Changes in the Expression of α-Fodrin during Embryonic Development of *Xenopus laevis*

Dawn H. Giebelhaus, Bruce D. Zelus, Susan K. Henchman, and Randall T. Moon

Department of Pharmacology SJ-30, University of Washington, Seattle, Washington 98195

Abstract. Fodrin (nonerythroid spectrin) and its associated proteins have been previously implicated in the establishment of specialized membrane-cytoskeletal domains in differentiating cells. Using antiserum which is monospecific for the α-subunit of fodrin, we demonstrate that α-fodrin is present in oocytes and adult tissues of *Xenopus laevis*. Analyses of the de novo synthesis of α-fodrin during embryonic development reveal that α-fodrin is synthesized in oocytes, but not during early development. To investigate the level of control of α-fodrin expression, we isolated two cDNA clones for oocyte α-fodrin. The oocyte cDNA clones were identified as encoding portions of α-fodrin based on DNA sequence analysis and on the comparison of the predicted amino acid sequence of the cDNAs with the known sequence of human erythro-cyte α-spectrin. The *Xenopus* α-fodrin cDNAs hybridize to a transcript of ~9 kb on RNA blots, and probably to a single gene type on genomic DNA blots. Both RNA blot analyses and S1 nuclease protection assays with the *Xenopus* α-fodrin cDNAs demonstrate that the observed decline in the de novo synthesis of α-fodrin polypeptides is controlled by a dramatic decrease in the abundance of α-fodrin transcripts after fertilization. In contrast, levels of actin transcripts do not decrease during this period. Inasmuch as steady-state levels of α-fodrin transcripts rise by the neurula stage of development, these results suggest that the synthesis of α-fodrin polypeptides during embryonic development of *Xenopus* is regulated, rather than constitutive, and that the primary level of control is the steady-state abundance of mRNA.

THE cortex of *Xenopus* oocytes and eggs is often defined as a 1–10-μm-thick yolk-free region of cytoplasm lying beneath the plasma membrane. Pigment and cortical granules are enriched in the cortex relative to the deeper cytoplasm, although some mitochondria, ribosomes, cytoskeletal elements, and other vesicles are also found in the cortex (reviewed by Gerhart, 1980; Vacquier, 1981). Regarding cytoskeletal elements in the cortex, previous studies have shown that the cortex contains an intricate web of actin bundles (Franke et al., 1976; Gall et al., 1983), microtubules (Franke et al., 1976), and intermediate filaments (Franz et al., 1983; Gall et al., 1983; Godsave et al., 1984*a,b*), although little is presently known about the specific roles of these submembranous filaments in the formation of asymmetries in the oocyte. That asymmetries exist in the membrane of *Xenopus* oocytes is evidenced by the segregation of pigment granules to the cortex of the animal hemisphere, the segregation of 1-μm a-cortical granules to the animal cortex, the segregation of 2.5-μm v-cortical granules to the vegetal pole (reviewed by Gerhart, 1980), and the asymmetric distribution of intramembranous particles in the plasma membrane of *Xenopus* oocytes (Bluemink, and Tertoolen, 1978). In contrast to the uncertain roles of submembranous filaments in the formation of membrane asymmetries, the post-fertilization roles of these filaments in the waves of surface contractions, in the extrusion of the polar body, and in the formation of the cleavage furrow are well established (reviewed by Gerhart, 1980).

Interest in the submembranous cytoskeleton or "membrane skeleton" of nonerythroid cells has blossomed after the detection of spectrin in numerous cell types (reviewed by Nelson and Lazarides, 1984). Spectrin is composed of distinct α- (240,000 M_r) and β- (220,000 M_r) subunits in human erythroid cells. In nonerythroid cells, spectrin (often referred to as fodrin, after Levine and Willard, 1981) is also composed of two nonidentical subunits, although the erythroid β-subunit is replaced with a subunit of 235,000 M_r (Glenney and Glenney, 1984; also referred to as γ-spectrin by Lazarides et al., 1984). Fodrin and other constituents of the membrane skeleton may play an active role in the establishment of specialized membrane-cytoskeletal domains, based on suggestions that membrane skeletons containing fodrin are involved in localizing integral membrane proteins to specific regions of cells (e.g., Nelson and Lazarides, 1984; Koenig and Repasky, 1985), and based on suggestions that fodrin may be involved in the active mobility of membrane proteins during receptor capping (Levine and Willard, 1983; Nelson et al., 1983). Because variants of fodrin are differentially expressed during differentiation of neuronal and myogenic tissues (reviewed by Nelson and Lazarides, 1984), and because β- and γ-spectrin are segregated to distinct regions within single cells (Lazarides et al., 1984), it is of consider-

able interest to determine the timing of expression of spectrinlike polypeptides, and to determine the spatial distribution of membrane skeleton proteins during embryonic development.

Immunofluorescence analyses have shown that gametes and embryos of sea urchins (Schatten et al., 1986) and mice (Sobel and Alliegro, 1985; Schatten et al., 1986) express spectrinlike polypeptides which change in spatial distribution during fertilization and cleavage. We have initiated experiments on the expression and function of fodrin during embryonic development of *Xenopus laevis*. In the present study we show that the de novo synthesis of α-fodrin declines markedly during early embryogenesis in *Xenopus laevis*. To determine the level of control of α-fodrin expression, we have isolated cDNA clones for *Xenopus* oocyte α-fodrin, and used them to demonstrate that there are dramatic decreases in the steady-state level of α-fodrin transcripts after fertilization, and that the level of α-fodrin transcripts increases late in embryonic development. These results suggest that fodrin may play a role in membrane functions in oocytes and during organogenesis, but that early development proceeds normally without the constitutive expression of this membrane skeleton polypeptide. Whether the fodrin that is synthesized during oogenesis is utilized during early embryogenesis remains unknown.

Materials and Methods

Isolation and Culture of Xenopus Eggs and Embryos

Xenopus laevis females (purchased from NASCO, Fort Atkinson, WI) were induced to lay eggs with an injection of 1,000 IU of human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO). Embryos were obtained by artificial insemination, cultured in OR-2 at 18°C (YoungLai et al., 1982), and staged according to Nieukoop and Faber (1967). To monitor the de novo synthesis of α-fodrin, oocytes were labeled by incubation in [^{35}S]methionine (1 mCi/ml, 1095-1103 Ci/mmol, from New England Nuclear, Boston, MA) and embryos were microinjected with 2 × 10^6 dpm of [^{35}S]methionine in a volume of 20 nl (Wasserman et al., 1982). After labeling for 6 h, eggs and embryos were homogenized and boiled in 1% SDS and diluted 10-fold in immunoprecipitation buffer (Moon et al., 1985), and the insoluble residue was removed by centrifugation in a microfuge. The incorporation of [^{35}S]methionine into hot trichloroacetic acid-insoluble polypeptides was determined as previously described (Moon et al., 1982).

To monitor the levels of fodrin transcripts after oocyte maturation in vitro, oocytes were dissected manually into modified Barth's solution, incubated in 10 μg/ml progesterone (Sigma Chemical Co.) in modified Barth's solution for 15 min, washed extensively with modified Barth's solution, and cultured for 12 h at 18°C (Gurdon and Wickens, 1983). Oocytes were then processed for either RNA extraction, described below, or assayed for germinal vesicle breakdown by light microscopy of intact oocytes or by light microscopy of oocytes which had been fixed in formalin and then dissected. Virtually all oocytes underwent germinal vesicle breakdown in these experiments.

Immunoprecipitation and Protein Blotting with α-Fodrin Antiserum

Polyclonal antibodies specific for α-spectrinlike polypeptides were prepared by isolation of chicken erythroid α-spectrin from polyacrylamide gels and injection of the gel bands into rabbits (Repasky et al., 1982). The resulting antiserum detects α-fodrin in different tissues and divergent species (data not shown) including *Xenopus*, as monitored by protein blotting methods (Repasky et al., 1982). That the antiserum is specific for the membrane-associated α-spectrin used as immunogen was confirmed by immunofluorescence of erythroid cells and by protein blotting–soluble and cytoskeletal fractions (data not shown). To monitor the steady-state levels of α-fodrin, protein blots were performed with α-fodrin antiserum as previously described (Repasky et al., 1982), with the fluorograph in Fig. 1 *b* being exposed for 3 h. To follow the de novo synthesis of α-fodrin, α-fodrin was

immunoprecipitated from identical counts (2 × 10^6 cpm) of [^{35}S]methionine-labeled polypeptides of oocytes and embryos, using methods similar to those previously described (Moon et al., 1985). Immunoprecipitates were separated on SDS 10% PAGE gels and processed for fluorography (Moon et al., 1985), with Fig. 2 being exposed for 18 h.

Isolation of Xenopus Oocyte α-Fodrin cDNAs

Approximately 10^6 plaques of a *Xenopus laevis* oocyte cDNA library in λgt10 (Rebagliati et al., 1985) were screened with Hα-fod 3, a 1.15-kb cDNA clone encoding several repeat units of human α-fodrin (McMahon et al., 1987). Nitrocellulose filter lifts were prehybridized and hybridized at 42°C in 50% formamide, 5× SSC, then washed at 42°C in 0.2× SSC, 0.2% SDS (Maniatis et al., 1982). The ^{32}P-hybridization probe was prepared by hexanucleotide primer extension (Feinberg and Vogelstein, 1984) of Hα-fod 3 to a specific activity of 1-3 × 10^9 cpm/μg cDNA. Plaques that initially screened positive were isolated and rescreened to homogeneity.

Two cDNA inserts from unique recombinant λgt10 were obtained by digestion of the bacteriophage DNA with Eco RI, followed by isolation of the cDNA inserts from low melting point agarose gels (Moon et al., 1985). The inserts were ligated into the Eco RI site of pGEM-1 (Promega Biotec, Madison, WI) with T4 DNA ligase, and used to transform *Escherichia coli* strain RRI (Maniatis et al., 1982). These two cDNA clones, designated Xen α1 and Xen α2, were then characterized as described below.

Analysis of cDNA Sequences and Restriction Endonuclease Sites

The DNA sequences of Xen α1 and Xen α2 were determined by sequencing (Sanger et al., 1980) cDNA restriction fragments which had been subcloned into pGEM1. In all cases, the sequences were confirmed by multiple-sequencing reactions for both strands of DNA. Restriction enzyme sites based on DNA sequencing were confirmed by simultaneous digestion of cDNA clones with one or more restriction endonucleases (Moon et al., 1985).

The predicted amino acid sequence, sequence homologies, and restriction endonuclease sites were generated on a MacIntosh computer by analysis of the nucleotide sequence with the DNA Inspector II computer program (Textco, W. Lebanon, NH). Predictions of polypeptide secondary structure were performed according to Chou and Fasman (1978a,b).

Hybridization of cDNA Probes to RNA and Genomic DNA Blots

For the analysis of α-fodrin sequences in genomic DNA, *Xenopus* liver DNA was isolated and digested with restriction endonucleases, separated on 0.9% agarose gels, and transferred to nitrocellulose (Maniatis et al., 1982). For the analysis of α-fodrin transcripts present in developing embryos, poly(A)-containing [poly(A)$^+$] RNA was isolated as follows. Oocytes were digested with 10 mg/ml collagenase (Sigma Chemical Co.) for 15 min at 20°C, and eggs and embryos were dejellied for 1-2 min with 2% L-cysteine, pH 7.8 (Gurdon and Wickens, 1983). Oocytes, eggs, and embryos were then homogenized in extraction buffer (100 mM Tris, pH 8, 50 mM NaCl, 5 mM EDTA, and 2% SDS) which had been mixed 1:1 with liquified phenol (equilibrated with 100 mM Tris, pH 8). Chloroform/isoamyl alcohol (24:1) was then added in a volume equivalent to that of the phenol; the sample was then vortexed and centrifuged. The resulting aqueous phase was reextracted with the chloroform-isoamyl alcohol solution, and finally the aqueous phase was precipitated with 2 vol of ethanol at −20°C. Total nucleic acids in the aqueous phase were digested with DNase I (RQ DNase, Promega Biotec) in the presence of RNasin (Promega Biotec), and the poly(A)-containing RNA was purified by chromatography on oligo(dT) cellulose (Aviv and Leder, 1972). This poly(A)$^+$ RNA was then separated on denaturing 0.9% agarose formaldehyde gels, and transferred to nitrocellulose (Moon et al., 1985). Both RNA and DNA blots were hybridized as previously described (Moon et al., 1985), except that cDNA probes were labeled by primer extension to specific activities of 1-3 × 10^9 cpm/μg DNA (Feinberg and Vogelstein, 1984), and filters were washed at 68–70°C in 0.2× SSPE, 0.2% SDS before exposure to x-ray film.

S1 Nuclease Protection Assay

Our strategy for preparing probes for S1 nuclease protection assays was to end label the cDNA strands which were complementary to their mRNAs, and to incorporate 1.5 kb of plasmid DNA into the probe. Since the plasmid

DNA would not hybridize to *Xenopus* RNA, digestion of hybridized probe with S1 nuclease would result in the degradation of the plasmid sequence. This would generate a smaller protected band on denaturing acrylamide gels, with the size of the band now corresponding to the cDNA region of the probe protected by hybridization with complementary regions of RNA. To construct the probes, Xen α1 and Xen α2 in pGem 1 were linearized by digestion at the 5′ Hind III site present in each cDNA. This Hind III site (and the Hind III site in the vector) was labeled by a fillout reaction with DNA polymerase I large fragment, in the presence of [α-³²P]dNTPs (each 3,000 Ci/mM). To obtain a DNA probe labeled only on the strand complementary to mRNA, and to remove the Hind III site labeled in the vector, each labeled plasmid was digested with Pvu I. Because Pvu I cuts in the vector, but not in either cDNA, this allowed for the isolation from low melting point gels of a 3.8-kb probe for Xen α1 (representing 2.3 kb of cDNA insert plus 1.5 kb of plasmid DNA) and of a 2.65-kb Xen α2 probe (containing 1.15 kb of cDNA insert plus 1.5 kb of plasmid DNA). For both cDNA probes, the 5′ Eco RI to Hind III sequences were, therefore, omitted from the final labeled probes. The probes (12,000 cpm; sp act ~6 × 10⁶ cpm/μg DNA) were resuspended with 2 μg of poly(A)⁺ RNA in 25 μl of 80% formamide, 40 mM Pipes, 0.4 M NaCl, 1 mM EDTA, and the probe was dena-

tured by heating the samples to 80°C for 5 min. For hybridization with 17 μg of total RNA, the sample volumes were increased 10-fold. The samples were then incubated at 52°C for 15 h, a temperature calculated to enable hybridization of the labeled cDNA strand with RNA, yet sufficient to prevent reannealing with the unlabeled strand of cDNA. After hybridization, the samples were digested with 1,000 U of S1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, IN; or Bethesda Research Laboratories, Gaithersburg, MD) for the poly(A)⁺ RNA hybridizations, and with 10,000 U for the total RNA hybridizations. After digestion with S1 nuclease, the samples were resolved on 0.3-mm × 40-cm 6% polyacrylamide gels, which contained 7 M urea. For calculation of the size of the protected DNA fragments, ³²P-markers were prepared by digestion of pBR322 with Pvu II or Bstn I, followed by end-labeling and electrophoresis on adjacent lanes.

Results

Synthesis of α-Fodrin during Development of Xenopus laevis

We have researched the expression of membrane skeleton proteins during embryonic development and cellular differentiation in *Xenopus laevis*. Nonerythroid spectrin (fodrin) is an attractive candidate for a plasma membrane-associated protein which may be involved in the specialization of membrane-cytoskeletal domains. Before investigating the expression of fodrin during development of *Xenopus laevis*, we first used protein blotting methods to determine whether α-fodrin was present in various tissues of adult *Xenopus*. As shown in Fig. 1 *b*, polypeptides immunologically related to chicken α-spectrin (lane *2*, see also lane *2* in Fig. 2) are present in adult *Xenopus* erythrocytes (lane *3*, with the lower molecular weight bands representing immunologically-reactive fragments generated through proteolysis), muscle (lane *4*), brain (lane *5*), and intestine (lane *6*). Although a band reacting with anti-α-spectrin antibodies was detected in *Xenopus* brain (Fig. 1 *b*, lane *5*), the signal was reproducibly lower than the signal obtained from erythroid cells. It is presently unknown whether this result reflects the relative abundance of α-spectrinlike polypeptides in these two tissues, or whether the different signal intensities are attributable to other factors, such as immunologic differences between the spectrinlike polypeptides present in different cell types. Given that α-fodrin was detected in adult tissues, we next investigated whether α-fodrin was expressed during embryonic development of *Xenopus laevis*. Preliminary protein blots demonstrated that α-fodrin was present in oocytes, but at very low levels which could not be monitored reproducibly (data not shown).

Because the low α-fodrin signals on protein blots precluded the use of this technique for monitoring the steady-state levels of α-fodrin during development, we next investigated whether de novo synthesis of α-fodrin could be detected at any stage in development. Oocytes and embryos were labeled with [³⁵S]methionine, and identical amounts of radioactive polypeptides (2 × 10⁶ cpm) were processed for immunoprecipitation with α-spectrin antiserum. These experiments revealed that oocytes synthesize detectable levels of α-fodrin (Fig. 2, lane *4*). Surprisingly, the amount of α-fodrin synthesized as a proportion of total protein synthesis declines markedly during early development, as shown by the greatly reduced levels of α-fodrin recovered by immunoprecipitation from labeled 16–64 cell embryos (lane *5*), blastula-stage embryos (lane *6*), and gastrula-stage (lane *7*) embryos. However, it is clear that the decrease in the expression of α-fodrin during early development is only transient
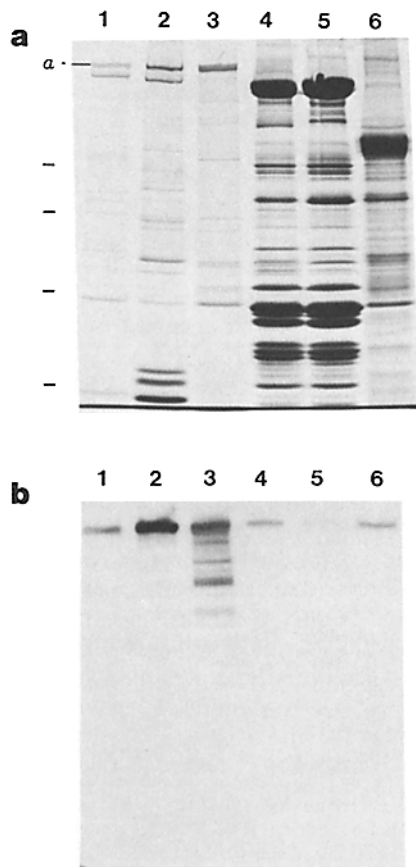
Figure 1. Detection of α-fodrin in tissues of adult *Xenopus laevis*. (*a*) Extracts of various tissues were separated by SDS-PAGE and stained for total protein with Coomassie Blue. To provide appropriate size markers for α-fodrin, erythrocytes of mouse (lane *1*), chicken (lane *2*), and *Xenopus* (lane *3*) were extracted with 0.5% Triton X-100 before separation of the detergent-insoluble cytoskeletons on the gel. Whole-tissue extracts of adult *Xenopus* skeletal muscle (lane *4*), brain (lane *5*), and intestine (lane *6*) were loaded on adjacent lanes. Size markers of 94,000, 67,000, 43,000, and 30,000 M_r are denoted to the left of the gel, and the position of α-fodrin (spectrin) is indicated (*a*). (*b*) A gel identical to that in *a* was blotted onto nitrocellulose and the proteins were probed with antiserum monospecific for α-fodrin (spectrin), followed by localization with ¹²⁵I-protein A. The band detected in all lanes comigrates with the α-fodrin (spectrin) in *a*.
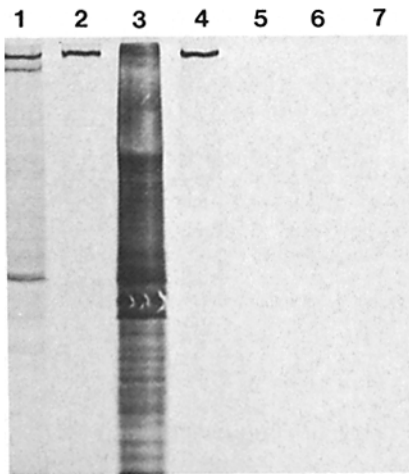
Figure 2. De novo synthesis of α-fodrin during early development of *Xenopus laevis*. To provide erythroid spectrin markers, chicken embryo erythroid cells were labeled with [³⁵S]methionine, and 100,000 cpm of the Triton X-100–insoluble cytoskeleton was applied to lane 1. Immunoprecipitation of the chicken erythroid cytoskeletal fraction with antisera raised against chicken erythroid α-spectrin recovers a single polypeptide species (lane 2). Oocytes and embryos of *Xenopus laevis* were labeled with [³⁵S]methionine, and 160,000 cpm of labeled oocyte polypeptides (lane 3) were electrophoresed to indicate the spectrum of polypeptides synthesized. α-Fodrin was then immunoprecipitated from 2 × 10⁶ cpm of labeled protein from oocytes (lane 4), 16–64-cell embryos (lane 5), blastula-stage embryos (lane 6), and gastrula-stage embryos (lane 7).

in that protein blots of various tissues of adult frogs, probed with this antiserum, then with ¹²⁵I-protein A, demonstrate the presence of α-fodrin in adult tissues of frogs (Fig. 1 *b*). Because the unexpected decrease in the expression of fodrin could be regulated at several levels, the following experiments were pursued.

Characterization of cDNAs for Xenopus Oocyte α-Fodrin

To investigate whether the steady-state level of α-fodrin mRNAs is a contributing factor in the observed reduction

in the synthesis of α-fodrin during development, we isolated two cDNA clones encoding portions of *Xenopus* oocyte α-fodrin. Using a cDNA for human α-fodrin which has been previously sequenced and characterized (McMahon et al., 1987), we screened a cDNA library in λgtl0 which had been prepared with poly(A)⁺ RNA from *Xenopus* oocytes (Rebagliati et al., 1985). Two putative α-fodrin cDNAs were obtained, designated Xen α1 (2.6 kb) and Xen α2 (1.4 kb). Before using these probes to monitor the steady-state levels of α-fodrin transcripts during embryonic development, the following analyses were undertaken to confirm the spectrinlike nature of the polypeptides partially encoded by these cDNAs.

Restriction enzyme sites in Xen α1 and Xen α2 were assigned by single- and double-enzyme digests, revealing restriction site similarities at the 5' ends of the cDNAs, yet with evident polymorphism in other restriction endonuclease sites (Fig. 3). To determine the molecular basis for this restriction site polymorphism, and to enable sequence comparisons with the amino acid sequence of human erythroid α-spectrin (Speicher and Marchesi, 1984), the entire sequence of Xen α2 and the related 5' Eco RI-Sal I restriction fragment of Xen α1 was determined, using the sequencing strategy depicted in Fig. 3. The nucleotide sequences of these two cDNAs are compared in Fig. 4. Of the 951 nucleotide overlap obtained in the best fit comparison of the two sequences, there are 79 nucleotide differences, designated by *solid dots*, and 872 identical nucleotides at the respective positions, designated by *colons*. The 83% identity between these sequences indicates that these are highly related cDNAs, as substantiated by the following comparison of the predicted amino acid sequences.

Both Xen α1 and Xen α2 encode portions of spectrinlike polypeptides, as determined by a comparison of the amino acid sequences encoded by the cDNAs with the amino acid sequence of human erythrocyte α-spectrin (Speicher and Marchesi, 1984). Human erythrocyte α-spectrin is composed of 20 repeated units, containing 106 amino acids on average. Within these repeat units are highly conserved residues at specific positions, indicated by the single-letter amino acid abbreviations in the top line of Fig. 5 *a* (e.g., I at position 1, W at position 12, etc.). When variations at these residues occur in human erythrocyte α-spectrin, there

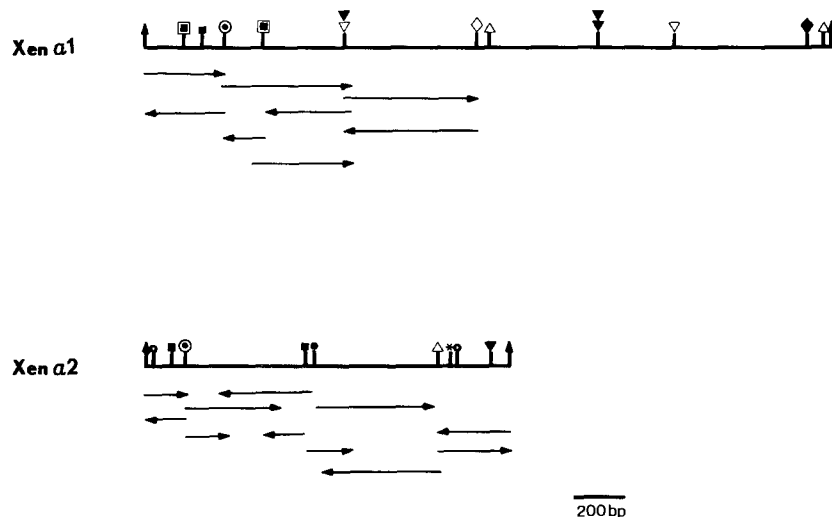Figure 3. Restriction endonuclease maps of the Xen α1 and Xen α2 spectrin cDNA clones. Restriction endonuclease sites are designated as follows: (●) Acc I; (▼) Ava I; (■) Bam HI; (▽) Bgl I; (△) Bgl II; (▲) Eco RI; (○) Hind III; (○) Pst I; (□) Pvu II; (*) Rsa I; (◇) Sal I; (◆) Sma I; (▽) Xba I; (▼) Xho I. The size marker is in base pairs (bp). *Arrows* indicate the lengths and directions of fragments which were sequenced.

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α1  GGAAAATTACAG CTCTGGATG AG TTTG CTACAAAG CTAATTCAG AATAACCA CTACG CCAAGG AAG ATG TGG CTACCCG T

α1  AG AG ATG CTCTG CTG AG CCG CCG CAATG CA CTTCACG AACG TG CACTCTACCG CCG CACTCAG CTGG CAG ACTCCTTTCA
                                                               :•::::::::::::::::::::::
α2                                                             CG CAG CTGG CAG ACTCCTTTCA

α1  CCTG CAG CAATTCTTAAG GG ACTCCG ACG AG CT AAAG AG CTGG ATTAATG AAAAG ATG AAG ACTG CAACAG ATG AGG CAT
    :::::::::::•::::•:::::::::•::•:::::•:::::::::::::::::::::::::::::::::::::::::::::::::
α2  CCTG CAG CAG TTCTTCAGGG ACT CG GATG AG CTG AAG AG CTGG ATTAATG AAAAG ATG AAG ACTG CAACAG ATG AGG CAT

α1  ACAAGG ATCCCTCCAATCT CCAGGG TAAGG TG CAG AAG CACAAG CTTATG AGG CTG AG CCT CTCTG CTAATCATAG CCG T
    ::::::::::::::::::::::::::::::::::::::::::•:::::::•::::•:::::•:::::::•:::•::::::
α2  ACAAGG ATCCCTCCAATCT CCAGGG TAAGG TG CAG AAG CACAAG CTTTTG AGG CCG AG ACT CTCCG CTAACCAAAG CCG T

α1  ATAG ATG CCTTGG AG AACT CTGG CCAG AAATTAATTG ATG TCAACCA TTACG CATCTG ATG AGG TGG CAG CCCG TATG AA
    :::::::::::::::::::::::::::::::::::::::::::•:::::::::::::::::::::::::::::::::::::
α2  ATAG ATG CCTTGG AG AACT CTGG CCAG AAATTAATTG ATG TCAACCA TTATG CATCTG ATG AGG TGG CAG CCCG TATG AA

α1  TG AGG TCATCAATTTG TGG AAG AAG CTG CTGG AGG CCA CTG AATTG AAAGGG ATCAAG CTG CG TG AAG CCAACCAACAG C
    :::•::•::::•::::::::::::::::::•::::::::::::::::::::::::::::::::::•:::::•:::::
α2  TG ATG TTATCA CTTTG TGG AAG AAG CTG CT AG AGG CCA CTG AATTG AAAGGG ATCAAG CTG CG CG AAG CG AACCAACAG C

α1  AG CAG TTCAATCG TAATG TGG AAG ACATAG AACTG TGG CTG TATG AAG TTG AGGGG CATTTGG CTTCAG ATG ACTATGG A
    :::::::::•::::::•:::::::::•:::::::::••::::::::::::•:::::::::::::::::::::::::::::::
α2  AG CAG TTTAATCG GAATG TGG AGG ACATAG AG TTG TGG CTG TATG AGG TTG AGGGG CATTTGG CTTCAG ATG ACTATGG A

α1  AAAG ACCTG ACCAATG TG CAAAATCTG CAG AAAAAACATG CCTTG TTGG AGG CTG ATG TAG CGG CTCACCAGG ACCG TAT
    :::::::::::::::::•::::•::•:•::::::::::::::::::•::::::::::•::::::::::::::::::::::
α2  AAAG ACCTG ACCAATG TTCAG AACCCG CAG AAAAAACA TG CCTTG CTGG AGG CTG ATATAG CAG CTCACCAGG ACCG TAT

α1  TG ATGG CATCA CTATCCAAG CTCG TCAG TTCCAAG AGG CCGG TCATTTTG ATG CTG ACAATATCAAG AAG AAA CAGG AGG
    •::::::•:::::::::::::::•:::::::::::::::::::::::::::::::::•::::::::::::::::::•::::
α2  CG ATGG TATCA CTATCCAAG CA CG TCAG TTCCAAG AAG CCGG TCATTTCG ATG CTG ACAATATCAAG AAG AAG CAGG AGG

α1  CCCTTG TTTCT AG ATATG AAG CTCTG AAGG AACCCA TGG CTG CACG CAAG CAG AAG TTATCCG ATTCTCTG CG TCTCCAA
    :::::::::••:::::::::•::::::::::•:::::•::•::•:::•:::::•:::::::•:::::::::::•:::
α2  CCCTTG TTAG TAG ATATG AGG CTCTG AAGG ATCCCA TGG TTG CG CG TAAGG AAAAG TTG TCAG ATTCTCTG CG TCTACAA

α1  CAG CTTTTCAGGG ATG TTG AAG ATG AGG AG ACCTGG ATAAGG AG AAGG AACCCA TTG CAG CCTCCA CCAA CAG AGG CAA
    :::•:::::::•:::::::::•:::::::::::::::::::::::::::::::::::::::::::::::::::::::::
α2  CAG ATTTTCAG AG ATG TTG AAG ACG AGG AG ACCTGG ATAAGG AG AAGG AACCCA TTG CAG CCTCCA CCAA CAG AGG CAA

α1  GGATTTG ATTGG AG TCCAG AATATC CTG AAG AAACA CCAGG CCCTG CAGG CTG AAATTG CTGG TCATG AACCCCG CATCA
    :::::::::::::::::::::•::•:::::::::::::::::::::::::::::•::::::::::::::::::::::::
α2  GGATTTG ATTGG AG TCCAG AATCT CTTG AAG AAACA CCAGG CCTTG CAGG CTG AAATTG CTGGG CATG AACCCCG CATCA

α1  AAG CGG TTACCCAG AAAGGGG AATCT ATG ATCACCG AAGG CCA CTTTG CCTCTG AAG AAG TG ACAGG TAAATTAAATG AG
    :::::•:::::::::::::•::::::::::::::::::::::::::::::::::::::::::::::::::::••::::::
α2  AAG CTG TTACCCAG AAAGG AG ATTCT ATG ATCACCG AAGG CCA CTTTG CCTCTG AAG AAG TG ATG GG TAAATTAAAGG AG

α1  CTG AATG ATAAG TGGG CCA CTCTG AAG AACAAAG CATCACAACG TCG AC
    :::::•:::::::::::::::::•:::::::::::::::::::::•:::::•::::
α2  CTG ATTG ATAAG TGGG CCA CTCTAAAG AACAAAG CATCG CAACG CCG ACAGG ACCTTG AAG ATTCG CTACAAG CT CAG CA

α2  GTATTTTG CTG ATG CAAACG AGG CGG AG TCTTGG ATG AGGG AG AAGG AG CCTATTG TTG GAAG CA CAG ACTATGG CAAAG

α2  ATG AAG ACTCTG CTG AGG CCCTTCTG AAG AAACA TG AAG CTCTTATG TCAG ATCTCCG AG CATACG GAAG CAG TATCCAG

α2  GGACTG CG TG TACG AG CCCAG TCCTG CAG ACAACAAG TTG CTCCAACGG ATG ATG AAACTGG AAAGG AG CTGG TG CTGG C

α2  ATTG TATG ATTACCAAG AG AAG AG CCCG AGGG AGG TG ACCATG AAG AAAGG AG ACATCTTG ACCTTG CTG AACAG CACAA

α2  ACAAGG ATTGG TGG AAGG TGG AAG TG AATG ATCG TCAGGG CTTTG TG CCTG CTG CATATG TG
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Figure 4. Nucleotide sequences for *Xenopus* α-fodrin cDNAs. The nucleotide sequences for the 5′ Eco RI to Sal I restriction fragment of Xen α1 (see Fig. 2) and for all of Xen α2 are aligned. *Colons* (:) denote nucleotides which are identical at that position, whereas *dots* (·) indicate nonidentical nucleotides. The sequences of the Eco RI linkers have been omitted.

are preferred substitutions, designated by the amino acids in parentheses on line 2 of Fig. 5 *a* (e.g., Y, R, or F instead of W at position 12; after Speicher and Marchesi, 1984). A comparison of those amino acids conserved within the spectrin repeat units with the predicted amino acid sequences of the *Xenopus* putative α-fodrin cDNAs reveals that those residues conserved in human erythrocyte α-spectrin are also highly conserved in the sequences of the *Xenopus* cDNAs (e.g., L at positions 17 and 26, W at position 45, E at position 48, H at position 72). Furthermore, most deviations from these conserved amino acids are consistent with the substitutions that also occur in human erythrocyte α-spectrin (e.g., K for R at position 22, E for D at position 42, and K for G at position 59). Finally, within the overlap region of 318 amino acids, Xen α1 and Xen α2 are 94% identical on the amino acid level (83% identical on the nucleotide level).

About half of the differences between the two *Xenopus* amino acid sequences result from the substitution of an amino acid which is found at that position in human erythrocyte α-spectrin (e.g., D vs. E at position 7 in repeat 7).

When the entire amino acid sequence of Xen α2 is compared with the human erythrocyte spectrin repeats, the greatest homology (53% identical residues, not including many evident conservative changes) is with repeat units 6–9 of human erythrocyte α-spectrin. Note that the best fit to repeat 6 of human α-spectrin requires that the corresponding *Xenopus* repeat unit be composed of 105 amino acids, one less than in the other repeat units. As the next best fit of the *Xenopus* repeat units with any other repeat units of human erythrocyte α-spectrin was <30%, these data strongly argue that Xen α2 is homologous with repeat units 6–9 of human α-spectrin, and is hence a cDNA encoding an α-spectrinlike
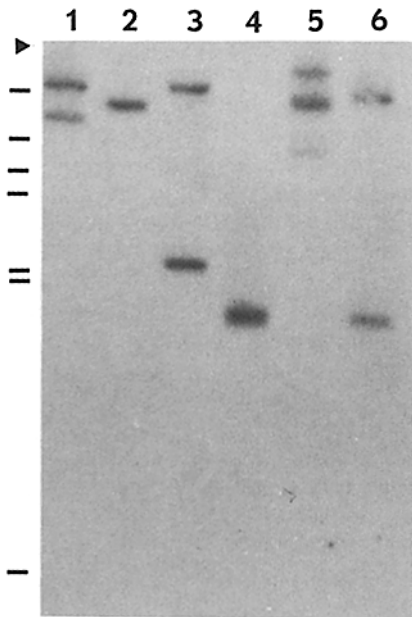
a

```
          10        20        30        40        50        60        70        80        90       100
           :         :         :         :         :         :         :         :         :         :
           :         :         :         :         :         :         :         :         :         :
      I         : W  L   : R   L   :  F  D : D  W I  : E     G:      K  : H     :         :         :H
     (TV)     (YRF)(FI)  :(K) (Y)  :(LIY)(NEK)      (ML)'(DKR)(PDK)  (SRF)'(FR) :         :       (LPY)
      |         : |  |   : |   |   :    |  | (EQV)| | [  :    |:        :||       :         :       :|
      |         : |  |   : |   |   :    |  |   | || |   | :   |:        :||       :         :       :|
      |         : |  |   : |   |   :    |  |   |:| ||| | :    |:        :||       :         :       :|
α1    |         | |  |   | |   |   |    |  |   | || || |      |         ||                  KITAL DE FATKL IQNNH YAKED
      |         | |  |   | |   |   |    |  |   | || || |      |         ||                  •:•••:••:::::•••::••:•
αSpec5 |        | |  |   | |   |   |    |  |   | || || |      |         ||                  EI ITV DKT ATKL IG DDH YDS EN
       |        | |  |   | |   |   |    |  |   | || || |      |         ||
α1    VATRRDALLSRRNAL HERAL YRRTQLADS FHLQQ FL RDS DEL KSW IN EKMKT ATDE AYKDPS NLQ GKVQ KH KL MRL SL SANH SRI DAL EN SGQ KL IDV NH YAS DE
                       :::::::::•::•:::::::::::::::::::•••:::•••:::::•::::::•:::::::•:
α2             QLADS FHLQQ FFRDS DEL KSW IN EKMKT ATDE AYKDPS NLQG KVQ KH KLL RPRL SANQ SRI DAL EN SGQ KL IDV NH YAS DE
      ••••:::•::•::•:::•:•:••::••:::••:••::••:::••••:::•::•:::•:
αSpec6 IKA IRDG LLARRDAL REKA ATRRRLL KES LLLQ KL YE DS DDL KNW INKK

α1    VAARMNEV INL WKKLL EATEL KG IKL REANQQQQ FN RNV EDI EL WL YEV EG HL AS DDYG KDL TNVQ NLQ KKH ALL EADV AAHQ DR IDG IT IQ ARQ FQ EAG HFDA DN
      :::::::•:•:::::::::::::::::::::::::::::::::::::::::::::•:::::::::::::•:::::::::::::
α2    VAARMNDV ITL WKKLL EATEL KG IKL REANQQQQ FN RNV EDI EL WL YEV EG HL AS DDYG KDL TNVQ NFQ KKH ALL EADI AAHQ DR IDG IT IQ ARQ FQ EAG HFDA DN
      ••::••:::••:::::••::••:•:::::•::••:•::•••::••::•••••:•::::•:••:::•••:::•:::••:::•::••:••:••:••:•:•::•:•••
αSpec7 LSEV ASL WEEL LEATKQ KG TQL HEANQQLQ FENNA EDLQ RW LEDV EWQ VTS EDYG KG LAEVQ NRL RKHG LLES AV AARQ DQ VDIL TDL AAYFEE IG HPDS KD

α1    IKKKQ EALV SRYE AL KEPMAARKQ KL SDSL RLQQL FRDV EDEE TW IR EKE PI AAS TN RG KDL IGVQN IL KKHQ ALQ AE IAG HEPR IKAV TQ KG ESM ITEG HFAS EE
      :::::::::::::::::::::•::•::•::::::::::::•:::::::::::::::::::::•:::::::::::::::::::::•:::::::::::
α2    IKKKQ EALV SRYE AL KDPMV ARKEKL SDSL RLQQ IFRDV EDEE TW IR EKE PI AAS TN RG KDL IGVQN LL KKHQ ALQ AE IAG HEPR IKAV TQ KG DSM ITEG HFAS EE
      :•••::•::•::•:::•:•:•:•::•:::::•:•:•::•:•:••:::::•:::•::•::••:::•:::::•••::••:••:••:•
αSpec8 IR ARQ ESLV CRFE AL KEPL ATRKKKLL DLL HLQL IL RDT EDEE AW IQ ETEPS ATS TYLG KDL IAS KKLL NRH RV IL EN IASHEPR IQ EITERG NKMV EBG HFAA ED

α1    VTG KL NEL NDKW ATL KNKASQ RR
      :•::•::•:::::::::::::::
α2    VMG KL KEL IDKW ATL KNKASQ RRQ DL EDSLQ AQQ YFA DAN EAESW MR EKE PIV GSTDYG KDE DS AE ALL KKH EAL MS DL RAYG SSIQGLRV RAQ SCRQQV APT DDE
      :••••::•::•••••:•••:••:••:::•••:•:::•:::••::•:•::•:•::•••:•::•::•••:•:::::::::•••:•••:•:•••:
αSpec9 VAS RV KSL NQ NMESL RARA ARRQ NDL EANVQ FQQ YL ADL HEAE TW IR EKE PIV DNTN YG ADEE AAG ALL KKH EAFLL DL NSFG DS MkAL

α2    TG KELV LAL YDYQ EKS PREV TMKKG DIL TLL NSTNKDWW KV EV NDRQ GFV PAA YV
```

b

c

Figure 5. Analysis of the predicted amino acid sequences of the *Xenopus* α-fodrin cDNAs. (*a*) The predicted amino acid sequences of the cDNAs sequenced in Fig. 3 are arranged into 106 amino acid repeating units, and compared with the known sequence of human erythrocyte α-spectrin (denoted α-Spec 5 to α-Spec 9 to designate the number of the repeat unit; designation from Speicher and Marchesi, 1984). The position of the conserved residues in the repeating structure of human erythrocyte α-spectrin are indicated in the top line (e.g., I, W, L, etc.) and the preferred amino acid substitutions at these positions are indicated in parentheses in the second line (e.g., TV, YRF, etc.). (*b*) Positions of hydrophobic ([•] A, F, I, L, M, V, W, Y); polar ([o] E, H, K, Q, R); small polar ([◗] D, N, S, T), and glycine and proline ([o] G, P) residues in the predicted amino acid sequence of Xen α2. (*c*) Secondary structure predictions of repeat units 7–9 of Xen α2, with positions indicated for predicted helical (*hatched*), β-turn (*stippled*), and nonhelical connecting regions (*open bar*).

polypeptide (as is the highly homologous Xen α1). This conclusion is further supported by a comparison of the hydrophobic and polar residues in the α-fodrin repeats of Xen α2 (Fig. 5 *b*) with the comparable data presented by Speicher and Marchesi (1984) for human erythrocyte α-spectrin. Moreover, analysis of the predicted secondary structure (Chou and Fasman, 1978*a,b*) of repeat units 7–9 in the protein encoded by Xen α2 indicates that these *Xenopus* repeat units share a common secondary structure with human erythrocyte α-spectrin. The repeat units of both *Xenopus* α-fodrin (Fig. 5 *c*) and human α-spectrin (Speicher and Marchesi, 1984) are composed of three large α-helical domains, linked together by short stretches of β turns. Each repeat unit is then connected with the next repeat unit by a nonhelical region. The excellent agreement in the positions of these secondary structures within both *Xenopus* α-fodrin and

human α-spectrin repeat units confirms that we have isolated *Xenopus* cDNAs encoding α-fodrin.

Genome Blot Analysis

Previous work has raised interesting questions concerning the number of α-fodrin and α-spectrin genes that have evolved in various species (Glenney and Glenney, 1984). Fig. 6 shows the hybridization pattern obtained on a genome blot with *Xenopus* liver DNA, using as a probe an ∼500-bp sequence derived from the 5' Hind III to Xba I restriction fragment of Xen α1. These bands represent a subset of the bands obtained when the entire Xen α1 cDNA is used as a probe; the bands not detected with the 5' probe are detected by a 3' probe containing the Xba I to Sal I restriction fragment (data not shown). A homologous 5' probe was prepared from the 5' Hind III to Bam HI restriction fragment of Xen α2, which

Figure 6. Genome blot analysis using *Xenopus* α-fodrin cDNA probes. 3 μg of *Xenopus* liver genomic DNA were digested with Bgl II (lane *1*), Sac I (lane *2*), Kpn I (lane *3*), Hind III (lane *4*), Eco RI (lane *5*), and Bam HI (lane *6*), then electrophoresed on a 0.9% agarose gel, and blotted onto nitrocellulose. The blot was then hybridized with a ^{32}P-labeled probe prepared by primer extension of the 0.5-kb 5' Hind III to Xba I restriction fragment of Xen α1 cDNA. *Bars* indicate the relative mobilities of markers (23, 9.4, 6.56, 4.36, 2.32, 2.03, and 0.564 kb) prepared by digestion of λ DNA with Hind III.

was hybridized to identical enzyme digests coelectrophoresed on the same gel shown in Fig. 6. The only difference between these two genome blots was that the lowermost of the three bands in lane *5* (Eco RI digest) was not obtained with the Xen α2 probe (data not shown), which may reflect differences in the number of Eco RI sites in the introns spanned by the two cDNAs.

Our cloning of two highly related *Xenopus* oocyte cDNAs, both homologous to the same repeat units of human erythrocyte α-spectrin, raises questions about the number of α-fodrin genes in *Xenopus*. It is clear that the nucleotide differences between the two cDNAs are not due to differential splicing of a single primary transcript, inasmuch as the nucleotide differences are sporadic and present throughout the regions which have been sequenced. Two possibilities are that these cDNAs represent different alleles of the same gene, or that *Xenopus* oocytes express two distinct α-fodrin genes which happen to be highly related in the regions that we have cloned. Future analysis of the nucleotide sequences of the untranslated regions of the transcripts complementary to the Xen α1 and Xen α2 cDNAs may resolve this issue. Because *Xenopus laevis* has been shown to have duplicated much of its genome during evolution (reviewed by Kobel and Du Pasquier, 1986), it is likely that Xen α1 and Xen α2 are encoded by distinct genes, which arose through duplication of a unique α-fodrin gene.

Developmental Changes in the Abundance of α-Fodrin Transcripts

The primary purpose for the isolation and characterization of *Xenopus* α-fodrin cDNAs was to determine whether the dramatic changes in the de novo synthesis of α-fodrin polypeptides during development (Fig. 2) were controlled at the level of mRNA abundance. To address this issue, poly(A)$^+$ RNA was isolated from oocytes and from several stages of embryonic development, separated on 0.9% agarose-formaldehyde gels, blotted onto nitrocellulose, and hybridized with ^{32}P-probes prepared from Xen α1 and Xen α2 cDNAs. As shown in lane *1* of Fig. 7 *a*, the Xen α1 probe hybridizes to a transcript of ∼9 kb in poly(A)$^+$ RNA isolated from mature oocytes. Upon comparing this signal with the signals obtained from embryonic RNA, it is evident that a dramatically lower α-fodrin signal is detected in RNA prepared from cleavage-stage (16–64 cells) embryos (lane *2*), from gastrula-stage embryos (lane *4*), and from neurula-stage embryos (lane *5*). Fodrin transcripts were detected in
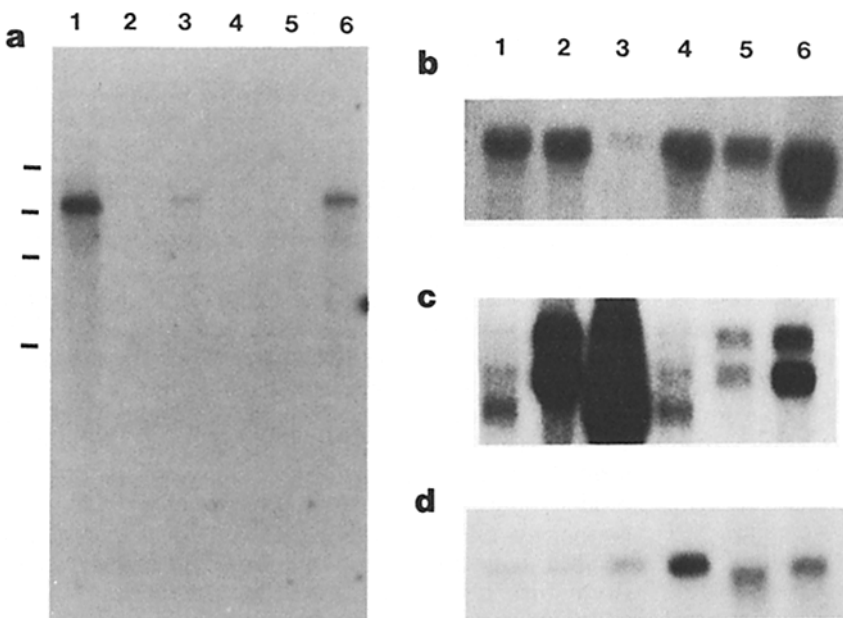
Figure 7. Developmental changes in the abundance of α-fodrin transcripts. (*a*) Poly(A)$^+$ RNA (5 μg per lane) was denatured and electrophoresed on a 0.9% agarose formaldehyde gel, blotted onto nitrocellulose, and hybridized with a ^{32}P-labeled cDNA probe prepared by primer extension of Xen α1 cDNA. Stage 6 oocyte (lane *1*), 16–32-cell embryos (lane *2*), blastula-stage embryos (lane *3*), gastrula-stage embryos (lane *4*), neurula-stage embryos (lane *5*), and hatching tadpoles (lane *6*). The nitrocellulose blot shown in *a* was rehybridized with a probe for *Xenopus* 27S ribosomal RNA (*b*), mouse α-actin (*c*), and the moderately abundant poly(A)$^+$ transcript SPr5B (*d*), with the six lanes oriented as in *a*.
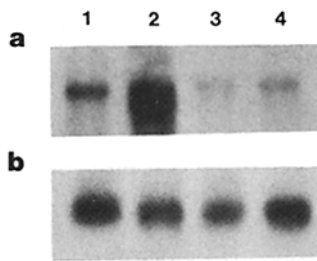
Figure 8. Expression of α-fodrin in early oocytes and adult tissues. (a) Poly(A)⁺ RNA (3 μg) from stage 2–3 oocytes (lane 1), stage 6 oocytes (lane 2), adult skeletal muscle (lane 3), and adult brain (lane 4) were electrophoresed and hybridized as in Fig. 7 a. Only the region corresponding to the α-fodrin signal is shown. (b) The nitrocellulose blot shown in a was rehybridized with a probe for 27S ribosomal RNA. Only the 27S region is shown.

both blastula-stage embryos (lane 3) and hatched tadpoles (lane 6), though at lower levels than in oocytes (lane 1). This pattern of hybridization and relative signal intensities was consistently obtained with three different RNA blots, undertaken with RNA isolated on three different occasions from developing embryos. Identical patterns and relative signal intensities were obtained when the Xen α2 cDNA was used as the probe for hybridization.

The reproducibility of the above results argues strongly for the conclusion that α-fodrin transcripts are less abundant in embryos than in oocytes. Additional experiments support this conclusion. First, all three RNA blots similar to that in Fig. 7 a were hybridized with a probe prepared from a cDNA for *Xenopus* 27S ribosomal RNA (clone pXlrlO1A, kindly provided by Dr. A. Bakken, University of Washington). All three blots gave the pattern shown in Fig. 7 b. Since the poly(A)⁺ RNA fractions contain comparable levels of ribosomal RNA, which is present in poly(A)⁺ RNA fractions at similar levels during early development of *Xenopus* (Dworkin et al., 1981), we conclude that the efficiency of recovery of poly(A)⁺ RNA relative to ribosomal RNA was similar for each sample, with the reproducible exception that less ribosomal RNA was present in the poly(A)⁺ RNA isolated from blastula-stage embryos (lane 3). Therefore, potential differences in RNA isolation cannot account for the observed data. With regard to the levels of ribosomal RNA in the poly(A)⁺ RNA fractions, it is presently unknown whether the lower 27S signal in blastula RNA is due to a change in adenylation of this ribosomal RNA. The lower abundance of 27S RNA relative to poly(A)⁺ RNA in blastula RNA may, however, bias signals for both α-fodrin (Fig. 7 a, lane 3) and actin (described below); hence we cannot conclude that there is a transient increase in the abundance of α-fodrin transcripts at the blastula-stage.

To determine whether fodrin transcripts are specifically degraded during early development, the RNA blot shown in Fig. 7 a was stripped and rehybridized with a clone for mouse α-actin (Minty et al., 1981), which has previously been shown to detect at least two transcripts in *Xenopus* (Heikkila et al., 1985). As shown in Fig. 7 c, this actin probe detects greater levels of actin transcripts in cleavage-stage (lane 2) and blastula-stage (lane 3) embryos than in stage 6 oocytes (lane 1), which demonstrates that not all poly(A)⁺ transcripts decline during early development as shown for α-fodrin in Fig. 7 a. The marked increase in actin transcripts in embryos relative to oocytes (Fig. 7 c) in the absence of new transcription is probably due to changes in the adenylation

of actin transcripts. Next, we rehybridized the blot shown in Fig. 7 a with the clone SPr5B (kindly provided by Sargent and Dawid), which hybridizes to a moderately abundant maternal poly(A)⁺ transcript. As shown in Fig. 7 d (lanes 1–3), low but constant levels of this transcript are present during those developmental stages when levels of α-fodrin transcripts decline, further supporting the conclusion that there is a preferential degradation of fodrin transcripts during early development. The last set of experiments that demonstrate that α-fodrin transcripts decrease in abundance during embryonic development involves the use of S1 nuclease protection assays to monitor the level of α-fodrin in total RNA. These experiments are described in a subsequent section.

The greater abundance of α-fodrin transcripts in mature stage 6 oocytes relative to developing embryos led us to test whether the relative abundance of α-fodrin transcripts in oocytes was greater than in any other cell type. To partially address this question, we examined the level of α-fodrin transcripts in previtellogenic oocytes and in two tissues from adult frogs. As shown in the RNA blot in Fig. 8 a, α-fodrin transcripts are less abundant in previtellogenic stage 2–3 oocytes (lane 1) than in mature vitellogenic stage 6 oocytes (lane 2), suggesting that transcripts for α-fodrin accumulate during oogenesis. Interestingly, the α-fodrin cDNAs isolated from the oocyte cDNA library also hybridize to transcripts present in adult *Xenopus* skeletal muscle (Fig. 8 a, lane 3) and brain (lane 4), indicating that the decline in α-fodrin transcripts during early development is followed by an increase in the expression of α-fodrin transcripts during tissue differentiation. These data also confirm the immunologic analyses (Fig. 1) demonstrating that adult tissues of *Xenopus* contain α-fodrin. The RNA blot shown in Fig. 8 a was rehybridized with the ribosomal cDNA probe to demonstrate that there was comparable contamination of the poly(A)⁺ RNA with 27 S ribosomal RNA (Fig. 8 b).

S1 Nuclease Protection Assay

We undertook S1 nuclease protection assays with both the Xen α1 and Xen α2 cDNAs as an independent test of the number of transcripts complementary to each cDNA (Fig. 9 a) and as a further investigation of the level of expression of α-fodrin transcripts during development (Fig. 9, b and c). ³²P-labeled DNA probes were prepared as described in Materials and Methods, and hybridized either to poly(A)⁺ RNA or to total RNA under conditions that allow hybridization of the probe to RNA, but not to the unlabeled complementary DNA strand present in the assay. For both the Xen α1 (Fig. 9 a, lane 1) and Xen α2 cDNAs (lane 5), the undigested probes migrated as single bands of 3.8 and 2.6 kb, respectively, on denaturing acrylamide gels. Each of these probes contained 1.5 kb of plasmid sequence, which could be digested with S1 nuclease to yield a smaller band corresponding to the length of the probe protected by hybridization to RNA. As expected, the control lanes showed that digestion of these probes with S1 nuclease in the absence of RNA (lanes 2 and 6) or in the presence (lanes 4 and 8) of 2 μg of tRNA resulted in the complete digestion of the ³²P-probes. Hybridization of these probes to 2 μg of poly(A)⁺ oocyte RNA, followed by digestion with S1 nuclease, resulted in the protection of a 2.3-kb fragment for Xen α1 (lane 3) and of a 1.2-kb fragment for Xen α2 (lane 7). The sizes of the protected probes represent full-length protection
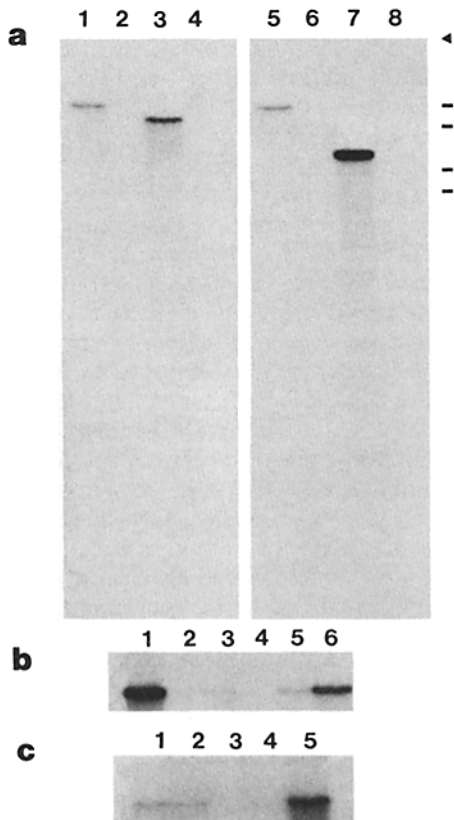
Figure 9. S1 nuclease protection of Xen α1 and Xen α2 cDNA by *Xenopus* RNA. [³²P]DNA probes were prepared and hybridized to 2 μg of poly(A)⁺ RNA as described in Materials and Methods and Results. (*a*) Lanes *1* (Xen α1 probe) and *5* (Xen α2 probe) contain end-labeled probes which were not digested with S1 nuclease. Lanes *2* (Xen α1 probe) and *6* (Xen α2 probe) demonstrate complete digestion of the probes by S1 nuclease. Lanes *3* (Xen α1 probe) and *7* (Xen α2 probe) show the protection of each probe by 2 μg of poly(A)⁺ RNA isolated from *Xenopus* stage *6* oocytes. Lanes *4* (Xen α1 probe) and *8* (Xen α2 probe) demonstrate that the probes are not protected from digestion after hybridization with 2 μg of tRNA. *Bars* indicate the relative mobilities of end-labeled DNA markers of 3.4, 1.85, 1.1, and 0.929 kb. The *arrowhead* denotes the origin of electrophoresis. (*b*) S1 nuclease protection assay of poly(A)⁺ RNA from developing embryos. Poly(A)⁺ RNA (2 μg per lane) from stage 6 oocytes (lane *1*), 16–64-cell embryos (lane *2*), blastula-stage embryos (lane *3*), gastrula-stage embryos (lane *4*), neurula-stage embryos (lane *5*), and hatching tadpoles (lane *6*) were hybridized with the Xen α1 probe, digested with S1 nuclease, and electrophoresed as in *a*. Identical results were obtained with the Xen α2 probe. Only the relevant region of the gel is shown. (*c*) S1 nuclease protection assay of total RNA from selected stages of developing embryos. Either 2 μg of poly(A)⁺ RNA from oocytes (lane *1*) or 17 μg of total RNA from stage 6 oocytes (lane *2*), gastrula-stage embryos (lane *3*), neurula-stage embryos (lane *4*), or hatching tadpoles (lane *5*) were hybridized with the Xen α1 probe and processed as in *b*. In the experiments shown in *b* and *c*, control lanes demonstrated that the bands shown represent full-length protection of the cDNA region of the probe, and not undigested probe (note the size differences between the input probe and the protected probe in *a*), and that hybridization of the probe to tRNA did not result in a protected band (data not shown).

of each cDNA, indicating that both cDNAs are complementary to endogenous α-fodrin transcripts in the oocyte. While these data appear to indicate that only a single species of

mRNA is complementary to each probe under these experimental conditions, it is presently unknown whether the 83% identical Xen α1 and Xen α2 cDNA probes are specific for their own transcripts, or hybridize with each others transcripts as well.

The conclusion that *Xenopus* α-fodrin transcripts are much less abundant in the poly(A)⁺ RNA of early embryos than in oocytes, and that the level of α-fodrin RNA increases by the tadpole stage, was substantiated using the Xen α1 probe in the S1 nuclease protection assay (Fig. 9 *b*). Similar S1 protection patterns were obtained using Xen α2 as the hybridization probe, with no indication that Xen α1 and Xen α2 are expressed differentially during embryonic development (data not shown). Significantly, a similar developmental pattern of expression was obtained using 17 μg of total RNA as the input RNA for the S1 nuclease protection assay (Fig. 9 *c*). Because this RNA was not passed over an oligo(dT)-cellulose column, this result eliminates the formal possibility that the apparent decline in α-fodrin transcripts was due to the deadenylation of these transcripts, followed by a failure to detect these transcripts in poly(A)⁺ RNA fractions. The greater fodrin signal in total RNA from tadpoles (Fig. 9 *c*, lane *5*) relative to total RNA from oocytes (lane *2*) suggests that a considerable amount of fodrin RNA in tadpoles is not adenylated, given that a greater fodrin signal was obtained with poly(A)⁺ RNA of oocytes than with the poly(A)⁺ of tadpoles (Fig. 9 *B*).

In that the decrease in abundance of α-fodrin transcripts involves the degradation rather than deadenylation of these maternal transcripts, we next investigated whether this degradation occurred after oocyte maturation or after fertilization. Poly(A)⁺ RNA isolated from stage 6 oocytes (Fig. 10, lane *1*), from oocytes 12 h after in vitro maturation with progesterone (lane *2*), from zygotes 30–60 min after fertilization (lane *3*), and from 16–64-cell embryos (lane *4*) was separated on denaturing agarose gels, blotted onto nitrocellulose, and probed with a ³²P-labeled pXen α1 probe. The data clearly demonstrate that degradation of α-fodrin tran-
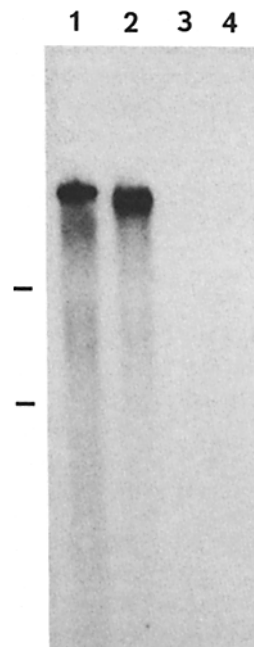

Figure 10. Destabilization of fodrin transcripts follows fertilization, but not oocyte maturation. Poly(A)⁺ RNA (5 μg/lane) from stage 6 oocytes (lane *1*), progesterone-stimulated oocytes (lane *2*), fertilized eggs (lane *3*), and 16–32-cell embryos (lane *4*) was prepared, blotted onto nitrocellulose, and probed as in Fig. 7 to determine whether the decline in steady-state levels of α-fodrin occurs after maturation or following fertilization.

scripts does not occur within the 12 h after in vitro maturation with progesterone; rather, these transcripts are degraded within 30–60 min after fertilization. (Fig. 10). The apparent decrease in the size of the α-fodrin transcripts in hormonally matured oocytes (lane 2) as compared with stage 6 oocytes (lane 1) may reflect a modification of the α-fodrin transcripts, such as deadenylation, though this has not yet been determined.

Discussion

We are investigating the expression and functions of spectrin during embryonic development and cellular differentiation in *Xenopus laevis*. In the present study we use antiserum raised against avian α-spectrin to show that α-spectrinlike polypeptides (referred to as α-fodrin in nonerythroid tissues) are present in *Xenopus* adults and oocytes, but that the de novo synthesis of α-fodrin declines during early development. RNA blot analyses and S1 nuclease protection assays with cDNAs encoding portions of *Xenopus* oocyte α-fodrin demonstrate that this decline in the synthesis of α-fodrin polypeptides is controlled at the level of mRNA abundance. RNA blot analyses also show that this dramatic decrease in steady-state levels of α-fodrin transcripts occurs within 30–60 min after fertilization, but not within 12 h after progesterone-mediated maturation. Although α-fodrin transcripts decrease in abundance after fertilization and remain low throughout early development, α-fodrin transcripts increase in abundance by the tadpole stage of development, and are expressed in adult skeletal muscle and brain.

The dramatic decline in α-fodrin transcripts during embryonic development of *Xenopus* is quite distinct from the levels of many other transcripts present during development of *Xenopus*. For example, levels of most abundant poly(A)$^+$ RNA species do not change appreciably until after the blastula-stage (Dworkin and Dawid, 1980; Colot and Rosbash, 1982), in distinct contrast to the levels of α-fodrin transcripts monitored in the present study. Also, the level of histone mRNAs increases somewhat during the period when α-fodrin transcripts decrease in abundance (Ruderman et al., 1979), demonstrating that known mRNAs do not follow the pattern of fodrin transcripts. That many maternal mRNAs persist during those embryonic stages when levels of α-fodrin transcripts decline is well established and consistent with the fact that embryonic protein synthesis requires maternal transcripts until transcription from the embryonic genome occurs at the mid-blastula transition (Newport and Kirschner, 1982). Furthermore, we have shown that the levels of two maternal poly(A)$^+$ transcripts (actin and SPr5B) remain constant or increase in the poly(A)$^+$ fraction during those developmental stages when levels of fodrin transcripts decline. Hence, we suggest that the loss of α-fodrin transcripts after fertilization of *Xenopus* may involve the preferential destabilization of α-fodrin transcripts that had been synthesized during oogenesis, independent of the overall stability of many abundant maternal poly(A)$^+$ mRNAs. Although it is likely that α-fodrin transcripts are indeed subject to preferential degradation in early embryos, the mechanisms responsible for this proposed degradation may not be specific for fodrin transcripts. A more generalized mechanism for enhanced turnover of certain transcripts may be operative during early development since several other tran-

scripts have been reported to decline markedly during early development, including XOC-2-7 transcripts (Colot and Rosbash, 1982), GS17 transcripts (Krieg and Melton, 1985), and homeo-box transcripts (Muller et al., 1984).

It is intriguing to speculate whether this transitory decrease in fodrin expression indicates that fodrin serves little function during early development, and that it reappears only when tissue differentiation requires specific membrane properties influenced by fodrin. Two alternative speculations are that the expression of fodrin may decline during early development because fodrin may be deleterious to certain membrane functions in the developing embryo, or conversely, because there is a sufficient pool of fodrin in the oocyte to supply the needs of the developing embryo. Microinjection of fodrin into developing embryos may distinguish between these possibilities. Although the functional significance of the observed decline in fodrin expression during early embryogenesis is presently unknown, the data demonstrate that the constitutive expression of fodrin is not a prerequisite for normal early development in *Xenopus*, and that the regulation of fodrin expression occurs principally at the level of mRNA abundance. Similarly, we have previously shown that there are signficant changes in spectrin expression during myogenesis which are controlled at the level of mRNA abundance (Moon et al., 1985).

Previous studies on the expression of fodrin during the development of mice (Sobel and Alliegro, 1985; Schatten et al., 1986) and sea urchins (Schatten et al., 1986) have been based on the use of immunofluorescence to define the spatial localization of fodrin in embryos. These studies have shown that fodrin is nonrandomly distributed in oocytes and embryos. In mouse oocytes, fodrin appears to be enriched at the cortical region near the meiotic spindle. In cleaving mouse embryos, the cleavage furrow stains more intensely than the outer membranes of the blastomeres. Although this pattern of staining is not observed in cleaving sea urchin embryos, it is interesting to note that an increase in fodrin fluorescence is detected at the site of sperm entry in sea urchins (Schatten et al., 1986). Collectively, these studies have clearly shown that fodrin changes in localization during early development, and that it is probably involved in the specialized properties of plasma membranes in discrete regions of oocytes and of the blastomeres of early embryos. In light of the unexpected reduction in the synthesis of α-fodrin during early development in *Xenopus*, we are presently studying the spatial organization of fodrin in oocytes of *Xenopus*, and we are following the developmental fate of the maternal fodrin which is either preexisting or synthesized in the oocyte. We speculate that preexisting fodrin may be nonrandomly distributed in cortex of the oocyte, and that this asymmetric distribution may persist in blastomeres during early cleavages.

Relatively little is known about the structure of the plasma membrane of *Xenopus* oocytes (reviewed by Gerhart, 1980), and whether a membrane-associated fodrin network plays a role in development by influencing pattern formation or the establishment of functionally specialized membrane-cytoskeletal domains. Our demonstration that α-fodrin is synthesized in oocytes of *Xenopus*, that this synthesis declines after fertilization, and that this decline is controlled at the level of abundance of fodrin mRNA, raises questions concerning the functions of fodrin and the control of fodrin mRNA stability during early development in vertebrates.

References

Aviv, H., and P. Leder. 1972. Purification of biologically active globin mRNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA.* 69:1408–1412.

Bluemink, J. G., and L. G. J. Tertoolen. 1978. The plasma-membrane IMP pattern as related to animal/vegetal polarity in the amphibian embyro. *Dev. Biol.* 62:334–343.

Chou, P. Y., and G. D. Fasman. 1978a. Empirical predictions of protein conformation. *Annu. Rev. Biochem.* 47:251–276.

Chou, P. Y., and G. D. Fasman. 1978b. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol. Relat. Areas Mol. Biol.* 47:45–148.

Colot, H. V., and M. Rosbash. 1982. Behavior of maternal pA⁺ RNAs during embryogenesis of *Xenopus laevis*. *Dev. Biol.* 94:79–86.

Dworkin, M. B., and I. B. Dawid. 1980. Use of a cloned library for the study of abundant poly(A)⁺ RNA during *Xenopus laevis* development. *Dev. Biol.* 76:449–464.

Dworkin, M. B., B. K. Kay, J. W. B. Hershey, and I. B. Dawid. 1981. Mitochondrial RNAs are abundant in the poly(A)⁺ RNA population of early frog embryos. *Dev. Biol.* 86:502–504.

Feinberg, A. P., and B. Vogelstein. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to a high specific activity. *Anal. Biochem.* 132:6–13.

Franke, W. W., P. C. Rathke, E. Seib, M. F. Trendelenburg, M. Osborn, and K. Weber. 1976. Distribution and mode of rearrangement of microfilamentous structures and actin in the cortex of the amphibian oocyte. *Cytobiologie.* 14:111–130.

Franz, J. K., L. Gall, M. A. Williams, B. Picheral, and W. W. Franke. 1983. Intermediate-size filaments in a germ cell: expression of cytokeratins in oocytes and eggs of the frog *Xenopus*. *Proc. Natl. Acad. Sci. USA.* 80:6254–6258.

Gall, L., B. Picheral, and P. Guonon. 1983. Cytochemical evidence for the presence of intermediate filaments and microfilaments in the egg of *Xenopus laevis*. *Biol. Cell.* 47:331–342.

Gerhart, J. C. 1980. Mechanisms regulating pattern formation in the amphibian egg and early embryo. *In* Biological Regulation and Development, vol. 2. R. F. Goldberger, editor. Plenum Publishing Corp., New York. 133–316.

Glenney, J. R., Jr., and P. Glenney. 1984. Comparison of spectrin isolated from erythroid and nonerythroid sources. *Eur. J. Biochem.* 144:529–539.

Godsave, S. F., B. H. Anderton, J. Heasman, and C. C. Wylie. 1984a. Oocytes and early embryos of *Xenopus laevis* contain intermediate filaments which react with anti-mammalian vimentin antibodies. *J. Embryol. Exp. Morphol.* 83:169–187.

Godsave, S. F., C. C. Wylie, E. B. Lane, and B. H. Anderton. 1984b. Intermediate filaments in the *Xenopus* oocyte: the appearance and distribution of cytokeratin-containing filaments. *J. Embryol. Exp. Morphol.* 83:157–167.

Gurdon, J. B., and M. P. Wickens. 1983. The use of *Xenopus* oocytes for the expression of cloned genes. *Methods Enzymol.* 101:371–386.

Heikkila, J. J., M. Kloc, J. Bury, G. A. Schultz, and L. W. Browder. 1985. Acquisition of the heat-shock response and thermotolerance during early development of *Xenopus laevis*. *Dev. Biol.* 107:483–489.

Kobel, H. R., and L. Du Pasquier. 1986. Genetics of polyploid *Xenopus*. *Trends Genet.* 2:310–315.

Koenig, E., and E. Repasky. 1985. Regional analysis of α-spectrin in the isolated Mauthner neuron and in isolated axons of the goldfish and rabbit. *J. Neurosci.* 5:705–714.

Krieg, P. A., and D. A. Melton. 1985. Developmental regulation of a gastrula-specific gene injected into fertilized *Xenopus* eggs. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:3463–3471.

Lazarides, E., W. J. Nelson, and K. Kasamatsu. 1984. Segregation of two spectrin forms in the chicken optic system: a mechanism for establishing restricted membrane cytoskeletal domains in neurons. *Cell.* 36:269–278.

Levine, J., and M. Willard. 1981. Fodrin: axonally transported polypeptides associated with the internal periphery of many cells. *J. Cell Biol.* 90:631–643.

Levine, J., and M. Willard. 1983. Redistribution of fodrin (a component of the cortical cytoplasm) accompanying capping of cell surface molecules. *Proc. Natl. Acad. Sci. USA.* 80:191–195.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

McMahon, A. P., D. H. Giebelhaus, J. E. Champion, J. A. Bailes, S. Lacey, B. Carritt, S. K. Henchman, and R. T. Moon. 1987. cDNA cloning, sequencing, and chromosome mapping of a non-erythroid spectrin, human α-fodrin. *Differentiation*. In press.

Minty, A. J., M. Caravatti, B. Robert, A. Cohen, P. Daubas, A. Weydert, F. Gros, and M. E. Buckingham. 1981. Mouse actin messenger RNAs: construction and characterization of a recombinant plasmid molecule containing a complementary DNA transcript of mouse α-actin mRNA. *J. Biol. Chem.* 256:1008–1014.

Moon, R. T., M. V. Danilchik, and M. B. Hille. 1982. An assessment of the masked message hypothesis: Sea urchin egg messenger ribonucleoprotein complexes are efficient templates for in vitro protein synthesis. *Dev. Biol.* 93:389–403.

Moon, R. T., J. Ngai, B. J. Wold, and E. Lazarides. 1985. Tissue-specific expression of distinct spectrin and ankyrin transcripts in erythroid and nonerythroid cells. *J. Cell Biol.* 100:152–160.

Muller, M. M., A. E. Carrasco, and E. M. DeRobertis. 1984. A homeo-box-containing gene expressed during oogenesis in Xenopus. *Cell.* 39:157–162.

Nelson, W. J., C. A. L. S. Colaco, and E. Lazarides. 1983. Involvement of spectrin in cell-surface receptor capping in lymphocytes. *Proc. Natl. Acad. Sci. USA.* 80:1626–1630.

Nelson, W. J., and E. Lazarides. 1984. Assembly and establishment of membrane-cytoskeleton domains during differentiation. *In* Cell Membranes, vol. 2. E. Elson, W. Frazier, and L. Glaser, editors. Plenum Publishing Corp., New York. 219–246.

Newport, J., and M. Kirschner. 1982. A major developmental transition in early *Xenopus* embryos: characterization and timing of cellular changes at the midblastula stage. *Cell.* 30:675–686.

Nieukoop, P. D., and J. Faber. 1967. Normal Table of *Xenopus laevis* (Daudin). Second edition, North Holland Publishing, Amsterdam.

Rebagliati, M. R., D. L. Weeks, R. P. Harvey, and D. A. Melton. 1985. Identification and cloning of localized maternal RNAs from *Xenopus* eggs. *Cell.* 42:769–777.

Repasky, E. A., B. L. Granger, and E. Lazarides. 1982. Widespread occurrence of avian spectrin in nonerythroid cells. *Cell.* 29:821–833.

Ruderman, J. V., H. R. Woodland, and E. A. Sturgess. 1979. Modulations of histone messenger RNA during the early development of *Xenopus laevis*. *Dev. Biol.* 71:71–82.

Sanger, F., A. R. Coulson, B. G. Barrell, A. J. H. Smith, B. A. Roe. 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol.* 143:161–178.

Schatten, H., R. Cheney, R. Balczon, M. Willard, C. Cline, C. Simerly, and G. Schatten. 1986. Localization of fodrin during fertilization and early development of sea urchins and mice. *Dev. Biol.* 118:457–466.

Sobel, J. S., and M. A. Alliegro. 1985. Changes in the distribution of a spectrin-like protein during development of the preimplantation mouse embryo. *J. Cell Biol.* 100:333–336.

Speicher, D. W., and V. T. Marchesi. 1984. Erythrocyte spectrin is comprised of many homologous triple helical segments. *Nature (Lond.).* 311:177–180.

Vacquier, V. D. 1981. Dynamic changes of the egg cortex. *Dev. Biol.* 84:1–26.

Wasserman, W. J., R. D. Richter, and L. D. Smith. 1982. Protein synthesis during maturation promoting factor- and progesterone-induced maturation in *Xenopus* oocytes. *Dev. Biol.* 89:152–158.

YoungLai, E. V., F. Godeau, B. Mulvihill, and E. E. Baulieu. 1982. Effects of cholera toxin and actinomycin on synthesis of [³⁵S]methionine-labeled proteins during progesterone-induced maturation of *Xenopus laevis* oocytes. *Dev. Biol.* 91:36–42.