Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 2056–2060, March 1994
Developmental Biology

An3 mRNA encodes an RNA helicase that colocalizes with nucleoli in *Xenopus* oocytes in a stage-specific manner

RAJ GURURAJAN*, LORI MATHEWS†, FRANK J. LONGO†, AND DANIEL L. WEEKS*‡

Departments of *Biochemistry and †Anatomy, University of Iowa, Iowa City, IA 52242

Communicated by Joseph G. Gall, December 10, 1993 (received for review October 18, 1993)

ABSTRACT **An3 is a maternal mRNA localized to the animal hemisphere of oocytes and early embryos. We have analyzed the enzymatic activity and the subcellular localization of the protein encoded by An3 mRNA during *Xenopus* oogenesis. Antibodies raised using recombinant full-length and truncated An3 protein recognized a single protein in *Xenopus* and single proteins from HeLa cells, *Drosophila*, mouse testes, and *Saccharomyces cerevisiae*. An3 protein immunoprecipitated from stage IV and stage VI oocytes had ATP-dependent RNA helicase activity. The subcellular location of An3 protein changed during oocyte development. In previtellogenic oocytes, An3 was present throughout the nucleus; cytoplasmic localization was relatively sparse. Nuclear localization in mid-vitellogenic oocytes was primarily nucleolar; cytoplasmic staining increased relative to earlier stages. In stage VI oocytes, An3 protein was detected only in the cytoplasm. The temporal change in An3 protein localization is consistent with a role in the production of large maternal pools of rRNA during oogenesis.**

In *Xenopus laevis*, oocyte development may take several months and is divided into six stages based on size, morphology, and biological activity (1). Some hallmarks of oogenesis have been recently reviewed (2) and can be summarized as follows. At stage I, oocytes are 50–300 μm in diameter and transparent. As oogenesis proceeds, vitellogenesis begins early in stage III (size, 450–600 μm), as does cortical pigmentation. By stage VI, the oocyte measures 1200–1300 μm in diameter. The dark pigmentation which marks the animal hemisphere of the oocyte is an obvious visual indicator of the animal/vegetal axis. Different embryonic tissues arise from specific areas along the animal/vegetal axis and in oocytes, yolk platelets, some maternal RNA, protein, and even the oocyte nucleus, referred to as the germinal vesicle (GV), are unevenly distributed with respect to this axis. Maternal mRNA synthesis begins in late stage I, increases during stages II and III, and declines to maintain equilibrium levels of mRNA thereafter. rRNA pools are generated by the transcription of amplified copies of rRNA genes. As many as a million copies of these genes are present in distinctive nuclear structures called extranucleoli. Transcription from the extranucleoli begins in stage II, increases to its highest rate in stages III and IV, and declines as the oocyte reaches stage VI.

Among the maternal mRNAs present in oocytes and passed to developing embryos are a small number that are unevenly distributed along the animal/vegetal axis. Maternal mRNAs localized to the vegetal hemisphere include two transforming growth factor β (TGFβ)-like proteins [Vg1 (3) and TGFβ5 (4)] and a protein with homology to *Drosophila* nanos (5). Although the function of these localized mRNAs is still not certain, the Vg1 protein may serve as the maternal inducer of embryonic mesoderm formation. This process may be controlled both by the vegetal localization of the maternal pool of Vg1 protein and by spatially restricted activation of the protein (6).

mRNAs localized to the animal hemisphere include those encoding G proteins (7), a histone-like protein (8), an unusual ubiquitin-fusion zinc finger protein [An1 (9)], a subunit of mitochondrial ATPase [An2 (10, 11)], and An3 (12). The protein encoded by the An3 mRNA has an ATP-binding domain that includes aspartic acid (D), glutamic acid (E), alanine (A), another aspartic acid (D), and other amino acid sequence motifs characteristic of the DEAD-box family of proteins (13). Some members of this family have ATP-dependent RNA helicase activity (13, 14) and cellular functions ranging from translation initiation to RNA processing. The role of the An3 protein during development has not been established.

We have used antibodies specific for An3 protein to show that it is present throughout oogenesis and has homologues in yeast, *Drosophila*, mouse, and human. An3 protein isolated by immunoprecipitation from *Xenopus* oocytes has ATP-dependent RNA helicase activity. An3 protein is both nuclear and cytoplasmic from oocyte stage I through stage V and colocalizes with extranucleoli between stages III and V. In stage VI, An3 protein is undetectable in the nucleus, even though extranucleoli are present, but is readily found in the cytoplasm. The timing, enzymatic activity, and localization of the An3 protein suggest a role in the processing or production of large stores of mature rRNA during oogenesis.

MATERIAL AND METHODS

Materials. Frogs (*X. laevis*) were purchased from Xenopus I (Ann Arbor, MI). Experiments using stage VI oocytes used animals that were injected with 200 units of pregnant-mare serum gonadotropin (PMSG; Sigma) at least 3 days prior to surgical removal. Injection with PMSG increases the number of stage VI oocytes by stimulating oocyte growth.

Western Blot Analysis. Oocytes from different stages (1) were treated with collagenase (0.2% collagenase in 0.1 M sodium phosphate, pH 7.4) and then manually defolliculated in MMR medium (15), homogenized by sonication in 0.1 M NaCl/1% (vol/vol) Triton X-100/1 mM phenylmethanesulfonyl fluoride/20 mM Tris·HCl at pH 7.6 [oocyte homogenization buffer (16)], and centrifuged. Appropriate volumes of the supernatant were mixed with 10% glycerol/25 mM EDTA/2% SDS/0.1 M dithiothreitol/0.2 M Tris·HCl, pH 6.8/0.1% bromophenol blue, boiled for 5 min, and loaded onto an SDS/10% polyacrylamide gel. The tissue samples from HeLa cells, whole *Drosophila*, mouse testes, and *Saccharomyces cerevisiae* were processed in a similar manner and 50–150 μg of total protein was loaded onto the SDS/

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GST, glutathione *S*-transferase; GV, germinal vesicle; PMSG, pregnant-mare serum gonadotropin.

‡To whom reprint requests should be addressed at: Department of Biochemistry, 4-403 Bowen Science Building, University of Iowa, Iowa City, IA 52242-1109.

polyacrylamide gel. The proteins were transferred to nitrocellulose by semidry electrophoretic transfer (ABN poly blot) and blocked with 10% nonfat milk in phosphate-buffered saline. Identical results were obtained when the primary antibody used was mouse polyclonal antiserum made against an An3 N-terminal glutathione *S*-transferase (GST) fusion protein or against full-length, nonfusion An3. The secondary antibody was anti-mouse IgG horseradish peroxidase conjugate (Sigma) at 1:10,000 dilution. The proteins were detected with the enhanced chemiluminescence (ECL) kit from Amersham.

Immunoprecipitation. Immunoprecipitations used protein A-Sepharose beads (Zymed) that had been preincubated in 0.1% bovine serum albumin and washed with oocyte homogenization buffer. Anti-An3 serum and control mouse serum were bound to protein A-Sepharose beads for 1 hr at 4°C, washed with oocyte homogenization buffer, and incubated with the oocyte supernatant for 4 hr at 4°C. The pelleted protein A-Sepharose beads were washed four times each with oocyte homogenization buffer and 1× helicase buffer (17 mM Hepes, pH 7.5/0.5 mM magnesium acetate/2 mM dithiothreitol) with or without 1 mM ATP.

Helicase Assays. The RNA substrate was transcribed from the plasmid PRP40 (ref. 17, plasmid and substrate preparation conditions provided by A. Pause and N. Sonenberg, McGill University, Montreal) by SP6 polymerase (Promega) as suggested by the supplier in the presence of 2 mM nonradioactive CTP in addition to 50 μCi of [α-^{32}P]CTP (800 Ci/mmol; 1 Ci = 37 GBq) and eluted from the gel with 2× standard saline citrate containing 1% SDS. A 10-bp duplex with 5′ and 3′ overhangs formed spontaneously during elution. RNA helicase activity was assayed using 0.18 ng of RNA substrate (specific activity, 2.2×10^7 cpm/μg) in a reaction mixture (20 μl) containing 4% (vol/vol) glycerol, 75 mM KCl, 40 units of RNasin (Promega), and 5 μg of tRNA in the presence or absence of 1 mM ATP. After incubation at 37°C for 40 min (15 min for the bacterial protein), the reaction was stopped by addition of 2.5 μl of a solution containing 50% glycerol, 2% SDS, and 20 mM EDTA, and products were analyzed by separation in an SDS/15% polyacrylamide gel and autoradiography.

Immunohistochemistry. Portions of ovaries were removed from PMSG-stimulated *Xenopus*, flash frozen in liquid nitrogen, cryosectioned at 5 μm, and fixed in cold (−20°C) acetone or methanol. After blocking with 1% bovine serum albumin in phosphate-buffered saline for 30 min, sections were incubated for 30 min each with polyclonal antibodies to An3 or monoclonal antibodies to fibrillarin (17C12 from M. Pollard, Salk Institute, La Jolla, CA). Secondary antibodies were rhodamine-conjugated (anti-An3) or fluorescein isothiocyanate-conjugated (anti-fibrillarin) (Pierce). Specimens were washed twice (5 min each) between and following antibody treatments, mounted in glycerol, and viewed with a Nikon fluorescence microscope with ×10 and ×60 (oil) objectives. For double labeling (Fig. 3 *B*, *C*, *G*, and *H*), blocked specimens were reacted according to the following scheme: primary antibody (e.g., anti-An3), wash, rhodamine-conjugated secondary antibody, wash, reblock (with mouse serum), primary antibody (anti-fibrillarin), wash, fluorescein-conjugated secondary antibody, wash, and mount.

RESULTS

Antibodies Specific for An3 Establish Its Production During Oogenesis and the Existence of Similar Protein in Other Species. To raise antibodies that recognize the *Xenopus* An3 protein, both the full coding sequence and the first 708 nucleotides of the coding sequence of An3 were cloned into bacterial expression plasmids. The full coding sequence was cloned in pET3b and the protein was synthesized in *Escherichia coli* (DE3)/pLysS (18). The sequence encoding the N-terminal region was cloned as a GST fusion into pGEX-2T (19) and the fusion protein was overproduced in *E. coli* TB1. After purification, the proteins were injected into mice. Polyclonal mouse serum resulting from each protein recognized a single 77-kDa protein in *Xenopus* oocytes and embryos. Protein extracts from oocytes fractionated by SDS/PAGE showed a gradual increase in the amount of An3 protein during oogenesis (Fig. 1). The specificity of the antibody in *Xenopus* led us to examine whether proteins with shared epitopes, and possibly shared functions, were present in other organisms. The recognition of a single prominent protein in yeast, *Drosophila*, mouse, and human suggests that An3 homologues are present in these organisms (Fig. 1).

An3 Protein Isolated as Fusion Proteins from Bacteria or Immunoprecipitated from Oocytes Has ATP-Dependent RNA Helicase Activity. The An3 protein from stages IV and VI oocytes was isolated by immunoprecipitation and assayed for RNA helicase activity (17, 20). Immunoprecipitation with both antibodies gave similar results. For comparison, purified GST–An3 fusion protein was also assayed (Fig. 2, lanes 11 and 12). The RNA substrate, in duplex form, was two 92-nucleotide RNA strands with a 10-nucleotide region of complementary sequence, containing both 3′ and 5′ single-stranded regions (17). The An3 protein from extracts of 10 stage IV or stage VI oocytes was able to unwind the duplex structure of an RNA substrate (Fig. 2, lanes 4 and 7). Helicase activity was ATP dependent, as An3 protein was unable to unwind the duplex structure in the absence of ATP (Fig. 2, lanes 5, 8, and 12). An3 isolated from oocytes and recombinant GST–An3 fusion protein unwound >80% of the duplex RNA during the assay.

An3 Protein Is Found in Oocyte Nucleoli During the Most Active Period of rRNA Synthesis. DEAD-box proteins may be cytoplasmic, like translation initiation factor eIF-4A, or nuclear, like the yeast PRP proteins involved in RNA splicing (13, 14). The subcellular localization of the An3 protein was examined during oogenesis by immunohistochemistry to give clues to its function. An3 protein localization changed during oogenesis. Nuclear localization was diffuse in stage I and II oocytes (data not shown), localized with the extranucleoli of vitellogenic stages (stages III–V), and undetectable in the nucleus of stage VI oocytes. Cytoplasmic staining of An3

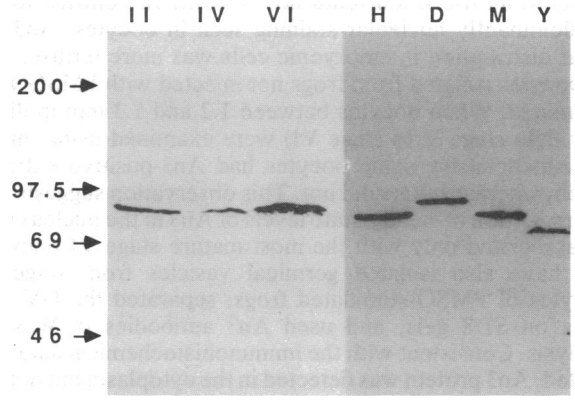

FIG. 1. An3 protein is present in *Xenopus* oocytes and other organisms. Lanes II, IV, and VI contained *Xenopus* oocyte protein from stages II (25 oocytes), IV (12 oocytes), and VI (12 oocytes), respectively. Protein extracts from various organisms were in lanes H, HeLa cell nuclear proteins; D, whole *Drosophila*; M, mouse testis; and Y, yeast (*S. cerevisiae*) cells. Proteins were detected with mouse polyclonal antisera made to either An3 N-terminal GST fusion protein or full-length An3. The secondary antibody was antimouse IgG horseradish peroxidase conjugate at a dilution of 1:10,000. The proteins were detected with the ECL kit from Amersham. Molecular size (kDa) markers are at left.

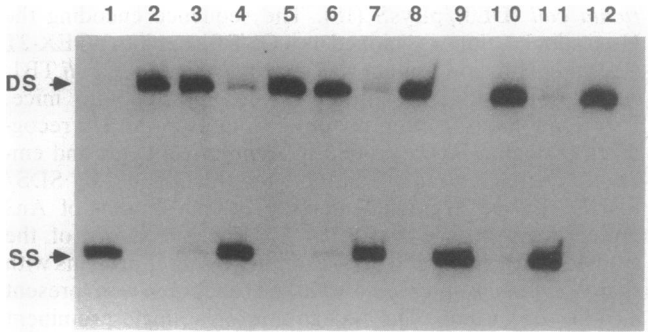

FIG. 2. Anti-An3-immunoprecipitated protein from oocytes has ATP-dependent helicase activity. Lanes 1 and 9, heat-denatured RNA substrate (single-stranded, SS); lanes 2 and 10, duplex RNA substrate (DS). Helicase assays used An3 protein from 10 stage VI oocytes (lanes 3–5); 10 stage IV oocytes (lanes 6–8); or 50 ng of purified GST–An3 fusion protein (lanes 11 and 12). Proteins were immunoprecipitated with control mouse serum (lanes 3 and 6) or An3 antiserum (lanes 4, 5, 7, and 8). ATP was omitted from the assays in lanes 5, 8, and 12.

protein was relatively sparse in stage I and II oocytes and more evident in later stages. To confirm the spatial and temporal changes in An3 protein localization, nuclear An3 staining was compared with other studies that examined nuclear components, as well as with the localization of the nucleolar protein fibrillarin (21, 22). An3 and fibrillarin staining were coincident in stage III–IV oocytes (Fig. 3 *B* and *C*). At higher magnification, the distinctive structure of the extranucleoli was seen with both the anti-An3 antibody and the anti-fibrillarin antibody (Fig. 3 *D* and *E*) (21, 22). Although the majority of An3 protein colocalized with extranucleoli, some appeared to associate with smaller nuclear structures (Fig. 3*D*). These structures were of the approximate size of A and B snurposomes (21), but definitive identification has not been made. By stage VI, nuclear An3 protein was no longer detected, despite the continued presence of extranucleoli in the oocyte recognized by anti-fibrillarin (Fig. 3 *G* and *H*); however, An3 protein was detected in the cytoplasm (Fig. 3*I*). Preliminary observations indicated that cytoplasmic staining for An3 persisted throughout embryogenesis; nuclear staining reappeared post midblastula transition (data not shown). In contrast to the predominantly nucleolar staining seen in oocytes, An3 nuclear distribution in embryonic cells was more diffuse.

Oocytes isolated from frogs not injected with PMSG were examined. When oocytes between 1.2 and 1.3 mm in diameter (late stage V to stage VI) were examined using immunohistochemistry some oocytes had An3-positive extranucleoli, whereas others did not. This observation suggests that the reduction of steady-state levels of An3 in the nucleus may be associated only with the most mature stage VI oocytes. We have also isolated germinal vesicles from stage VI oocytes of PMSG-stimulated frogs, separated the GV proteins on SDS gels, and used An3 antibodies in Western analysis. Consistent with the immunohistochemical data presented, An3 protein was detected in the cytoplasm but not the GV of stage VI oocytes (data not shown).

DISCUSSION

Using specific antibodies, we have examined the steady-state level, activity, and subcellular localization of An3 protein in developing oocytes of *X. laevis*. A number of DEAD-box proteins have been identified in *Xenopus* (23). However, the antibodies used in this study detected a single protein, suggesting that the epitopes being recognized lie outside the conserved domains of the DEAD-box family. Consistent with this suggestion is the detection of single proteins from mouse, human, fruit fly, and yeast by antibodies directed against the *Xenopus* An3 protein. The recognition of single proteins in these other species provides evidence for conservation of a protein with An3-like activity.

We show that An3 protein has ATP-dependent RNA helicase activity. Although proteins that have DEAD-box family motifs are often assumed to be ATP-dependent RNA helicases, this activity has been documented for only a few members. ATPase activity has been reported for several members of the family; however, attempts to show related helicase activity have sometimes been unsuccessful (24, 25). The difficulty in showing both activities may be due to substrate specificity requirements not fulfilled by the *in vitro* substrates or by the lack of a necessary auxiliary factor. The demonstration of ATP-dependent helicase activity in the case of An3 establishes its potential enzymatic role during oogenesis and embryogenesis.

Our immunohistochemical studies provide a cellular context for utilization of An3 RNA helicase activity. An3 protein was detected in both the cytoplasm and the nucleus in oocyte stages I–V but was detected only in the cytoplasm in stage VI. Therefore, at least prior to stage VI conditions exist that allow An3 entry into the nucleus. Although stage VI oocytes are not transcriptionally silent, RNA synthesis is more active during earlier stages, stages when An3 protein is nuclear. The extranucleoli are formed during the amplification of rRNA genes and can easily be detected in all stages of oocytes (2). Extranucleoli are the sites for the transcription and processing of precursors for 5.8S, 18S, and 28S rRNA and the initial steps of ribosome assembly. Transcription of rRNA is most active from late stage II through stage V, consistent with the stages when An3 protein is associated with extranucleoli.

Several DEAD-box proteins have been identified in connection with nucleoli or with rRNA processing. p68, isolated from HeLa cells, is associated with the nucleoli in a cell cycle-dependent manner and is proposed to be involved in nucleolar assembly (26). Mutations of SPB4 or DRSI in *S. cerevisiae* result in the incomplete processing of rRNA precursors (27, 28). A comparison of the An3 protein with other members of the DEAD-box gene family does not show substantial similarity with p68, SPB4, or DRSI outside of the conserved regions that define the family. However, the nucleolar location of An3 suggests that it may have a function like these proteins. Greater similarity is found between An3, the mouse PL10 protein, and the yeast DED1 protein (13, 14). The function of the testis-specific PL10 product is unknown, but the DED1 protein was proposed to unwind the U4/U6–U5 small nuclear RNA (snRNA) complex involved in splicing of RNA polymerase II transcripts (29). Although present in the nucleus, U4, U5, and U6 snRNAs were not detected in amphibian oocyte nucleoli (21), making the interaction of these snRNAs with the bulk of An3 protein present in the nucleus during oogenesis unlikely. The identification of An3 protein with smaller nuclear structures, putative snurposomes, provides a possible site of association with U4, U5, and U6. The localization pattern seen for An3 protein may suggest that multiple substrates are recognized by this RNA helicase. The disappearance of the An3 protein from the nucleus in stage VI oocytes indicates that whatever role this protein is playing, neither nuclear nor nucleolar localization is required in stage VI. In addition, although An3 is in the nucleus in post-midblastula-transition embryonic cells, the strong nucleolar staining found in stage III–VI oocytes is not reiterated. If An3 is involved in processing a variety of RNA transcripts, its association with the extranucleoli may reflect its participation in the major RNA processing event during that time in development, that involving rRNA. At other developmental stages, where RNA transcripts other than rRNA are a more prevalent part of the

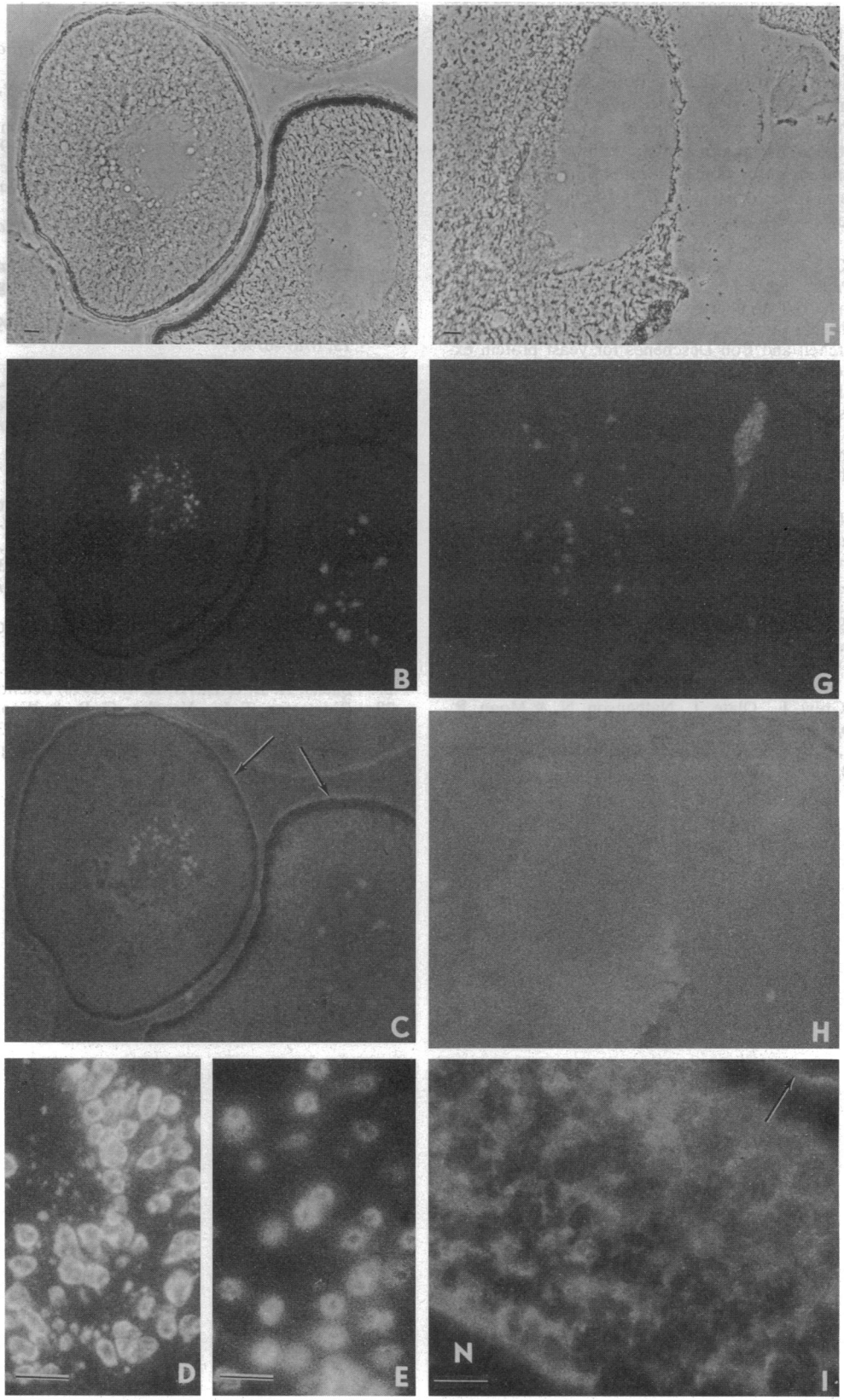

FIG. 3. Distribution of An3 protein during oogenesis. Indirect immunoreactivity of cryostat sections of oocytes demonstrates the localization of An3 (*C*, *D*, *H*, and *I*) and fibrillarin (*B*, *E*, and *G*). Phase-contrast images *A* and *F* are of preparations shown in *B*, *C* and *G*, *H*, respectively. The extranucleoli of stage III–IV (*A–C*) oocytes react with anti-fibrillarin (*B*) and anti-An3 (*C*). Cytoplasmic staining of An3 is also present in oocytes and follicle cells (arrows). High magnification of extranucleoli (*D*) of a stage III–IV oocyte shows immunoreactivity to only anti-An3 and secondary antibody. A similar staining pattern is seen by indirect staining with only anti-fibrillarin (*E*). The extranucleoli of stage VI (*F–H*) oocytes react with antifibrillarin (*F*) but not with anti-An3 (*G*). Cytoplasmic immunoreactivity to An3 is apparent in stage VI oocytes (*H* and *I*) and follicle cells (arrow in *I*). Nuclear staining (N) is absent in stage VI oocytes. Because the numerical aperture of the ×10 objective is much less than the ×60 (0.25 and 1.4, respectively), cytoplasmic staining is seen to better advantage in *I* than in *C* and *H*. (Bars = 10 μm.)

processing pool, the concentration of the An3 protein in the nucleolus may not occur.

The key issues that remain to be resolved concerning An3 protein include identification of its cellular substrate(s) and how An3 protein is directed to different cellular compartments during the course of oogenesis. A more detailed investigation of the dynamics of the subcellular localization of An3 protein and an extension of studies that examine the An3 homologues in other organisms may help lead us to the *in vivo* role of An3.

We thank Joe Gall and Michael Pollard for the anti-fibrillarin antibody; Arnim Pause and Nahum Sonenberg for plasmid PRP40; Marty Stoltzfus and Brad Amendt for HeLa cell protein extracts; Pam Geyer, Dan Mallin, and Scott Patton for *Drosophila* protein extracts; Dave Mitchell and Bob Deschenes for yeast protein extracts; and Ray Runyan for help with the generation of the An3 antibody. We thank the members of the Weeks and Longo labs for helpful suggestions. This work was supported by grants from the National Institutes of Health (F.J.L. and D.L.W.). This work was done during the tenure of an Established Investigatorship (D.L.W.) of the American Heart Association.

1. Dumont, J. N. (1972) *J. Morphol.* **136,** 155–179.
2. Hausen, P. & Riebesell, M. (1991) *The Early Development of Xenopus laevis: An Atlas of Histology* (Springer, Berlin).
3. Weeks, D. L. & Melton, D. A. (1987) *Cell* **51,** 861–867.
4. Schwartz, S. P., Aisentahal, L., Elisha, Z., Oberman, F. & Yisraeli, J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11895–11899.
5. Mosquera, L., Forristall, C., Zhou, Y. & King, M. L. (1993) *Development* **117,** 377–386.
6. Thomsen, G. & Melton, D. A. (1993) *Cell* **74,** 433–442.
7. Otte, A., McGrew, L., Olate, J., Nathanson, N. & Moon, R. (1992) *Development* **116,** 141–146.
8. Smith, R. C., Dworkin, M. B. & Dworkin-Rastl, E. (1988) *Genes Dev.* **2,** 1296–1306.
9. Linnen, J., Bailey, C. & Weeks, D. L. (1993) *Gene* **128,** 181–188.
10. Weeks, D. L. & Melton, D. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2798–2802.
11. Rebagliati, M. R., Weeks, D. L., Harvey, R. P. & Melton, D. A. (1985) *Cell* **42,** 769–777.
12. Gururajan, R., Perry-O'Keefe, H., Melton, D. A. & Weeks, D. L. (1991) *Nature (London)* **349,** 717–719.
13. Schmid, S. R. & Linder, P. (1992) *Mol. Microbiol.* **6,** 283–292.
14. Wassarman, D. A. & Steitz, J. A. (1991) *Nature (London)* **349,** 463–464.
15. Kay, B. K. & Peng, H. B. (1991) *Methods Cell Biol.* **36,** 657–662.
16. Colman, A. (1986) in *Transcription and Translation: A Practical Approach*, eds. Hames, B. D. & Higgens, S. J. (IRL, Oxford, U.K.), pp. 271–302.
17. Pause, A., Méthot, N. & Sonenberg, N. (1993) *Mol. Cell. Biol.* **13,** 6789–6798.
18. Rosenburg, A. H., Lade, B. N., Chui, D., Lin, S.-W., Dunn, J. J. & Studier, F. W. (1987) *Gene* **56,** 125–135.
19. Smith, D. B. & Johnson, K. S. (1988) *Gene* **67,** 31–40.
20. Jaramillo, M., Dever, T., Merrick, W. & Sonenberg, N. (1991) *Mol. Cell. Biol.* **11,** 5992–5997.
21. Wu, Z., Murphy, C., Callan, H. G. & Gall, J. G. (1991) *J. Cell Biol.* **113,** 465–483.
22. Caizergues-Ferrer, M., Mathieu, C., Mariottini, P., Amalric, F. & Amaldi, F. (1991) *Development* **112,** 317–326.
23. Watanabe, M., Itoh, K., Abe, K., Akizawa, T., Ikenishi, K. & Furusawa, M. (1992) *Dev. Growth Differ.* **34,** 223–231.
24. Schwer, B. & Guthrie, C. (1991) *Nature (London)* **349,** 494–499.
25. Lee, C.-G. & Hurwitz, J. (1993) *J. Biol. Chem.* **268,** 16822–16830.
26. Iggo, R. D. & Lane, D. P. (1989) *EMBO J.* **8,** 1827–1831.
27. Sachs, A. B. & Davis, R. W. (1990) *Science* **247,** 1077–1079.
28. Ripmaster, T. L., Vaughn, G. P. & Woolford, J. L. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11131–11135.
29. Jamieson, D., Rahe, B., Pringle, J. & Beggs, J. (1991) *Nature (London)* **349,** 715–717.