gained their normal appearance. Within a few hours the chromosomes began to lengthen (Figure 3, C and G). At the same time, they became covered with short barely detectable loops, losing the smooth contours characteristic of the most contracted state. By 12–24 h, the chromosomes in many nuclei had expanded to their normal lengths and were covered with loops as long as those in control oocytes (Figure 3, D and H). An unexpected feature of the recovery process was that after 24–36 h the loops on chromosomes from injected oocytes were frequently *longer* than those on control chromosomes. In fact, chromosomes as "loopy" as those in Figures 5B, 7B, and 8B are rarely encountered in control oocytes. The rate of recovery and the final condition of the chromosomes were somewhat variable, even within a single batch of oocytes injected at the same time. Sometimes the chromosomes failed to lengthen completely, but nevertheless bore loops of normal size (Figure 7D). We emphasize that all aspects of the recovery process just described, like the initial loss of loops,

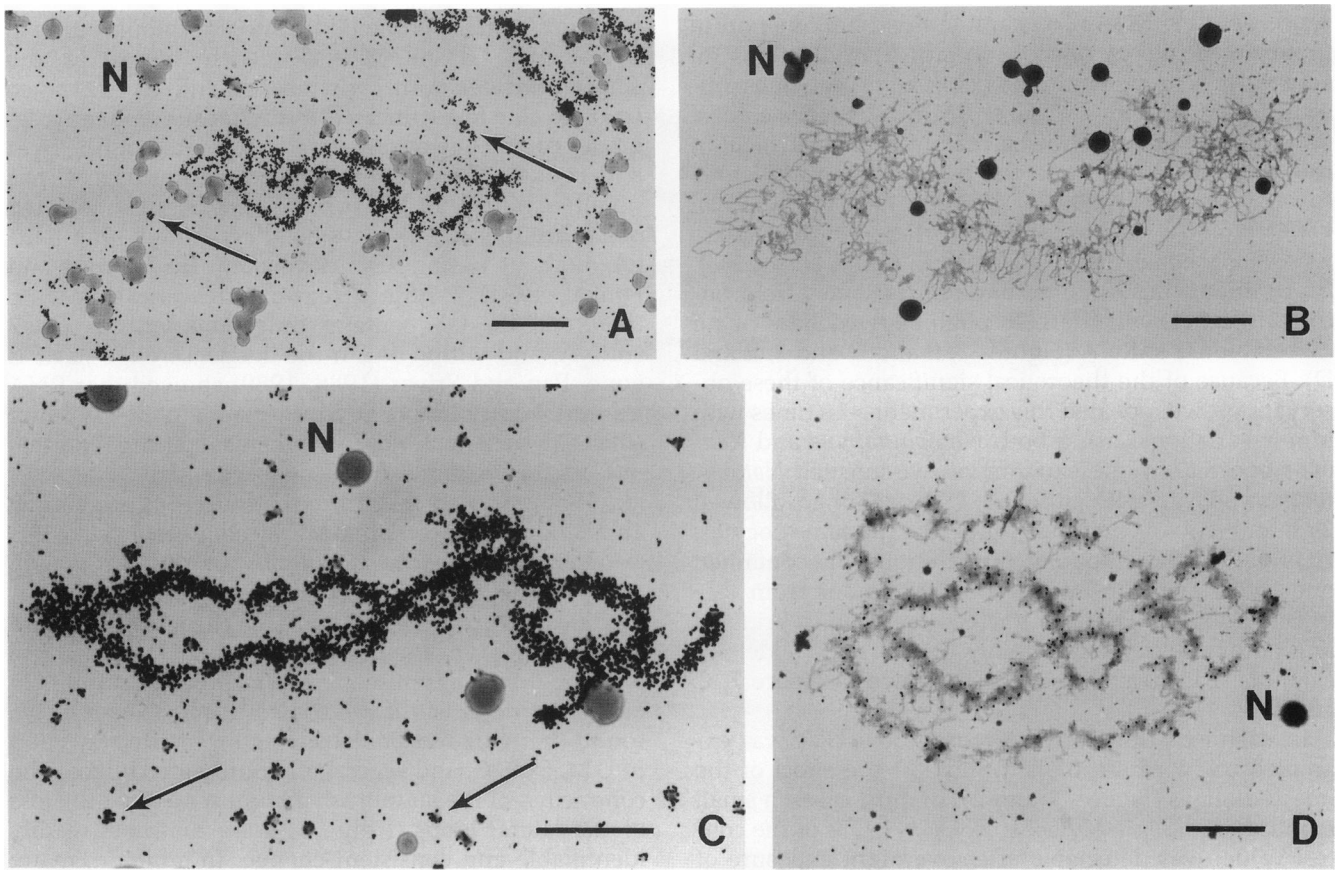


Figure 8. (A) Spread GV contents from an uninjected control oocyte of *Xenopus* hybridized in situ with an antisense probe against U2 snRNA. The chromosomes and B snurposomes (arrows) are heavily labeled, but the nucleoli (N) are at background level. Photographed at 470 nm. 10-d exposure. (B) Spread GV contents from a *Xenopus* oocyte injected 24 h previously with the U2b oligo hybridized as in A. Nothing is labeled above background level, although this fact is obscured by the stain in the B snurposomes and nucleoli. (C) Spread GV contents from a control *Xenopus* oocyte hybridized as in A. Heavily labeled chromosome and B snurposomes (arrows), unlabeled nucleoli. 17-d exposure. (D) Spread GV contents from a *Xenopus* oocyte injected 24 h previously with the U2b oligo. Photographed at 500 nm. Very weak label is detectable above the chromosome, but B snurposomes and nucleoli are at background level. 17-d exposure. Bars, 20 μm.

were unrelated to the specific oligo injected into the oocyte.

During the recovery process, B snurposomes reappeared. Again this was easier to assess in *Xenopus* than in *Notophthalmus*. By 24 h after injection of an oligo, the majority of *Xenopus* GVs contained an apparently normal complement of B snurposomes, both free and on the surface of C snurposomes. In the case of *Notophthalmus*, many GVs from injected oocytes showed normal chromosomes after 24 h but few or no detectable B snurposomes. This was particularly evident as the absence of Bs from the surface of the C snurposomes. The greater variability in the number of B snurposomes in recovered *Notophthalmus* GVs correlates with the variability in controls.

Because a lampbrush chromosome loop consists almost exclusively of nascent RNA chains with associated proteins, the existence of loops is taken as prima facie evidence for transcription (Callan, 1986). To prove that recovered chromosomes from injected oocytes are no exception to this rule, we injected ^{3}H-UTP into oocytes at various times after injection of the oligos. Autoradiographs of chromosomes from such oocytes showed incorporation of ^{3}H into the RNA of the loops (Figure 4). As in control oocytes, the amount of incorporation was related to the general size of the loops and the length of incubation after injection of the radioactive precursor.

In Situ Hybridization of snRNAs

The morphological studies showed that oocytes injected with the U1a, U2b, or C oligos underwent essentially similar changes and in each case recovered a more or less normal structure after incubation in a saline solution. At the same time, Northern blots showed that U2 snRNA was reduced to negligible levels after U2b but not after C oligo injection. During the recovery process, therefore, lampbrush chromosome loops and snurposomes reappeared equally well in the presence or ab-

sence of U2 snRNA. To examine this phenomenon in more detail, we carried out in situ hybridizations on spread nuclear preparations using an ^{3}H-labeled antisense probe specific for U2 snRNA. GVs from control (uninjected) oocytes showed readily detectable label in the lampbrush loops and the B snurposomes (Figures 5A; 6, A and B; 7A; and 8, A and C) (Wu *et al.*, 1991). GVs from oocytes injected 24 h earlier with the C oligo were also well labeled (Figures 5B; 6, C and D; and 7C). By contrast, chromosomes and snurposomes from oocytes injected with the U2b oligo showed little or no detectable U2 snRNA (Figures 7, B and D and 8, B and D). Because of the theoretical significance of these observations, we repeated this experiment >10 times with minor variations, using both *Notophthalmus* and *Xenopus* oocytes. In one experiment, we injected *Notophthalmus* oocytes with either the U2b or C oligo, allowed 24 h for recovery, and then reinjected the same oocytes. At 48 h we made "double" preparations that contained one GV from a U2b-injected oocyte and one from a C-injected oocyte. The two nuclei were then hybridized and autoradiographed together on the same slide. An example from this experiment is shown in Figure 7, C and D.

In each experiment, RNA from a few GVs was examined on Northern blots to monitor the effect of the injected oligos (Figures 1 and 2). In most cases, a small amount of undegraded U2 snRNA, ~1–2% of the control value, was detectable after overnight exposure of the blots. Similarly, in cytological preparations exposed for several weeks, a few silver grains can be seen over the chromosome loops and B snurposomes from oocytes injected with the U2b oligo; after comparable exposures, control chromosomes are heavily labeled and the emulsion above B snurposomes is essentially saturated with silver grains. Thus, although *some* U2 snRNA is detectable by in situ hybridization in U2b injected oocytes, the reduction in the chromosome loops and snurposomes parallels the reduction in the Northern blots.

An occasional nucleus from a batch of U1a or U2b injected oocytes had an exceptionally high level of undegraded snRNA by Northern blotting (Figure 1B, lane 9). Likewise, a few GV preparations from U2b-injected oocytes had easily detectable U2 snRNA by in situ hybridization. We assume that such nuclei resulted from injection of an inadequate amount of the oligo.

DISCUSSION

Earlier experiments by Pan and Prives (1988, 1989) and Hamm *et al.* (1989) showed that antisense oligos injected into *Xenopus* oocytes can modify or eliminate U1 and U2 snRNAs. The mechanism of action is presumably through base pairing with homologous sequences in the RNA followed by cleavage of the hybridized region by an endogenous ribonuclease H (Wassarman *et al.*, 1974; Cazenave *et al.*, 1987). The reaction takes only a few minutes whether the oligos are injected into the GV, as in the original experiments, or into the cytoplasm, as in our studies. U1 snRNA is cleaved by oligo U1a to a stable truncated form, whereas U2 snRNA is cleaved by oligo U2b to a truncated form that subsequently degrades completely over a period of several hours (Prives and Foukal, 1991). The unrelated control oligo C has no effect on any of the splicing snRNAs. The injected oligos are themselves unstable, degrading with a half-life of about 10 min (Cazenave *et al.*, 1987; Prives and Foukal, 1991). Within a few minutes after injection, therefore, the GV contains only modified U1 or U2 snRNA, and within 1–2 h the antisense oligo itself is gone. Pan and Prives (1988, 1989) showed that RNA transcribed from SV40 DNA injected into the GV is not spliced in such depleted GVs, whereas splicing is normal in GVs that received the C oligo. Similarly, Hamm *et al.* (1989) showed that injected transcripts containing an adenovirus intron are not spliced in depleted GVs.

When we began these experiments our aim was to examine the cytological effects of U1 or U2 snRNA modification on the lampbrush chromosomes and snurposomes. On the basis of the biochemical studies, we thought we might see an effect of the U1a and U2b oligos but no effect of the control C oligo. Instead we found dramatic morphological changes after injection of U1a, U2b, C, and several other unrelated oligos. The contraction of the lampbrush chromosomes and the loss of the lateral loops (Figure 3) were the most readily identifiable and consistent change. In addition, many nuclei showed loss of some or all of the B snurposomes. The changes in the chromosomes resembled those seen after incubation of oocytes in actinomycin or α-amanitin (Izawa *et al.*, 1963; Mancino *et al.*, 1971). Not surprisingly, therefore, the chromosomes in treated oocytes failed to incorporate ^{3}H-UTP, a sign that polymerase II-dependent RNA synthesis was shut down (Schultz *et al.*, 1981). Interestingly, the multiple nucleoli continued to incorporate the precursor, presumably into pre-rRNA.

We have not tried to determine the cause of the nonspecific effects of injected oligos. One possibility is that the negatively charged oligos interact with basic proteins on the chromosome loops that are normally complexed with the pre-mRNA (DiMario *et al.*, 1989). Likely candidates would be the A and B group hnRNPs, which are well-known to bind both RNA and single-stranded DNA (Kumar *et al.*, 1986; Riva *et al.*, 1986; Piñol-Roma *et al.*, 1988). Another possibility is that the oligos interact with metal ions such as Ca^{++} or Mg^{++}, whose sequestering might lead to secondary effects on the chromosomes. Whatever the mechanism(s) may be of the nonspecific effects, these observations suggest caution in interpreting the action of antisense oligonucleotides on cell structure and function (Colman, 1990; Smith *et al.*, 1990).

When injected oocytes are held for 12–24 h in physiological saline (OR2), the GV can recover essentially normal structure. Complete recovery involves extension

of the chromosomes from their contracted state; reappearance of the lampbrush loops, which reflects the resumption of transcription; and reformation of B snurposomes, both free and on the surface of C snurposomes. Surprisingly, the loops on recovered chromosomes are often longer than those from control uninjected oocytes. Recovery is sometimes only partial, as when lampbrush loops of typical size reappear on short chromosomes (Figure 7D). In the case of *Xenopus* oocytes, B snurposomes nearly always return, whereas in *Notophthalmus* their reappearance is more variable.

When oocytes are treated with an inhibitor of RNA synthesis, such as actinomycin (Izawa *et al.*, 1963) or α-amanitin (Mancino *et al.*, 1971), or are subjected to γ-rays (Loones, 1979) or to a heat shock of 32–37°C (Flannery and Hill, 1988; Rodriguez-Martin *et al.*, 1989), the lampbrush loops retract and the chromosomes shorten essentially as they do after oligo injections. Snow and Callan (1969) showed that the effect of actinomycin is reversible. In their experiments, *Triturus* oocytes, still in the ovary, regained their normal structure 1–2 d after removal of the inhibitor. Later studies by Scheer and his colleagues (Scheer, 1987; Scheer *et al.*, 1984) showed that isolated oocytes could also recover from the effects of actinomycin when incubated 1–2 d in a medium without the inhibitor. Similarly, lampbrush chromosomes can recover their normal structure after γ-ray treatment (Loones, 1979) or after heat shock, either in vitro (Flannery and Hill, 1988) or in vivo (Rodriguez-Martin *et al.*, 1989). Thus, the recovery of structure seen after oligo injections is probably a general physiological response that occurs after any of several inhibitors of polymerase II activity.

The immediate nonspecific effects of injected oligos appeared at first sight to preclude analysis of any specific effects that might result from the alteration of U snRNAs. However, the reappearance of the chromosome loops and B snurposomes after their initial disappearance, regardless of which oligo was injected, provided a unique opportunity to study the reformation of these structures under various conditions. Thus, the experiments reported here do not address the *stability* of preformed transcription units and B snurposomes after removal of U2 snRNA, as we had originally intended, but rather the *assembly* of these structure in the presence or absence of U2.

Our results show that transcription can initiate on lampbrush chromosome loops and continue for many hours in the virtual absence of U2 snRNA. In this respect, the chromosomes behave like the injected SV40 DNA in the experiments of Pan and Prives (1988, 1989), which is also transcribed in the absence of U2 snRNA. It is well documented that U2 is essential for splicing in vitro (Steitz *et al.*, 1988), and the experiments of Pan and Prives (1988, 1989) and Hamm *et al.* (1989) show that U2-depleted GVs are unable to splice SV40 or adenovirus transcripts. Thus, although we have no direct evidence about splicing of chromosomal pre-mRNA in our experiments, it seems reasonable to conclude that transcription can occur on lampbrush chromosome loops in the absence of splicing. This conclusion is perhaps not surprising on a priori grounds, because in many experimental situations, particularly in vitro transcription and in vitro splicing reactions, transcription and splicing are not obligately linked. Nevertheless, our immunofluorescence and in situ hybridization studies show that all five splicing snRNAs and a variety of associated proteins, including the Sm antigen, U1- and U2- specific proteins, and the non-snRNP splicing factor SC35 (Fu and Maniatis, 1990), occur normally on amphibian lampbrush chromosome loops (Gall and Callan, 1989; Wu *et al.*, 1991). snRNPs are also present on the actively transcribing puffs and Balbiani rings of *Chironomus* polytene chromosomes (Sass and Pederson, 1984; Vazquez-Nin *et al.*, 1990) and on transcripts from tissue culture nuclei (Fakan *et al.*, 1986). Strongly suggestive evidence that splicing itself can occur on nascent transcripts comes from the electron microscopic observations on *Drosophila* chromatin spreads by Beyer and her colleagues (Osheim *et al.*, 1985; Beyer and Osheim, 1988). Thus, although transcription can occur on lampbrush loops in the absence of U2 snRNA, there remains the question of what role the splicing components play normally, both on the loops and in the snurposomes.

Data from in vitro splicing experiments stress the sequential addition of splicing components to the pre-mRNA (Maniatis and Reed, 1987; Guthrie and Patterson, 1988; Steitz *et al.*, 1988; Ruby and Abelson, 1991). The U1 snRNP first binds to the 5′ splice junction; the U2 snRNP then associates with the lariat region; and finally, the U5 and U4/U6 snRNPs are added, building up the fully formed spliceosome. It is generally assumed that events occur in essentially the same fashion in vivo, and there is some experimental evidence to support this view for oocytes (Hamm *et al.*, 1989). If this is the case, the elimination of U2 snRNA from the GV should not affect the initial binding of the U1 snRNP to the nascent transcripts, but the U5 and U4/U6 snRNPs should not bind.

A somewhat different scheme was suggested by our discovery of B snurposomes in the GV (Wu *et al.*, 1991). B snurposomes contain the five splicing snRNAs plus a variety of proteins ordinarily present in snRNPs or functional spliceosomes. The composition of the Bs, as well as the fact that they consist of fairly uniform 20- to 30-nm particles (Callan and Gall, 1991; Gall, 1991), raised the possibility that a major part of the spliceosome might be preassembled in the B snurposomes and then transported to the nascent transcripts on the chromosomes. In this case, it would be hard to predict what effect the elimination of U2 snRNA would have on such "prespliceosome" particles. One could imagine that elimination of U2 snRNA from the GV would prevent the assembly of the particles altogether, or alternatively, a deficient particle might be formed that lacked U2 but contained some or all the other splicing snRNAs.

These speculations are tentative, but they highlight the need for additional in situ hybridization experiments to determine what effect the elimination of U2 snRNA has on the cytological distribution of the other splicing snRNAs. If spliceosomes are assembled on nascent transcripts as they are in vitro, we might expect the loops of recovered chromosomes to contain U1 snRNA but not U4, U5, and U6. On the other hand, if some type of prespliceosome particle is formed in the B snurposomes, the situation is less straightforward. We have shown that B snurposomes reform in U2-deficient nuclei, and it is therefore conceivable that a deficient particle is assembled that lacks only U2 snRNA. If deficient particles occur and are transported to the chromosomes during recovery, their composition would determine which snRNAs are present on the loops. In any case, useful information should come from additional in situ hybridization experiments on the loops and B snurposomes of recovered oocytes.

ACKNOWLEDGMENTS

We thank Alan Weiner and Iain Mattaj for clones of human and *X. laevis* U2 snRNA genes, respectively. Supported by research grant GM 33397 from the National Institute of General Medical Sciences (J.G.G.) and a Schrödinger Fellowship from the Austrian Science Foundation (M.J.). J.G.G. is American Cancer Society Professor of Developmental Genetics.

REFERENCES

Angelier, N., Bonnanfant-Jais, M.-L., Herberts, C., Lautredou, N., Moreau, N., N'Da, E., Penrad-Mobayed, M., Rodriguez-Martin, M.-L., and Sourrouille, P. (1990). Chromosomes of amphibian oocytes as a model for gene expression: significance of lampbrush loops. Int. J. Dev. Biol. *34*, 69–80.

Beyer, A.L., and Osheim, Y.N. (1988). Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. Genes & Dev. *2*, 754–765.

Bittner, M., Kupferer, P., and Morris, C.F. (1980). Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose or nitrocellulose sheets. Anal. Biochem. *102*, 459–471.

Callan, H.G. (1986). Lampbrush Chromosomes, New York: Springer-Verlag.

Callan, H.G., and Gall, J.G. (1991). Association of RNA with the B and C snurposomes of Xenopus oocyte nuclei. Chromosoma *101*, 69–82.

Cazenave, C., Chevrier, M., Thuong, N.T., and Hélène, C. (1987). Rate of degradation of [α] and [β]-oligodeoxynucleotides in *Xenopus* oocytes. Implications for anti-messenger strategies. Nucleic Acids Res. *15*, 10507–10521.

Chin, D.J., Green, G.A., Zon, G., Szoka, F.C., and Straubinger, R.M. (1990). Rapid nuclear accumulation of injected oligodeoxyribonucleotides. New Biologist *2*, 1091–1100.

Colman, A. (1990). Antisense strategies in cell and developmental biology. J. Cell Sci. *97*, 399–409.

DiMario, P.J., Bromley, S.E., and Gall, J.G. (1989). DNA-binding proteins on lampbrush chromosome loops. Chromosoma *97*, 413–420.

Fakan, S., Leser, G., and Martin, T.E. (1986). Immunoelectron microscope visualization of nuclear ribonucleoprotein antigens within spread transcription complexes. J. Cell Biol. *103*, 1153–1157.

Flannery, A.V., and Hill, R.S. (1988). The effect of heat shock on the morphology of amphibian lampbrush chromosomes. Exp. Cell Res. *177*, 9–18.

Fu, X.-D., and Maniatis, T. (1990). Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. Nature *343*, 437–441.

Gall, J.G. (1991). Organelle assembly and function in the amphibian germinal vesicle. In: Advances in Developmental Biochemistry, Vol. 1, ed. P. Wassarman, Greenwich, CT: JAI Press, 1–29.

Gall, J.G., and Callan, H.G. (1989). The sphere organelle contains small nuclear ribonucleoproteins. Proc. Natl. Acad. Sci. USA *86*, 6635–6639.

Gall, J.G., Callan, H.G., Wu, Z., and Murphy, C. (1991). Lampbrush chromosomes. In: Methods in Cell Biology, Vol. 36, ed. B.K. Kay and H.B. Peng, New York: Academic Press, 149–166.

Guthrie, C., and Patterson, B. (1988). Spliceosomal snRNA. Annu. Rev. Genet. *22*, 387–419.

Hamm, J., Dathan, N.A., and Mattaj, I.W. (1989). Functional analysis of mutant *Xenopus* U2 snRNAs. Cell *59*, 159–169.

Izawa, M., Allfrey, V.G., and Mirsky, A.L. (1963). The relationship between RNA synthesis and loop structure in lampbrush chromosomes. Proc. Natl. Acad. Sci. USA *49*, 544–551.

Kay, B.K. (1991). Appendix B. Injection of oocytes and embryos. In: Methods in Cell Biology, Vol. 36, ed. B.K. Kay and H.B. Peng, New York: Academic Press, 663–669.

Kumar, A., Williams, K.R., and Szer, W. (1986). Purification and domain structure of core hnRNP proteins A1 and A2 and their relationship to single-stranded DNA-binding proteins. J. Biol. Chem. *261*, 11266–11273.

Lacroix, J.C., Azzouz, R., Boucher, D., Abbadie, C., Pyne, C.K., and Charlemagne, J. (1985). Monoclonal antibodies to lampbrush chromosome antigens of Pleurodeles waltlii. Chromosoma *92*, 69–80.

Leser, G., and Martin, T.E. (1987). The major protein components of hnRNP complexes. In: DNA: Protein Interactions and Gene Regulations, ed. E.B. Thompson and J. Papaconstantinou, Austin, TX: Univ. of Texas Press, 97–106.

Loones, M. T. (1979). In vivo effects of γ-irradiation on the functional architecture of the lampbrush chromosomes in Pleurodeles (Amphibia, Urodela). Chromosoma *73*, 357–368.

Mancino, G., Nardi, I., Corvaja, N., Fiume, L., and Marinozzi, V. (1971). The effects of α-amanitin on Triturus lampbrush chromosomes. Exp. Cell Res. *64*, 237–239.

Maniatis, T., and Reed, R. (1987). The role of small nuclear ribonucleoprotein particles in pre-mRNA splicing. Nature *325*, 673–678.

Martin, T.E., and Okamura, C.S. (1981). Immunocytochemistry of nuclear hnRNP complexes. In: The Cell Nucleus, Vol. 9, ed. H. Busch, New York: Academic Press, 119–144.

Moreau, N., Angelier, N., Bonnanfant-Jais, M.-L., Gounon, P., and Kubisz, P. (1986). Association of nucleoplasmin with transcription products as revealed by immunolocalization in the amphibian oocyte. J. Cell Biol. *103*, 683–690.

Osheim, Y.N., Miller, O.L., and Beyer, A.L. (1985). RNP particles at splice junction sequences on Drosophila chorion transcripts. Cell *43*, 143–151.

Pan, Z.-Q., and Prives, C. (1988). Assembly of functional U1 and U2 human-amphibian hybrid snRNPs in *Xenopus laevis* oocytes. Science *241*, 1328–1331.

Pan, Z.-Q., and Prives, C. (1989). U2 snRNA sequences that bind U2-specific proteins are dispensable for the function of U2 snRNP in splicing. Genes & Dev. *3*, 1887–1898.

Piñol-Roma, S., Choi, Y.D., Matunis, M.J., and Dreyfuss, G. (1988). Immunopurification of heterogeneous nuclear ribonucleoprotein par-

ticles reveals an assortment of RNA-binding proteins. Genes & Dev. *2*, 215–227.

Piñol-Roma, S., Swanson, M.S., Gall, J.G., and Dreyfuss, G. (1989). A novel heterogeneous nuclear RNP protein with a unique distribution on nascent transcripts. J. Cell Biol. *109*, 2575–2587.

Prives, C., and Foukal, D. (1991). The use of oligonucleotides for antisense experiments in *Xenopus laevis* oocytes. In: Methods in Cell Biology, Vol. 36, ed. B.K. Kay and H.B. Peng, New York: Academic Press, 185–210.

Riva, S., Morandi, C., Tsoulfas, P., Pandolfo, M., Biamonti, G., Merrill, B., Williams, K.R., Multhaup, G., Beyreuther, K., Werr, H., Henrich, B., and Schäfer, K.P. (1986). Mammalian single-stranded DNA binding protein UP I is derived from the hnRNP core protein A1. EMBO J. *5*, 2267–2273.

Rodriguez-Martin, M.L., Herberts, C., Moreau, N., and Angelier, N. (1989). Effects of in vivo heat treatment on lampbrush chromosome structure in amphibian oocytes. Exp. Cell Res. *185*, 546–550.

Roth, M.B., and Gall, J.G. (1987). Monoclonal antibodies that recognize transcription unit proteins on newt lampbrush chromosomes. J. Cell Biol. *105*, 1047–1054.

Ruby, S.W., and Abelson, J. (1991). Pre-mRNA splicing in yeast. Trends Genet. *7*, 79–85.

Sass, H., and Pederson, T. (1984). Transcription-dependent localization of U1 and U2 small nuclear ribonucleoproteins at major sites of gene activity in polytene chromosomes. J. Mol. Biol. *180*, 911–926.

Scheer, U. (1987). Structure of lampbrush chromosome loops during different states of transcriptional activity as visualized in the presence of physiological salt concentrations. Biol. Cell. *59*, 33–42.

Scheer, U., Hinssen, H., Franke, W.W., and Jockusch, B.M. (1984). Microinjection of actin-binding proteins and actin antibodies demonstrates involvement of nuclear actin in transcription of lampbrush chromosomes. Cell *39*, 111–122.

Schultz, L.D., Kay, B.K., and Gall, J.G. (1981). In vitro RNA synthesis in oocyte nuclei of the newt Notophthalmus. Chromosoma *82*, 171–187.

Scott, S.E.M., and Sommerville, J. (1974). Location of nuclear proteins on the chromosomes of newt oocytes. Nature *250*, 680–682.

Smith, R.C., Bement, W.M., Dersch, M.A., Dworkin-Rastl, E., Dworkin, M.B., and Capco, D.G. (1990). Nonspecific effects of oligodeoxynucleotide injection in *Xenopus* oocytes: a reevaluation of previous D7 mRNA ablation experiments. Development *110*, 769–779.

Snow, M.H.L., and Callan, H.G. (1969). Evidence for a polarized movement of the lateral loops of newt lampbrush chromosomes during oogenesis. J. Cell Sci. *5*, 1–25.

Sommerville, J., Crichton, C., and Malcolm, D. (1978). Immunofluorescent localization of transcriptional activity on lampbrush chromosomes. Chromosoma *66*, 99–114.

Steitz, J.A., Black, D.L., Gerke, V., Parker, K.A., Krämer, A., Frendeway, D., and Keller, W. (1988). Functions of the abundant U-snRNPs. In: Structure and Function of Major and Minor Small Nuclear Ribonucleoproteins, ed. M. Birnstiel, New York: Springer, 115–154.

Vazquez-Nin, G.H., Echeverria, O.M., Fakan, S., Leser, G., and Martin, T.E. (1990). Immunoelectron microscope localization of snRNPs in the polytene nucleus of salivary glands of Chironomus thummi. Chromosoma *99*, 44–51.

Wallace, R.A., Jared, D.W., Dumont, J.N., and Sega, M.W. (1973). Protein incorporation by isolated amphibian oocytes: III. Optimum incubation conditions. J. Exp. Zool. *184*, 321–333.

Wassarman, P.M., Hollinger, T.G., and Smith, L.D. (1974). Ribonuclease H activity in germinal vesicles of oocytes from Rana pipiens. Exp. Cell Res. *89*, 410–412.

Wu, Z., Murphy, C., Callan, H.G., and Gall, J.G. (1991). Small nuclear ribonucleoproteins and heterogeneous nuclear ribonucleoproteins in the amphibian germinal vesicle: loops, spheres, and snurposomes. J. Cell Biol. *113*, 465–483.