The EMBO Journal vol.3 no.9 pp.1939–1943, 1984

Histone RNA in amphibian oocytes visualized by *in situ* hybridization to methacrylate-embedded tissue sections

M.Jamrich, K.A.Mahon, E.R.Gavis[1] and J.G.Gall[2]

Laboratory of Molecular Genetics, National Institutes of Health, Bethesda, MD 20205, [1]Department of Biochemistry, Stanford University, Stanford, CA 94305, and [2]Department of Embryology, Carnegie Institution of Washington, Baltimore, MD 21210, USA

Communicated by W.J.Gehring

We present an *in situ* hybridization method for detecting cellular RNAs in tissue sections using methacrylate as the embedding medium. The technique offers the advantage of superior morphological preservation compared with previously published procedures. Since sections can be cut 1 μm or less in thickness, full advantage is taken of the short path length of ^{3}H electrons. Applying this procedure to developing amphibian oocytes, we investigated the accumulation and localization of RNA complementary to the histone genes and their adjacent spacers. Histone RNA begins to accumulate in the cytoplasm of late pachytene-early diplotene oocytes, rapidly reaching a maximum concentration during Dumont stage 1. After this stage the concentration of histone RNA declines. RNA transcribed from histone coding regions is located almost exclusively in the cytoplasm of oocytes. Transcripts of the spacer regions, which are known to be synthesized on oocyte lampbrush chromosomes, do not accumulate in the oocytes. [^{3}H]RNA complementary to U2 small nuclear RNA, used in these experiments as a control, hybridized predominantly to the nucleus of the oocytes.

Key words: in situ hybridization/histone RNA/mRNA localization/oogenesis/tissue sections

Introduction

In situ hybridization to tissue sections enjoys an increasing popularity as a tool for localizing cellular RNAs (Harding *et al.*, 1977; Capco and Jeffery, 1978; Angerer and Angerer, 1981; Scheller *et al.*, 1982; Akam, 1983; Gee *et al.*, 1983; Hafen *et al.*, 1983; Levine *et al.*, 1983; Cox *et al.*, 1984). Most of the previously published methods focused on improving the sensitivity of the hybridization technique, using frozen or paraffin-embedded tissues. Here we describe an *in situ* hybridization procedure based on the use of methacrylate as the embedding medium. The morphological detail is superior to that of frozen or paraffin-embedded sections, although the sensitivity of RNA detection is somewhat reduced.

We used this technique to monitor the accumulation and distribution of histone RNA in amphibian oocytes. Amphibian oocytes store large quantities of RNA for use in early embryogenesis (reviewed by Davidson, 1977), and the histone RNAs contribute significantly to the population of non-ribosomal RNA in the cell. Several laboratories have shown that histones belong to the most prominent products of *in vitro* translated mRNA from oocytes in several developmental stages (Levenson and Marcu, 1976; Destree *et al.*, 1977; Ruderman and Pardue, 1978; Ruderman *et al.*, 1979). Van Dongen *et al.* (1981) found that a single oocyte contains

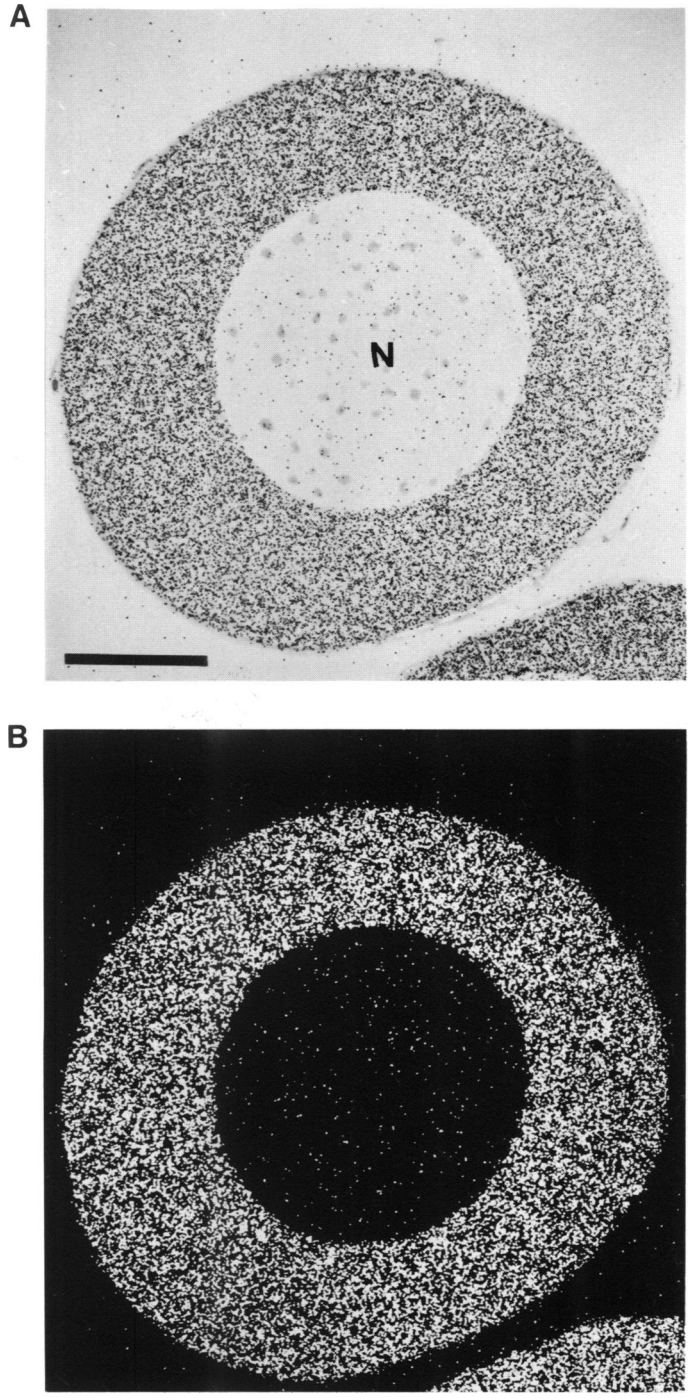

Fig. 1. (A) A 1 μm section of a *Xenopus* oocyte (Dumont stage 1) hybridized with ^{3}H-labeled RNA complementary to one strand of the histone gene clone XlhlC. The nucleus (N) is not detectably labeled above background, whereas the cytoplasm is uniformly and heavily labeled. Probe-specific activity 5 x 10^7 d.p.m./μg. Exposure 3 weeks. Brightfield illumination. Bar = 100 μm. **(B)** Same preparation viewed in darkfield to accentuate the silver grains.

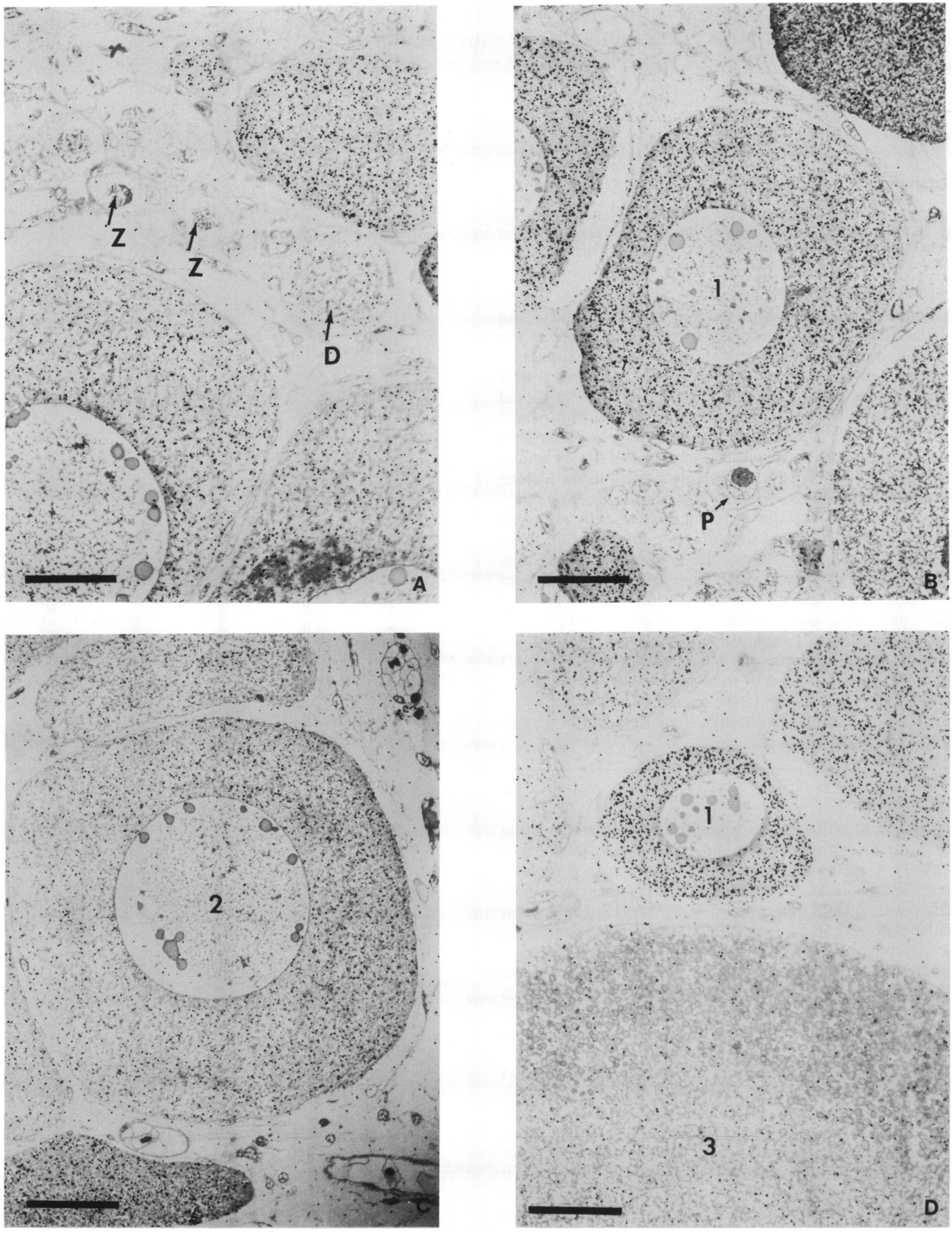

Fig. 2. (A–D) *Xenopus* oocytes hybridized with the same histone gene probe as in Figure 1. Exposure 10 days. The selected oocytes are from the same 1 μm section and are labeled as follows: Z, zygotene; P, pachytene; D, diplotene; 1,2,3, oocytes of Dumont stages 1, 2 and 3, respectively. Bar = 100 μm.

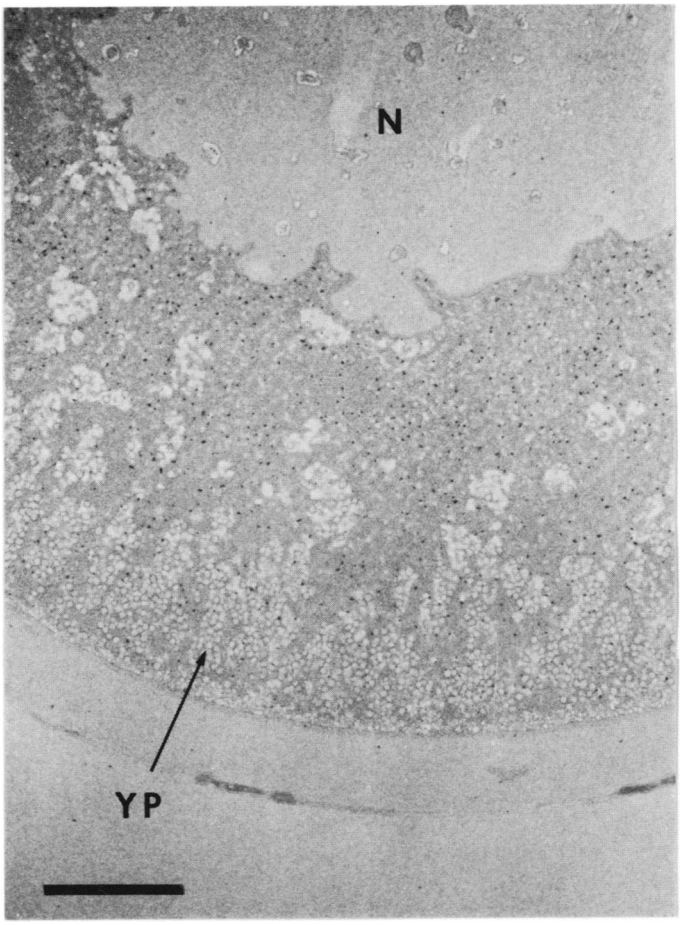

Fig. 3. *Xenopus* oocyte after the beginning of yolk deposition (Dumont stage 3). The yolk platelets (YP) occupy much of the peripheral cytoplasm, resulting in a dilution of histone RNA in that region. Histone gene probe as in Figure 1. Exposure 2 weeks. Brightfield illumination. Bar = 100 μm.

as many as 5 x 10^8 copies of histone H3 mRNA. They also concluded that the accumulation of histone RNA begins in oocytes at Dumont stage 1 (Dumont, 1972). This conclusion was based on the presence of histone RNA in a segment of ovary containing only clear, yolk-free oocytes. As pointed out by the authors, the presence of a large number of follicle cells in such preparations introduced uncertainty in the interpretation. A detailed analysis of different sizes of Dumont stage 1 oocytes was not performed, since manual isolation of such small oocytes for biochemical purposes is not feasible. To obtain a more detailed picture of the stage-specific accumulation of histone RNA in amphibian oocytes, we studied histone RNA in sections of developing oocytes of the toad, *Xenopus laevis* and the newt, *Notophthalmus viridescens*. We compared the accumulation and nucleocytoplasmic distribution of two coordinately transcribed sequences: RNA complementary to the histone coding regions and RNA complementary to the spacer sequences separating the individual histone genes. The spacer sequences are known to be transcribed on newt lampbrush chromosomes (Diaz *et al.*, 1981), but the significance of their expression is not understood.

Results

[^{3}H]RNA complementary to single-stranded M13 clones of *Xenopus* histone genes H2A, H2B, H3, H4 and their adjacent spacers was hybridized to sections of methacrylate embedded *Xenopus* ovaries. The M13 clones were derived from a genomic clone, pXlhlC (Zernik *et al.*, 1980) as described in Materials and methods. Histone genes H2B, H3 and H4 on this segment of DNA are in the opposite orientation to histone gene H2A (Perry and Roeder, personal communication). Consequently cRNA made from either of the two complementary strands should recognize some histone RNA in molecular hybridizations. This is indeed the case, and the results of hybridizing oocyte sections with cRNA complementary to the H2B, H3 and H4 sequences is shown in Figure 1. One can see that in Dumont stage 1 oocytes the histone RNA is localized almost exclusively in the cytoplasm. The pattern of hybridization is identical using cRNA made from the opposite strand of this sequence with the expected difference in intensity of labeling (not shown). We performed a similar hybridization to sections of *Notophthalmus* ovary using [^{3}H]cRNA to newt clone Nv51-9-18 (Diaz *et al.*, 1981) which contains the newt histone genes H1, H2A and H3 as well as their adjacent spacers (Stephenson *et al.*, 1981). We obtained a similar, predominantly cytoplasmic labeling. To exclude the possibility that the hybridization is due to spacer sequences, we hybridized cRNA made from *Xenopus* histone clones to sections of *Notophthalmus* ovary. The hybiridization resulted in an identical pattern of labeling (not shown). Since the *Xenopus* and *Notophthalmus* histone clones have similar coding regions but different spacers, we conclude that the hybridization is due to the histone coding regions. Additional evidence will be presented in a later part of this paper.

The predominantly cytoplasmic hybridization of the histone RNA is not a general feature of all RNAs at this developmental stage. We hybridized oocyte sections with [^{3}H]RNA complementary to the coding region of *Xenopus* U2 small nuclear RNA (Mattaj and Zeller, 1983). Most of the silver grains resulting from the hybridization were localized in the nucleus (not shown).

The accumulation of histone RNA starts very early in the development of the oocytes. Figure 2 shows a range of oocyte stages from a section of ovary hybridized with cRNA to *Xenopus* histone genes. Although zygotene oocytes do not show detectable accumulation of histone RNA, the late pachytene to early diplotene oocytes do. A rapid increase in histone RNA concentration is evident during stage 1. Later, however, the density of labeling declines. During all previtellogenic stages the histone RNA is evenly distributed in the cytoplasm of the oocytes. The situation changes after the onset of vitellogenesis. The deposition of yolk platelets in the peripheral cytoplasm results in a dilution of the histone RNA in that region (Figure 3). The difference in the concentration of histone RNA in stage 1 and in later vitellogenic oocytes is shown strikingly in Figure 4.

The transcription of histone genes on lampbrush chromosome loops is unusual in that the spacers between the coding regions are actively transcribed (Diaz *et al.*, 1981; Gall *et al.*, 1983). In order to trace the fate of the spacer transcripts, we hybridized newt oocyte sections with [^{3}H]RNA complementary to newt clone Nv51-7, which contains the spacer DNA separating histone genes H4 and H2A. We did not see labeling of oocytes above the background level using exposure times of 2 weeks (not shown). This suggests that the RNA resulting from transcription of spacer sequences, although readily demonstrable on isolated lampbrush chromosomes, does not accumulate significantly either in the nucleus or cytoplasm. Furthermore, this negative result provides additional support for the assumption that the labeling seen in Figures 1–4 is due to the coding regions of the clones.

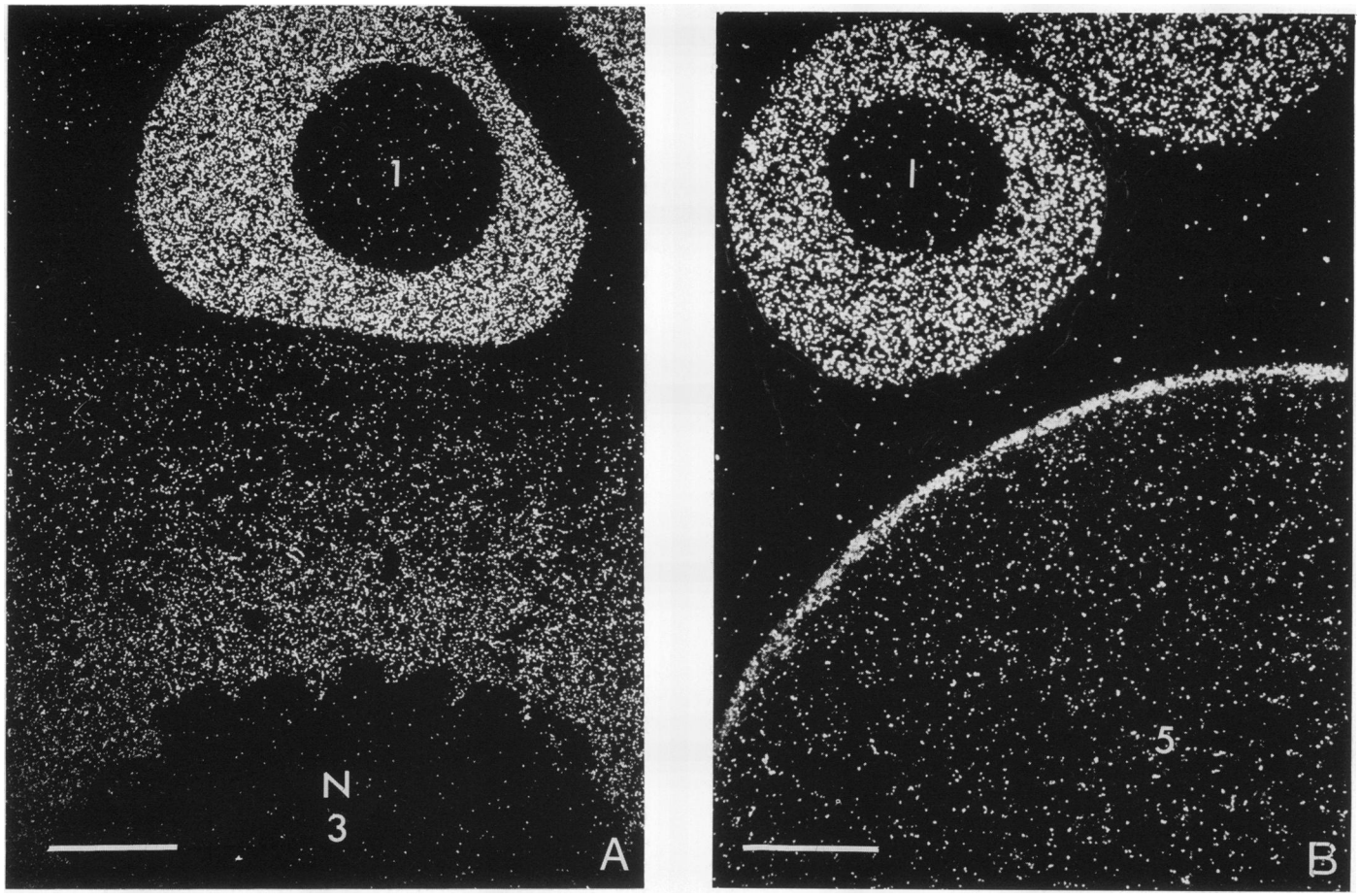

Fig. 4. (A,B) *Xenopus* oocytes of several sizes hybridized with a histone gene probe as in Figure 1. Exposure 3 weeks. Darkfield illumination. 1, 3 and 5 are oocytes in Dumont stages 1, 3 and 5, respectively. Note the dramatic reduction in histone RNA concentration in the cytoplasm as the oocyte enlarges beyond stage 1. The bright ring around the stage 5 oocyte is due to melanin granules in the cortex, not silver grains. Bar = 100 μm.

Discussion

Sections of methacrylate-embedded material have been used successfully in the past for structural analysis of biological materials. Our adaptation of this procedure for *in situ* hybridization resulted in improvement of the morphological detail of the hybridized sections compared with paraffin-embedded or frozen tissues. Utilizing this procedure we analyzed the accumulation of histone RNA in developing amphibian oocytes. We found that the accumulation of histone RNA starts in late pachytene to early diplotene oocytes. The rapid accumulation of histone RNA in stage 1 oocytes results in a strong autoradiographic signal at this stage. Subsequently there is a gradual decrease in the concentration of silver grains over the sections. Golden (1980) and van Dongen *et al.* (1981) found by Northern blot analysis that the amount of histone RNA per oocyte remains constant after stage 2. A constant amount of histone RNA in a growing oocyte should give a decreasing concentration of RNA and a decreasing intensity of hybridization, measured as silver grains per unit area of cytoplasm. Our observations are in qualitative agreement with a constant amount of histone RNA throughout later oogenesis, although we have not attempted to quantitate our autoradiographs. Several studies indicate that not only histone RNA but the total poly(A)$^+$ RNA population reaches a plateau early in oogenesis (Rosbash and Ford, 1974; Dolecki and Smith, 1979; Golden *et al.*, 1980). Nevertheless, the lampbrush chromosomes at this stage and well beyond produce large amounts of RNA, including histone RNA (Diaz *et al.*, 1981). As pointed out by Anderson *et al.* (1982), the store of poly(A)$^+$ RNA in an oocyte is so large that very active transcription is needed simply to counteract a low rate of turnover.

We can calculate the sensitivity of our autoradiographic technique by using the estimate of 5×10^8 histone H3 mRNA molecules per oocyte given by van Dongen *et al.* (1981). The volume of an oocyte reaches 5×10^8 μm^3 at a diameter of just under 1000 μm (Dumont stage 4–5). Since part of the total volume is nuclear and part of the cytoplasmic volume is occupied by yolk, the concentration of histone H3 mRNA in such an oocyte will be >1 molecule/μm^3 of cytoplasm but probably <2 molecules/μm^3. The oocytes in Figure 4A and B are approximately this size. Since our probe reacts with the mRNAs for histones H2B and H4 in addition to H3, and these mRNAs average ~500 nucleotides, the autoradiographs detect ~3–6 mRNA molecules/μm^3 or 4500–9000 nucleotides/μm^3 after an exposure of 3 weeks. In a recent paper Cox *et al.* (1984) demonstrated histone mRNA in paraffin sections of sea urchin eggs and embryos using an RNA probe transcribed from the bacteriophage SP6 promoter. In the 1–2 cell stage the total mRNA concentration is ~100 molecules/μm^3 (derived from their estimate of 1.27 pg of mRNA in a volume of 3.9×10^4 μm^3; the volume is that of the fixed egg). A readily detectable autoradiographic signal, comparable with our Figure 4A, was seen after a 9 h exposure (their Figure 9B). When allowance is made for differences in

exposure time, it appears that our method is less sensitive than that of Cox *et al.* by a factor of 2–4.

At no stage were we able to detect significant accumulation of histone RNA in the nucleus, although occasionally a cluster of grains was seen over a localized area of the nucleus, consistent with hybridization to lampbrush chromosome loops. These results suggest that the transcripts of histone genes are rapidly processed and transported into the cytoplasm. This conclusion is supported by unpublished S1 nuclease experiments from our laboratory. Under conditions where cytoplasmic RNA isolated from a single oocyte gave easily detectable protection of a histone gene probe, RNA from 300 germinal vesicles failed to do so. That the almost exclusively cytoplasmic location of histone RNA is not a technical artifact nor a general feature of all RNAs present at this stage was shown by our observations on U2 nuclear RNA. As expected, *in situ* hybridization with a U2 probe gave predominantly nuclear label. This observation agrees well with the work of Zeller *et al.* (1983), who found that antibodies against a small nuclear ribonucleoprotein particle (snRNP) reacted at this stage predominantly with the nucleus of the oocyte.

As mentioned in the Results section, the deposition of yolk granules in the cytoplasm of early vitellogenic oocytes causes a gradient of histone RNA. It should be emphasized, however, that this gradient does not reflect selective sequestration of histone RNA in a specific part of the cytoplasm. A similar gradient is well known from staining of the total RNA population at this stage.

Note

In recent experiments we have found that *Drosophila* ovaries fixed in formaldehyde and embedded in methacrylate hybridize much less efficiently than the same tissue prepared by freeze substitution in ethanol and embedded in methacrylate. The effect appears to be specific to the combination of formaldehyde and methacrylate, because tissue fixed in formaldehyde but embedded in polystyrene (Frangioni and Borgioloi, 1979) hybridizes at the higher efficiency.

Materials and methods

Preparation of sectioned tissue for hybridization

Segments of ovary from the toad, *X. laevis*, or the newt, *N. viridescens*, were fixed in 5% acetic acid, 2% formaldehyde and 250 mM NaCl for 30–60 min. Alternatively the fixation can be done for 15 min in 5% formaldehyde in phosphate-buffered saline. The latter fixative results in better retention of RNA but in less favorable morphology of oocytes. An equal volume of 95% ethanol was added dropwise to the fixative over a period of 15 min. The tissue was then treated for 1 h each in 70%, 95% and 100% ethanol, followed by 1 h incubation in a mixture of equal volumes of 100% ethanol and methacrylate solution (9 parts butyl methacrylate and 1 part methyl methacrylate). Finally the tissue was transferred into methacrylate solution containing 1% benzoyl peroxide. The specimens were incubated in this solution for 1 h at room temperature before being polymerized in an oven at 65°C for 16–24 h. This step was usually done in polyethylene capsules. The polymerized block was cooled to room temperature, removed from the capsule, trimmed, and sectioned on a Sorvall Porter-Blum microtome. Sections were cut at 0.5–1.0 μm thickness, transferred onto a drop of water on a subbed slide, spread with xylene or chloroform vapor, and dried on a warming plate at 65°C. The slides were dipped for 15 min into xylene, washed in 100% ethanol and dried. In some cases slides were passed through a decreasing ethanol series and held in water until just before adding the hybridization solution.

Freshly dissected *Drosophila* ovaries were immersed in 100% ethanol at −78°C (dry ice) and were held for 1–2 days at the low temperature. They were allowed to warm slowly to room temperature before infiltration with methacrylate monomer as described above. This much abbreviated freeze-substitution procedure gave excellent preservation of *Drosophila* oocytes, but will probably need to be modified for the larger amphibian oocytes.

Hybridization of ^{3}H-labeled RNA to tissue sections

[^{3}H]RNA was dried down and dissolved in 40% formamide, 4 x SSC, 10% dextran sulfate, and 1 x Denhardt's solution (Denhardt, 1966) at a concentration of ~10^5 c.p.m./μl (determined by scintillation counting on a nitrocellulose filter in toluene fluor). 5 μl of this solution was placed over the sections; a coverslip was added and sealed with rubber cement. The preparation was hybridized for 16–24 h at 42°C. After hybridization the slides were washed in 2 x SSC, 1% Triton-X for 2 h at room temperature, followed by 1 h in 0.2 x SSC at 60°C. The slides were dehydrated through an ethanol series and air dried. They were then dipped in Kodak NTB-2 liquid emulsion and exposed for various times as indicated in the figure legends. For further details of technique see Pardue and Gall (1975) and Angerer and Angerer (1981).

Preparation of cloned DNA and radioactive probes

The *Xenopus* histone clone, pXlhlC, was described in Zernik *et al.* (1980). The newt clones Nv51-9-18 and Nv51-7 were described in Gall *et al.* (1981). The M13 subclones were made by standard procedures and the cRNA was prepared as described in Pardue and Gall (1975) and Diaz *et al.* (1981).

Acknowledgements

We thank Dr. R.Roeder for providing clone pXlhlC and Dr. I.Mattaj for the *Xenopus* U2 clone. During part of this research M.J. was a fellow of the Jane Coffin Childs Memorial Fund for Medical Research. Supported by grants from the NIH (GM 33397) and American Cancer Society (CD 37) to J.G.Gall.

References

Akam,M.E. (1983) *EMBO J.*, **2**, 2075-2084.
Anderson,D.M., Richter,J.D., Chamberlin,M.E., Price,D.H., Britten,R.J., Smith,L.D. and Davidson,E.H. (1982) *J. Mol. Biol.*, **155**, 281-309.
Angerer,L.M. and Agerer,R.C. (1981) *Nucleic Acids Res.*, **9**, 2819-2840.
Capco,D.G. and Jeffery,W.R. (1978) *Dev. Biol.*, **67**, 137-151.
Cox,K.H., DeLeon,D.V., Angerer,L.M. and Angerer,R.C. (1984) *Dev. Biol.*, **101**, 485-502.
Davidson,E.H. (1977) *Gene Activity in Early Development*, 2nd ed., published by Academic Press, NY.
Denhardt,D.T. (1966) *Biochem. Biophys. Res. Commun.*, **23**, 641-646.
Destree,O.H.J., Haenni,A.-L. and Birnstiel,M.L. (1977) *Nucleic Acids Res.*, **4**, 801-811.
Diaz,M.O., Barsacchi-Pilone,G., Mahon,K.A. and Gall,J.G. (1981) *Cell*, **24**, 649-659.
Dolecki,G.J. and Smith,L.D. (1979) *Dev. Biol.*, **69**, 217-236.
Dumont,J.N. (1972) *J. Morphol.*, **136**, 153-180.
Frangioni,G. and Borgioli,G. (1979) *Stain Technol.*, **54**, 167-172.
Gall,J.G., Diaz,M.O., Stephenson,E.C. and Mahon,K.A. (1983) in Subtelny, S. and Kafatos,F. (eds.), *Gene Structure and Regulation in Development*, Alan R. Liss, NY, pp. 137-146.
Gee,C.E., Chen,C.-L.C., Roberts,J.L., Thompson,R. and Watson,S.J. (1983) *Nature*, **306**, 374-376.
Golden,L. (1980) Ph.D. Thesis, Brandeis University, Waltham, MA.
Golden,L., Schafer,U. and Rosbash,M. (1980) *Cell*, **22**, 835-844.
Hafen,E., Levine,M., Garber,R.L. and Gehring,W.J. (1983) *EMBO J.*, **2**, 617-623.
Harding,J.D., MacDonald,R.J., Przybyla,A.E., Chirgwin,J.M., Pictet,R.L. and Rutter,W.J. (1977) *J. Biol. Chem.*, **252**, 7391-7397.
Levenson,R.G. and Marcu,K.B. (1976) *Cell*, **9**, 311-322.
Levine,M., Hafen,E., Garber,R.L. and Gehring,W.J. (1983) *EMBO J.*, **2**, 2037-2046.
Mattaj,I.W. and Zeller,R. (1983) *EMBO J.*, **2**, 1883-1891.
Pardue,M.L. and Gall,J.G. (1975) *Methods Cell Biol.*, **10**, 1-16.
Rosbash,M. and Ford,P.J. (1974) *J. Mol. Biol.*, **85**, 87-101.
Ruderman,J.V. and Pardue,M.L. (1978) *J. Biol. Chem.*, **253**, 2018-2025.
Ruderman,J.V., Woodland,H.R. and Sturgess,E.A. (1979) *Dev. Biol.*, **71**, 71-82.
Scheller,R.H., Jackson,J.F., McAllister,L.B., Schwartz,J.H., Kandel,E.R. and Axel,R. (1982) *Cell*, **28**, 707-719.
Stephenson,E.C., Erba,H.P. and Gall,J.G. (1981) *Cell*, **24**, 639-647.
van Dongen,W., Zaal,R., Moorman,A. and Destree,O. (1981) *Dev. Biol.*, **86**, 303-314.
Zeller,R., Nyffenegger,T. and De Robertis,E.M. (1983) *Cell*, **32**, 425-434.
Zernik,M., Heintz,N., Boime,I. and Roeder,R.G. (1980) *Cell*, **22**, 807-815.

Received on 10 May 1984; revised on 15 June 1984