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Sequential Expression of Multiple POU Proteins during Amphibian Early Development

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The octamer motif is a common *cis*-acting regulatory element that functions in the transcriptional control regions of diverse genes and in viral origins of replication. The ability of a consensus octamer motif to stimulate transcription of a histone H2B promoter in frog oocytes suggests that oocytes contain a transcriptionally active octamer-binding protein(s). We show here that frog oocytes and developing embryos contain multiple octamer-binding proteins that are expressed in a sequential manner during early development. Sequences encoding three novel octamer binding-proteins were isolated from *Xenopus* cDNA libraries by virtue of their homology with the DNA binding (POU) domain of Oct-1. The predicted POU domains of these proteins were most highly related to mammalian Oct-3 (also termed Oct-4), a germ line-specific gene required for mouse early development. Transcripts from these amphibian POU-domain genes were most abundant during early embryogenesis and absent from most adult somatic tissues. One of the genes, termed Oct-60, was primarily expressed as a maternal transcript localized in the animal hemisphere in mature oocytes. The protein encoded by this gene was present in oocytes and early embryos until the gastrula stage of development. Transcripts from a second POU-domain gene, Oct-25, were present at low levels in oocytes and early embryos and were dramatically upregulated during early gastrulation. In contrast to the Oct-60 mRNA, translation of Oct-25 mRNA appeared to be developmentally regulated, since the corresponding protein was detected in embryos during gastrulation but not in oocytes or rapidly cleaving embryos. Transcripts from the third POU protein gene, Oct-91, were induced after the midblastula transition and reached their highest levels of accumulation during late gastrulation. The expression of all three genes decreased during late gastrulation and early neurulation. By analogy with other members of the POU-domain gene family, the products of these genes may play critical roles in the determination of cell fate and the regulation of cell proliferation.

Current models of embryonic development postulate that embryogenesis and morphogenesis are controlled by the sequential activation of a complex cascade of regulatory genes. In *Drosophila melanogaster*, many of the genes that control embryogenesis are expressed in a precise temporal and spatial pattern, and many of the encoded proteins share structural motifs that suggest a common function. The first motif identified was the homeobox which encodes a conserved 60-amino-acid homeodomain with similarity to the helix-turn-helix DNA binding domain of several prokaryotic transcriptional regulators. The realization that the homeodomain was a conserved motif in proteins involved in important aspects of pattern formation in *D. melanogaster* initiated searches for vertebrate homeodomain (Hox) genes. These studies led to the identification and analysis of numerous Hox genes with various degrees of structural homology to distinct *Drosophila* homeodomain genes. Although the precise function of Hox genes is not yet known, the available structural and functional data are consistent with a presumed role in patterning and the establishment of axial polarity during early development (reviewed in references 22 and 28).

The helix-turn-helix motif of the homeodomain is present in other DNA-binding proteins that otherwise do not have obvious homology to products of *Drosophila* homeotic genes or Hox genes. A family of recently characterized transcription factors contains a diverged homeodomain as part of a structure referred to as a POU domain (12). The POU domain is a 160-amino-acid bipartite structure that consists of a specialized homeodomain preceded by a highly conserved POU-specific region. The POU homeodomain alone is capable of weak interactions with DNA and participates in the formation of heteromeric protein complexes (42, 48). The POU-specific domain contributes to the specificity and strength of DNA binding by the POU homeodomain; it also participates in protein-protein interactions (15).

Members of the POU-domain gene family originally included three mammalian transcription factors (Pit-1, Oct-1, and Oct-2) and *unc*-86, a gene required for neuroblast differentiation in nematodes (9, 12). Subsequent studies showed that POU-domain proteins constitute a much larger family of tissue-specific transcriptional regulators, many of which bind to sequences related to the conserved octamer sequence and are differentially expressed early in mouse embryogenesis and in various adult tissues (for reviews, see references 29 and 33). The transcriptional regulatory properties of POU-domain proteins can be promoter specific and modified by interactions with other proteins (17, 42).

In addition to their role as transcriptional regulators, POU-domain proteins may be involved in regulating cell proliferation. Oct-1 appears to be identical to NF-III, a cellular DNA replication factor that binds to an octamer motif in the adenovirus origin of replication and that stimulates DNA replication in vitro (26). The POU domain of either Oct-1 or Oct-2 is sufficient to stimulate octamer-dependent adenovirus DNA replication (47). Recent evidence suggests a role for two other POU-domain proteins in cell proliferation. Pit-1 is expressed in the anterior pituitary, where it is responsible for activating growth hormone and prolactin gene transcription (2, 14). Dwarf mice (*dw* and *dw*^{J})

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contain naturally occurring mutations in the *pit*-1 gene that abolish its expression, resulting in a deficiency in growth hormone and prolactin synthesis and hypoplasia of cells that express Pit-1 in the normal pituitary (21). Antisense oligonucleotide-directed inhibition of Pit-1/GHF-1 synthesis in pituitary cell cultures leads to decreased growth hormone and prolactin expression and inhibition of cell proliferation (5). Similar experiments suggest a dual role for a maternally expressed POU protein, Oct-3, whose expression is restricted to the female germ line and to totipotent and pluripotent stem cells before gastrulation (31, 38). Studies with antisense oligonucleotides have shown that Oct-3 function is required for the first cleavage in mouse embryos, suggesting a possible role for this protein in DNA replication (30).

We have recently shown that an octamerlike motif in a *Xenopus* H2B histone gene promoter was not required for H2B transcription in frog oocytes (13). However, substitution of the variant octamer sequence with a consensus octamer site activated transcription of the H2B promoter. Furthermore, frog oocytes were found to contain octamer-binding proteins that formed at least two electrophoretically distinct complexes, one of which appeared to result from the binding of maternal Oct-1. Because Oct-1 is reportedly an inefficient transcriptional activator of mRNA-encoding genes (45), this finding raised the possibility that octamer-dependent activation of the H2B promoter in oocytes might be mediated by a protein distinct from Oct-1. To further explore this possibility, we analyzed protein-DNA complexes formed with the consensus octamer motif and extracts from oocytes and developing embryos. We show here that multiple maternal proteins (including Oct-1) were expressed in oocytes and early embryos and that they formed distinct complexes with the octamer site. A decline in the abundance of several of these complexes during early gastrulation coincided with the accumulation of additional complexes. To investigate whether any of these complexes were due to the temporally regulated expression of distinct POU proteins, *Xenopus* oocyte and embryo cDNA libraries were screened for sequences with homology to the POU domain of *Xenopus* Oct-1. We report here the isolation and analysis of three novel POU-domain genes related to Oct-3 that are transiently and sequentially expressed during early *Xenopus* development.

MATERIALS AND METHODS

Embryo and oocyte manipulations. Female *Xenopus laevis* were induced to ovulate by injection with 50 U of pregnant mare serum, followed 1 day later by an injection with 500 U of human chorionic gonadotropin. Eggs were fertilized in vitro with a solution of macerated testis, then dejellied with 2% cysteine, and washed extensively in 0.1× modified Barth's solution (MBS [8]). Embryos were allowed to develop at 23°C in 0.1× MBS and staged according to the method of Nieuwkoop and Faber (24).

Oocytes were surgically removed from mature female frogs and defolliculated by gentle swirling in 1× MBS containing 2 mg of collagenase per ml. Individual oocytes were arranged on a microscope slide with their animal-vegetal axis oriented horizontally and then frozen in situ by placing the glass slide on a metal block imbedded in dry ice. Frozen oocytes were cleaved with a scalpel at an equatorial plane perpendicular to the animal-vegetal axis. Animal and vegetal halves were collected and stored in separate tubes at −80°C.

Extract preparation. Staged embryos were transferred at appropriate intervals to 1.5-ml microcentrifuge tubes. The excess fluid was removed, and the tubes were frozen in dry ice before storage at −80°C. Extracts were prepared from frozen oocytes and embryos by homogenizing 30 oocytes or embryos per sample in 0.45 ml of 0.42 M NaCl–20 mM Tris (pH 7.8)–5 mM dithiothreitol (DTT)–1 mM EDTA–20% glycerol–1 mM phenylmethylsulfonyl fluoride–1 μg of aprotinin per ml–1 μg of leupeptin per ml–1 μg of pepstatin per ml. After incubation of the homogenates at 4°C for 30 min, samples were centrifuged at 13,000 rpm for 15 min, and then the supernatants were removed and stored at −80°C. Protein concentrations, measured with a dye-binding assay (Bio-Rad, Richmond, Calif.) using a bovine serum albumin standard, were typically 5 to 10 mg/ml. Before use in mobility shift assays, extracts were diluted with 2 volumes of homogenization buffer containing 50 mM NaCl, and the insoluble material was cleared by centrifugation.

Isolation and characterization of cDNA clones. A stage 11 cDNA library was screened with a 600-bp fragment containing the POU domain of a *Xenopus* Oct-1 cDNA, using low-stringency hybridization conditions as previously described (34). Clones encoding Oct-1 were identified by stripping the primary filters and rehybridizing them at high stringency (45°C, 50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) with a fragment specific to the 3′ end of the *Xenopus* Oct-1 cDNA. A λ ZAP oocyte cDNA library was screened at high stringency with a radiolabeled probe specific to Oct-60. Positive clones were converted to plasmids after plaque purification and characterized by partial DNA sequence analysis. The complete coding region of Oct-60 was reconstructed by fusing oocyte and embryo cDNAs at the unique *Xmn*I restriction site. The oocyte and embryo Oct-60 partial cDNAs were identical in a 340-bp region of overlap that spanned the POU-specific domain. A cDNA fragment encoding *Xenopus* mitochondrial cytochrome oxidase, fortuitously isolated during screening of the embryo cDNA library, was identified by DNA sequence analysis and used as a standard for Northern (RNA) and RNase protection assays. Clones with overlapping unidirectional deletions were prepared by using exonuclease III and S1 nuclease and then analyzed by dideoxynucleotide sequencing. Sequences were analyzed with the Beckman Microgenie and University of Wisconsin Genetics Computer Group sequence analysis packages.

Northern blot analysis and RNase protection studies. RNA was isolated from oocytes, eggs, and staged embryos as previously described (34). Total cellular RNA (20 μg) was subjected to electrophoresis on a 1.5% agarose gel containing 6% formaldehyde and blotted onto nylon filters. Filters were hybridized with probes prepared from polymerase chain reaction-amplified DNA. RNA was isolated from adult *Xenopus* tissues by homogenization of frozen tissue sections in guanidinium thiocyanate followed by phenol extraction and alcohol precipitation (6).

Antisense riboprobes were prepared by transcription of linearized plasmids with the appropriate T3 or T7 RNA polymerase and [α-^{32}P]UTP. After DNase I digestion, riboprobes were purified by electrophoresis on 6% polyacrylamide gels containing 7 M urea. Probes were eluted from gel slices by incubation at 37°C for 4 to 16 h in 10 mM Tris (pH 7.4)–1 mM EDTA–0.1% sodium dodecyl sulfate (SDS). Equal amounts (4 μg) of RNA from oocytes, eggs, and embryos and 10 μg of tissue RNA were hybridized with 10^5 cpm of riboprobe for 12 to 18 h at 45°C in 10 μl of 80% formamide–0.4 M NaCl–10 mM piperazine-*N*,*N*′-bis(2-

ethanesulfonic acid (PIPES; pH 6.8)–1 mM EDTA. Parallel hybridizations were performed with spectrophotometrically determined amounts of synthetic sense RNA to allow determination of absolute transcript numbers. RNase-resistant hybrids were purified by phenol extraction and ethanol precipitation and then analyzed by electrophoresis on 6% acrylamide gels containing 7 M urea. Quantitation was performed on dried gels with a Betascope 603 blot analyzer (Betagen Corp., Waltham, Mass.).

In vitro transcription and translation. Synthetic RNA was prepared by in vitro transcription of linearized plasmids, using T3 or T7 RNA polymerase as appropriate. Proteins were synthesized in a rabbit reticulocyte lysate (Promega, Madison, Wis.) programmed with 1 μg of synthetic RNA in a 25-μl reaction. Translation products were resolved by SDS-polyacrylamide gel electrophoresis. We note that the electrophoretic mobilities of the prestained molecular weight standards used in this analysis are approximately 90% of their expected mobility relative to standards that were not prestained. This factor was applied in the calculation of the apparent molecular weights of the in vitro translation products.

Construction and purification of Oct-60 fusion protein. To express an Oct-60 fusion protein, the Oct-60 cDNA was digested with *Bgl*II, and the ends were repaired with DNA polymerase I. After ligation with an excess of *Eco*RI linkers, the DNA was purified, digested with *Eco*RI, and inserted into the *Eco*RI site of pGEX-3X (39). BL21(DE3) cells were used for expression of the fusion protein. Cell growth and extract preparation was performed as previously described (18). The lysate supernatant from 8 liters of cells was applied to a 5-ml column of glutathione-agarose equilibrated in SPE (0.15 M NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 10 mM EDTA). After loading, the column was washed with SPE until the A_{254} returned to baseline. Bound protein was eluted with 50 mM Tris (pH 8.0)–5 mM glutathione. The protein eluted from the glutathione-agarose column was primarily a mixture of intact fusion protein and partially degraded protein containing the glutathione *S*-transferase moiety (approximately 27 to 30 kDa). The intact fusion protein was further purified by chromatography on a 2-ml heparin agarose column equilibrated in 50 mM Tris (pH 8.0)–5 mM DTT–1 mM EDTA. After washing with equilibration buffer, the bound protein was eluted with buffer containing 0.5 M NaCl. The peak protein fractions were pooled and stored at −80°C. The purified protein (approximately 1 mg) was about 90% homogeneous. New Zealand White rabbits were injected subcutaneously with 100 μg of fusion protein in complete Freund's adjuvant and then boosted twice at 2-week intervals with an additional 100 μg.

DNA binding studies. DNA binding reactions were performed on ice in a 20-μg reaction mixture containing 100 mM NaCl, 10 mM Tris (pH 7.8), 5 mM $MgCl_2$, 10 mM DTT, 20% glycerol, 0.5% Ficoll, 5 μg of extract from oocytes or embryos, 0.5 μg of poly(dI-dC), and 0.2 ng of 3′-end-labeled oligonucleotide (GTACCTTATTTGCATAAGCGTAC) containing the underlined consensus octamer sequence (13). Binding assays with in vitro-synthesized proteins were performed as described above except that 3 μl of synthetic protein was used in place of the whole cell extract. Protein-DNA complexes were resolved by electrophoresis on 6% polyacrylamide gels. Oligonucleotide competitions were performed with a 50-fold excess (10 ng) of oligonucleotides containing either the consensus octamer binding site or an ATF binding site (13).

Nucleotide sequence accession numbers. The nucleotide sequences of the Oct-25, Oct-60, and Oct-91 cDNAs were submitted to GenBank and assigned accession numbers M60074, M60075, and M60077, respectively.

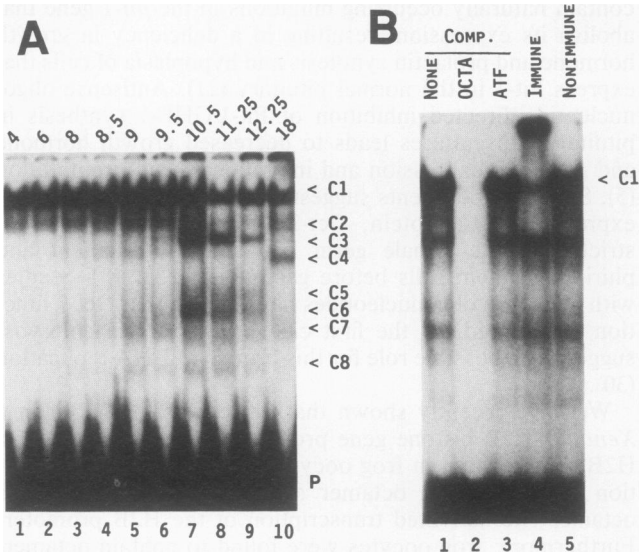


FIG. 1. Expression of novel octamer-binding proteins in developing frog embryos. (A) Extracts prepared from staged embryos were incubated with a radiolabeled oligonucleotide containing the consensus octamer motif (ATTTGCAT). Protein-DNA complexes were resolved by polyacrylamide gel electrophoresis, and the dried gel was exposed to film for 3 days. Individual complexes, C1 through C8, are indicated. The position of the unbound probe is shown (P). Labels above each lane indicate stages of development according to Nieuwkoop and Faber (24). (B) The specificity of protein-DNA interactions in complexes from gastrula-stage embryos (stage 11.25) was tested by oligonucleotide competition and with Oct-1 antisera. Lanes 1, no competitor added; 2, 50-fold molar excess of homologous oligonucleotide competitor (OCTA); 3, 50-fold excess of oligonucleotide containing an ATF/CREB binding site (TGACGTCA); 4, 1 μl of immune antiserum directed against human Oct-1; 5, 1 μl of normal rabbit serum.

RESULTS

Multiple octamer binding activities in *Xenopus* embryos. During studies of complexes formed with extracts from gastrula-stage embryos and a consensus octamer motif, ATTTGCAT, we observed the formation of multiple protein-DNA complexes. To further explore the nature and abundance of these complexes, we examined their formation by using the octamer site probe and extracts from embryos at various stages during early development (Fig. 1A). It was apparent that a complex array of electrophoretically distinct complexes was formed and that the abundance of several complexes changed during development. Several complexes, including C1, C2, C4, C7, and C8, were formed with embryo extracts from cleavage stage (stage 4) through the midblastula stage (stage 8.5). These complexes, which increased slightly in abundance during early development until gastrulation and then subsequently declined, appeared to result from the expression of maternally active genes, since the DNA-binding proteins responsible for their formation were present in oocytes and early embryos before transcriptional activation of the embryonic genome at the midblastula transition. This is more apparent for C4, C7, and C8 with longer autoradiographic exposures (shown below). Several

novel complexes were formed with extracts from embryos after the midblastula transition. The C3, C5, and C6 complexes appeared coordinately; they were first observed at stage 9.5, reached maximal levels between stages 10.5 and 12, then decreased during neurulation. The C2 and C4 complexes, present in extracts from cleavage-stage embryos, also decreased in abundance during gastrulation. A complex with an electrophoretic mobility similar to that of C4 was present at the neural groove stage, as was a complex migrating slightly more rapidly than C2 (lane 10).

The DNA sequence specificity of complex formation was tested by using oligonucleotide competitors in the gel shift assay. The addition of a 50-fold excess of the octamer site competitor to extracts from gastrula-stage embryos prevented the formation of complexes C1 through C8 (Fig. 1B, lane 2). In contrast, an equivalent molar excess of an oligonucleotide containing an ATF/CREB binding site had no effect on these complexes (lane 3). The most slowly migrating complex, C1, was the most abundant and contained Oct-1, since it alone was recognized by rabbit antisera directed against bacterially expressed human Oct-1 (lane 4). The inability of this antiserum to recognize the rapidly migrating complexes suggests that they are neither immunologically related to Oct-1 nor derived from Oct-1 by proteolysis. We concluded from these results that multiple octamer-binding proteins, distinct from Oct-1, are differentially expressed during early amphibian development.

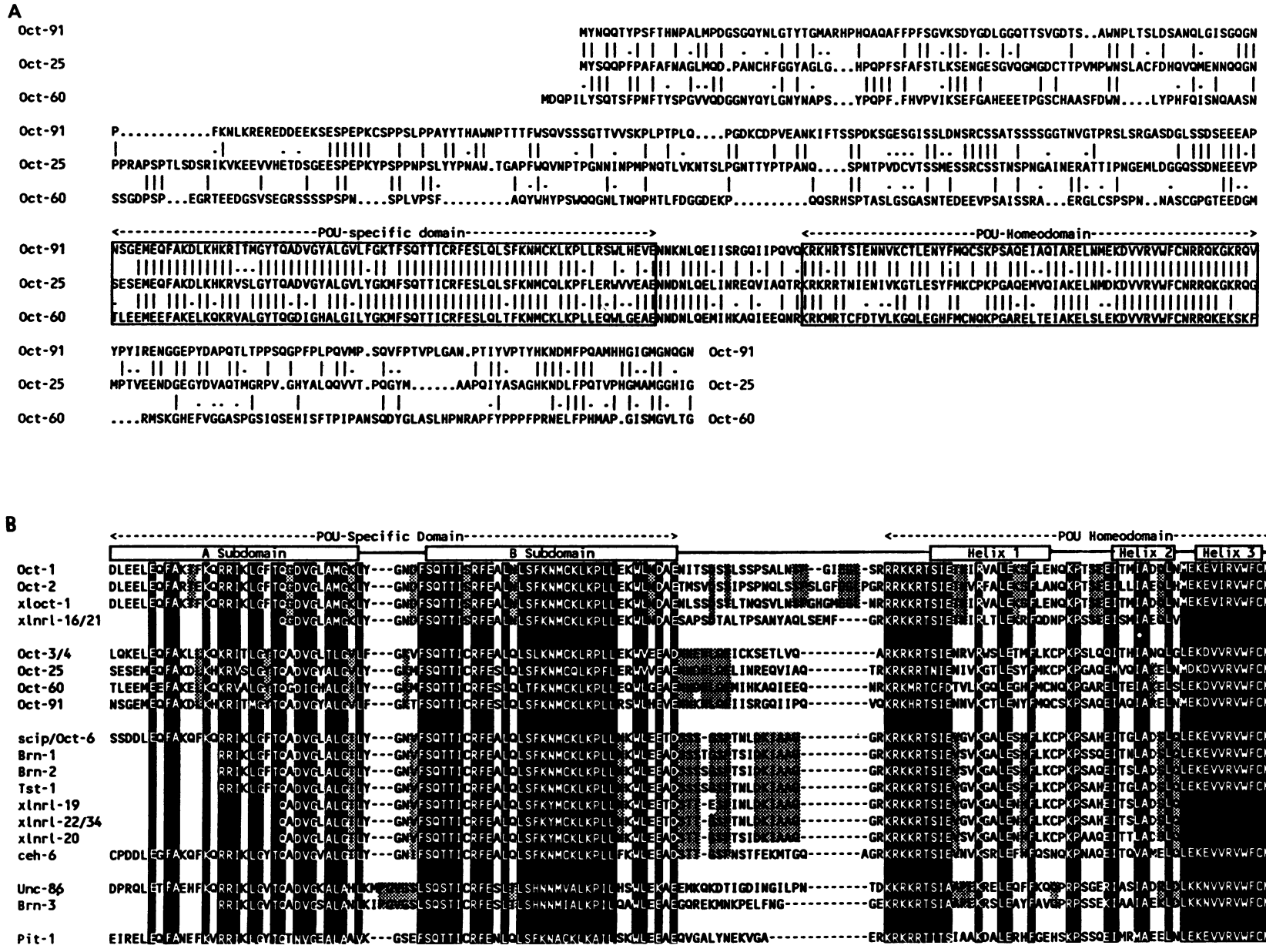
Isolation of multiple cDNAs encoding distinct POU-domain proteins. Because the complexes observed above were formed with the octamer motif, it seemed plausible that they might reflect the activity of POU-domain proteins. To investigate whether POU-domain sequences are expressed in early *Xenopus* development, we screened a stage 11 cDNA library with the *Xenopus* Oct-1 POU domain under low-stringency hybridization conditions. Clones distinct from Oct-1 were organized into groups according to their extent of cross-hybridization at high stringency with probes generated from bacteriophage DNA by polymerase chain reaction. Members from three groups were chosen for further analysis. Analysis of their nucleotide sequence revealed that they each have a single open reading frame, preceded by in-frame stop codons upstream of the presumed translation initiation site. None of these cDNAs appeared to contain the 3′ end of the mature transcript since they lacked a poly(A) tail. Northern analysis suggests that mature Oct-25, Oct-60, and Oct-91 mRNAs contain an additional 400, 1,000, and 800 nucleotides, respectively (discussed below).

The proteins encoded by the three cDNAs are similar in size (ranging from 47.3 to 49.5 kDa) and amino acid composition. The predicted protein sequences are more highly related to each other than they are to the sequences of other POU-domain proteins. The POU domain, located near the carboxy end of each protein, represents the primary region of amino acid sequence similarity between these proteins (Fig. 2A). Upon pairwise analysis, these DNA binding domains displayed approximately 75% amino acid identities relative to each other, 65% relative to the POU domain of murine Oct-3, and only 55% compared with the POU domain of amphibian Oct-1 (19, 40). In addition, there is a low degree of similarity that extends beyond the POU domain of these proteins. Several short regions of amino acid similarity are dispersed throughout the amino and carboxy ends, particularly between Oct-91 and Oct-25. Proline, glycine, and serine/threonine residues are the most abundant amino acids in each protein. Proline residues are distributed throughout the length of each protein, and several proline-rich regions are present at the amino and carboxy ends of each protein. Multiple potential phosphorylation sites are clustered in the amino half of each protein. Activation of transcription by a related POU-domain protein, Oct-2, requires a carboxy-terminal domain that also contains a high concentration of these amino acids (45). Computer searches of the GenBank and EMBL data bases using sequences outside of the POU domain failed to reveal significant similarities to previously reported nucleotide and protein sequences.

The POU domain consists of two highly conserved regions, the POU-specific domain and the POU homeodomain, joined by a linker region of variable length and sequence. The POU-specific domain may be further divided into an A subdomain and a B subdomain. Highly conserved amino acids are present throughout this region in most POU-domain proteins; however, the linker region is not generally conserved among all members of this family (Fig. 2B). From analysis of conserved and nonconserved residues, the POU domain family was divided into four classes: POU-I (Pit-1), POU-II (Oct-1, Oct-2), POU-III (Brn-1, Brn-2, and Tst-1), and POU-IV (Brn 3 and *unc*-86 [11]). Amino acids diagnostic of each class are located at variable positions throughout the POU domain and, particularly, in the linker region. For example, proteins in the POU-II class contain a relatively long linker region and unique amino acids at several positions in the POU-specific domain and the POU homeodomain. Similarly, proteins in the POU-III class possess a relatively conserved linker region and characteristic amino acids at several positions in the POU-specific domain and the POU homeodomain. A comparison of the sequences reported here with those of other members of the POU-domain family shows that Oct-25, Oct-60, and Oct-91 are most closely related to Oct-3. This relationship is evident from the presence of conserved amino acids in the linker region and at multiple locations in the POU-specific domain, as well as the absence of particular amino acid residues diagnostic of the other POU-domain classes. We conclude from the structural analysis that, together with Oct-3, the *Xenopus* POU-domain proteins reported here are new members of the POU-V subclass of the POU-domain gene family (46).

Sequential expression of *Xenopus* POU-domain genes. The accumulation of transcripts corresponding to the Oct-25, Oct-60, and Oct-91 cDNAs was examined by hybridizing Northern blots with probes that span each cDNA (Fig. 3A to C). As a control for sample loading and integrity, filters were also hybridized with a probe specific for cytochrome oxidase (Fig. 3D). The Oct-specific probes each hybridized to a major transcript and several smaller, less abundant RNA species. The Oct-60 probe hybridized predominantly to a 3.2-kb transcript that was most abundant in oocytes and fertilized eggs but barely detectable in embryos after gastrulation (Fig. 3B). Oct-25 and Oct-91 transcripts were present at low levels in oocytes, increased rapidly in abundance during gastrulation, and subsequently declined (Fig. 3A and C). Elevated levels of Oct-91 transcripts were present at later developmental stages than were the Oct-25 transcripts.

A detailed time course through early development was performed to examine more closely the kinetics of accumulation of Oct-25, Oct-60, and Oct-91 transcripts. Specific transcripts were analyzed in a quantitative RNase protection assay with gene-specific antisense probes. We found that maternal Oct-60 transcripts were maintained at a constant level of 6×10^5 molecules per embryo through early embryogenesis (Fig. 4). These transcripts began to disappear at the beginning of gastrulation with an apparent half-life of about 90 min so that very little Oct-60 mRNA (less than 5 ×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Oct-91
Oct-25
Oct-60
POU-specific domain
POU-Homeodomain
B
POU-Specific Domain
POU Homeodomain
A Subdomain
B Subdomain
Helix 1
Helix 2
Helix 3
Oct-1
Oct-2
xloct-1
xlnrl-16/21
Oct-3/4
Oct-25
Oct-60
Oct-91
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Brn-1
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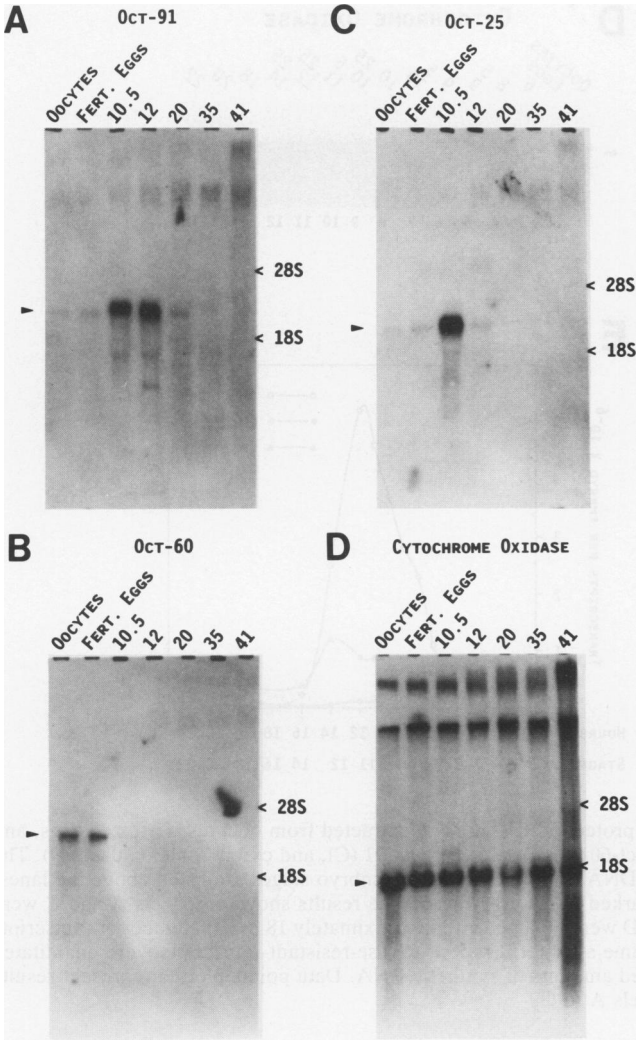

FIG. 3. Northern blot analysis of POU-domain gene expression during early development. Filters containing electrophoretically resolved total RNA from oocytes, fertilized egg, and staged embryos were hybridized with probes for Oct-91 (A), Oct-60 (B), Oct-25 (C), and mitochondrial cytochrome oxidase (D). Embryo stages are listed above the lanes. The positions of the detected transcripts and the rRNAs are indicated. The filters hybridized with the Oct-25 and cytochrome oxidase probes were exposed to film for approximately 18 h. The filters probed with Oct-91 and Oct-60 were exposed for 3 to 4 days.

10^4 transcripts per embryo) was detected during late gastrulation (stages 12 to 13).

In contrast, the other POU protein transcripts were most prevalent after the midblastula stage, with the maximal levels of accumulation occurring during gastrulation. Oct-25 transcripts were present in oocytes and cleavage-stage embryos (approximately 2×10^5 transcripts per embryo), although at lower levels than the maternal Oct-60 mRNA. Increased accumulation of Oct-25 transcripts was first detected at stage 8.5, reached a maximum at stage 11 during gastrulation, and then declined with an apparent half-life of about 2 h. The accumulation and decay of Oct-91 transcripts were similar to those of the Oct-25 mRNA except that the overall abundance of the Oct-91 mRNA was about fivefold lower, the maximal accumulation of the Oct-91 transcripts occurred during late gastrulation about 3 h after the peak of the Oct-25 transcripts, and they were present during neurulation at slightly higher levels than was the Oct-25 mRNA. Maternal Oct-91 transcripts were not detectable by the RNase protection assay (less than 10^4 copies per oocyte), although they were previously observed by Northern analysis. This might reflect the expression of a divergent allele due to pseudotetraploidy of the *Xenopus* genome, as recently described for *Xenopus* MyoD (35). The data from the Northern blot analysis and the RNase protection studies show that the Oct-60 mRNA was a maternal transcript that was maintained at a constant level through the rapid cleavage stages of development until early gastrulation. The Oct-60 transcripts were succeeded sequentially during early development, first by Oct-25 transcripts then by Oct-91 transcripts.

We were unable to detect expression of these genes by Northern blot analysis using total cellular RNA from adult tissues, including blood, brain, gut, heart, kidney, liver, lung, skeletal muscle, skin, spleen, and testis. However, in the more sensitive RNase protection assay, very low levels of Oct-25 and Oct-60 transcripts were found in kidney and low levels of Oct-91 RNA were observed in the brain (Table 1).

It is believed that cytoplasmic determinants localized along the animal-vegetal axis of the egg participate in pattern formation and cell-type determination during *Xenopus* development (for a review, see reference 32). Therefore, it was of interest to determine whether the maternal transcripts that encode potential transcriptional regulatory proteins are specifically localized in oocytes. To address this issue, mature oocytes were separated into animal and vegetal hemispheres (Fig. 5A) and the RNA in each half was isolated. An RNase protection assay with antisense riboprobes was performed to examine the distribution of the maternal Oct-60 transcripts relative to mRNA encoding Vg1, a transcript localized to the vegetal end of mature frog oocytes (27). The results show that greater than 90% of the Oct-60 transcripts were present in the animal hemisphere, whereas Vg1 mRNA was localized in the vegetal hemisphere (Fig. 5B). We conclude that transcripts encoding this potential transcriptional regulatory factor are not randomly distributed in mature oocytes but rather are preferentially localized in the region of the oocyte that will give rise to ectodermal and mesodermal cell lineages.

Oct-25, Oct-60, and Oct-91 cDNAs encode octamer-binding proteins. Proteins that bind specifically to the octamer motif

FIG. 2. Amino acid comparison of *Xenopus* POU-domain proteins. (A) Sequential alignment of the predicted amino acid sequences of Oct-91, Oct-25, and Oct-60, using the Genetics Computer Group Bestfit alignment program. Amino acid identities and similarities are represented by vertical lines and dots, respectively. The POU-specific domains and POU homeodomains are boxed. (B) Alignment of the POU-domain regions of known POU-domain genes. POU-domain sequences were organized into groups as previously described (11). Generally conserved amino acid residues are shown in blackened boxes. Amino acids that are relatively specific to each subclass are shaded. Sequences and references: Oct-1 (43); Oct-2 (7); xloct-1 (19, 40); xlnrl-16, xlnrl-19, xlnrl-20, xlnrl-21, xlnrl-22, and xlnrl-24 (1); Oct-3 (25, 31); Oct-4 (38); scip/Oct-6 (23, 44); Brn-1, Brn-2, Brn-3, Tst-1, and Tst-2 (11); ceh-6 (4); Unc-86 (9); Pit-1 (2, 14).

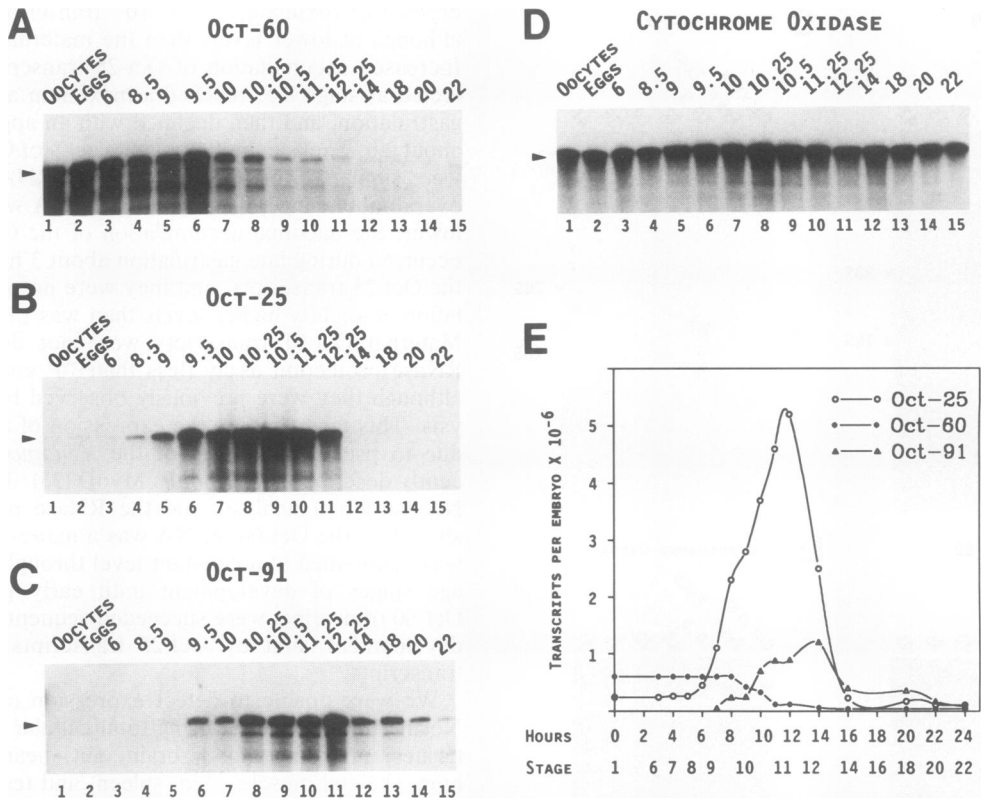

FIG. 4. Analysis of POU-domain transcript accumulation by RNase protection. Total RNA extracted from oocytes, fertilized eggs, and staged embryos was hybridized with antisense riboprobes specific for Oct-60 (A), Oct-25 (B), Oct-91 (C), and cytochrome oxidase (D). The location of each POU-domain riboprobe relative to the corresponding cDNA is shown in Fig. 2. Embryo stages are listed above the lanes. The fragment expected for complete protection of each riboprobe is marked by an arrowhead. The results shown in panels A and C were obtained after 3 days of autoradiography, whereas those in panels B and D were obtained after approximately 18 h. (E) Number of transcripts per embryo, expressed relative to the stage of early development and time after fertilization. RNase-resistant transcripts were quantitated with a Betascanner and normalized to the results obtained with measured amounts of synthetic RNA. Data points plotted represent results obtained from two separate spawnings, including the one shown in panels A to C.

have thus far contained a POU domain; however, not all POU proteins interact specifically with the octamer site, or even bind DNA (15, 46). To determine whether the cloned cDNAs encoded octamer-binding proteins that might account for the activities observed in embryo extracts, synthetic mRNA was used to program in vitro translation in a reticulocyte lysate system. Binding of the synthetic POU proteins to the octamer site was examined by using the electrophoretic mobility shift assay. Each transcript directed the synthesis of a protein that resulted in the formation of novel protein-DNA complexes that were absent from the unprogrammed reticulocyte lysate (Fig. 6A). Interestingly, multiple complexes were observed in binding reactions with Oct-25 (lanes 2 to 4) and Oct-60 (lanes 5 to 7). The major complex formed with Oct-25 had an electrophoretic mobility that was identical to that of the complex formed with Oct-91 (lanes 8 to 10), whereas the predominant complex formed with Oct-60 migrated slightly more rapidly. The binding of synthetic proteins to the octamer site was judged specific because the formation of the protein-DNA complexes in each reaction was inhibited by a 50-fold excess of the homologous unlabeled oligonucleotide and not by the ATF/CREB site. The complexes formed with the synthetic proteins comigrated with several of the complexes present in the gastrula stage extract (lane 11). The predominant complexes formed with synthetic Oct-25 (lanes 2 and 4) and Oct-91 (lanes 8 and 10) comigrated with the C3 complex. The kinetics of accumulation of Oct-25 and Oct-91 transcripts

TABLE 1. Expression of *Xenopus* POU-domain genes in adult tissues

Gene	Expression in[a]:											
	Blood	Brain	Gut	Heart	Kidney	Liver	Lung	Muscle	Ovary	Skin	Spleen	Testis
Oct-25	−	−	−	−	+	−	−	−	+	−	−	−
Oct-60	−	−	−	−	+	−	−	−	+	−	−	−
Oct-91	−	+	−	−	−	−	−	−	+/−	−	−	−

[a] Determined by Northern blot and by RNase protection analysis. +, expression; −, expression not detected, +/−, expression detected by Northern blot but not by RNase protection analysis.

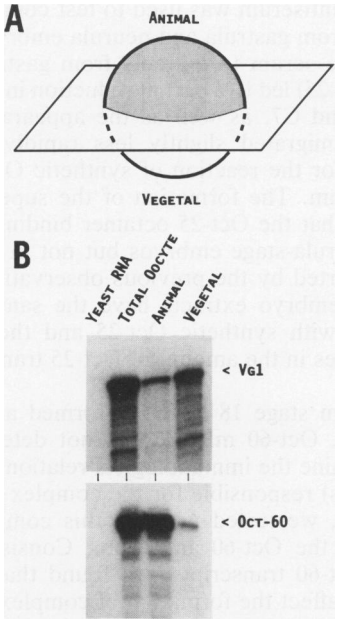

FIG. 5. Localization of Oct-60 transcripts in the animal region of mature oocytes. (A) Mature oocytes dissected on an equatorial plane into approximately equal animal and vegetal halves. (B) RNase protection analysis performed with RNA from yeast, total oocytes, animal poles, and vegetal poles, using riboprobes specific for Vg1 and Oct-60. The protected fragments expected for each probe are indicated.

coincided with the appearance of C3 in embryos (Fig. 1); they reach their highest levels during stages 11 through 13. Because Oct-25 and Oct-91 have overlapping kinetics of expression and they encode distinct proteins that form protein-DNA complexes with identical electrophoretic mobilities, it is likely that C3 from embryos is heterogeneous and consists of different protein-DNA complexes with similar electrophoretic mobilities.

As previously noted, synthetic Oct-25 formed several complexes with distinct electrophoretic mobilities. One of these complexes appeared to comigrate with C4; the others had mobilities that were indistinguishable from those of complexes C5 through C7 from gastrula-stage embryos. Changes in the amounts of C5, C6, and C7 formed in embryo extracts closely paralleled the accumulation and decline of Oct-25 transcripts, as expected if they were due to the binding of Oct-25 to the octamer site. The formation of multiple protein-DNA complexes with synthetic proteins might have resulted from several trivial possibilities, including partial proteolysis and translation initiation at multiple sites. To investigate these possibilities, translation reactions were performed by using [^{35}S]methionine and analyzed by SDS-polyacrylamide gel electrophoresis. We found that the products of each translation reaction contained one major polypeptide of the predicted mass (Fig. 6B). Several low-molecular-weight peptides were produced in the Oct-25 translation reaction; however, they were not present in sufficient quantities to account for the additional protein-DNA complexes formed. Identical results were obtained when the translation products were incubated under conditions used for the DNA binding studies and when the amino terminus of Oct-25 was replaced by a translation initiation site (from T7 gene 10) that functions more efficiently in the reticulocyte lysate system (data not shown). We conclude from these results that translation initiated primarily at one site for each gene analyzed and that the synthetic proteins were stable under the conditions used for the mobility shift assays. The basis for the formation of the multiple protein-DNA complexes is uncertain. They might be due to the formation of homomeric and heteromeric complexes as recently shown with other POU-domain proteins (48).

Maternal expression of the Oct-60 protein. Synthetic Oct-60 formed a complex that comigrated with C4 from oocytes and cleavage-stage embryos. With longer autoradiographic exposures, two other complexes were observed that appeared to comigrate with C6 and C7. To examine the expression of the Oct-60 protein in oocytes and embryos in further detail, antibodies were prepared against a fusion protein containing Oct-60 linked to glutathione *S*-transferase. The specificity of the polyclonal serum was examined by testing its ability to recognize protein-DNA complexes formed with synthetic proteins and the consensus octamer binding site. The Oct-60 antibody efficiently recognized the major complex formed with Oct-60 and either inhibited its formation or prevented it from entering the gel (Fig. 7A, lane 6). This effect was specific to the immune serum, since it was not observed with preimmune serum (not shown). The immune serum also showed some reactivity with Oct-25, generating a small amount of a supershifted protein-DNA complex (arrowhead, lane 5). Increased amounts of immune serum shifted more of the Oct-25 complexes into the larger complex, indicating that the Oct-60 immune serum had a low affinity for one or a few epitopes on complexes formed with Oct-25. The serum did not recognize complexes containing synthetic Oct-91 or the Oct-1 endogenous to the reticulocyte lysate.

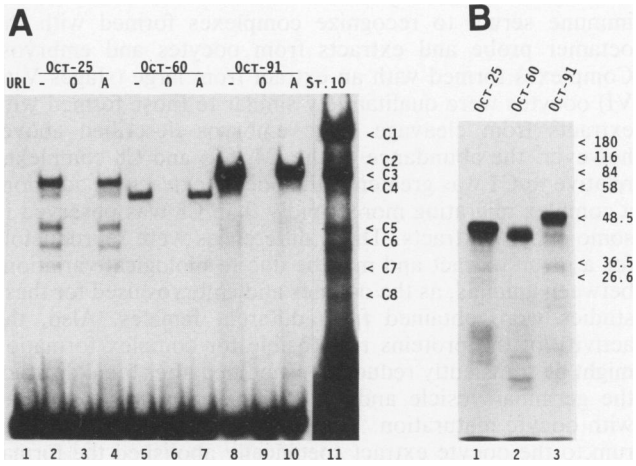

FIG. 6. In vitro translation and DNA binding of Oct-25, Oct-60, and Oct-91. (A) In vitro-synthesized proteins were incubated with the octamer binding site probe with a 50-fold molar excess of various oligonucleotide competitors. Lanes: 1, unprogrammed translation reaction (URL); 2 to 4, synthetic Oct-25 protein; 5 to 7, synthetic Oct-60 protein; lanes 8 to 10, synthetic Oct-91 protein; 11, gastrula-stage embryo extract; −, no competitor; 0, consensus octamer site competitor; A, ATF/CREB binding site competitor. (B) SDS-polyacrylamide gel electrophoresis of synthetic proteins labeled with [^{35}S]methionine. The translation products from a reticulocyte lysate programmed with synthetic Oct-25 mRNA (lane 1), Oct-60 mRNA (lane 2), and Oct-91 mRNA (lane 3) were analyzed by electrophoresis on SDS-polyacrylamide gels and subsequent autoradiography. The positions of prestained molecular weight markers are indicated at the right in kilodaltons.

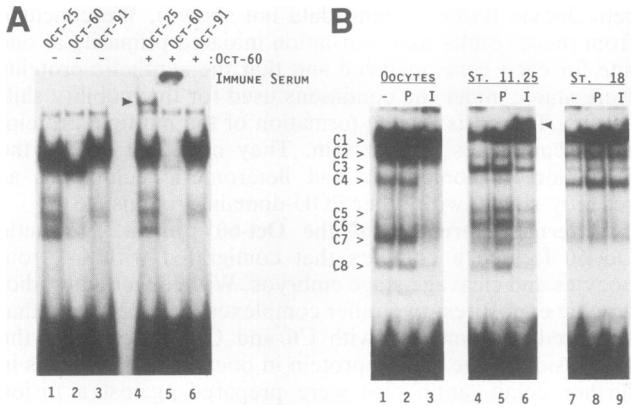

FIG. 7. Recognition by Oct-60 antiserum of specific complexes formed with extracts from oocytes and embryos. (A) Band shift assays were performed by using synthetic Oct-25, Oct-60, and Oct-91 protein with polyclonal antiserum directed against the glutathione *S*-transferase–Oct-60 fusion protein. Lanes: 1 to 3, control reactions without added serum; 4 to 6, reactions incubated with 0.1 μl of immune serum. The reaction of the antibody with complexes formed with synthetic Oct-25 (lane 4) resulted in the formation of a supershifted complex, indicated with an arrowhead. (B) Complexes formed with extracts from oocytes (lanes 1 to 3), gastrula-stage embryos (lanes 4 to 6), and neurula embryos (lanes 7 to 9) were incubated without serum (−), with 0.1 μl of preimmune serum (P), and with 0.1 μl of immune serum (I). The immune serum specifically inhibited the formation of C4, C7, and C8 in oocyte extracts (lane 3). This serum also resulted in the formation of a supershifted complex (indicated with an arrowhead) with the gastrula extract (lane 6).

To determine whether Oct-60 binding activity was present in oocytes and embryos, we examined the ability of the immune serum to recognize complexes formed with the octamer probe and extracts from oocytes and embryos. Complexes formed with an extract from large (stages V to VI) oocytes were qualitatively similar to those formed with extracts from cleavage stage embryos described above; however, the abundance of the C4, C7, and C8 complexes relative to C1 was greater in the oocyte extract. In addition, a complex migrating more rapidly than C8 was observed in some oocyte extracts. These differences were reproducible for a given extract and may be due to biological variations between animals, as the oocytes and embryos used for these studies were obtained from different females. Also, the activity of the proteins responsible for complex formation might be transiently reduced or modified after breakdown of the germinal vesicle and other nuclear events associated with oocyte maturation. The addition of the Oct-60 antiserum to the oocyte extract specifically abolished the formation of C4 and C7 (Fig. 7B, lanes 1 to 3). Other complexes formed with the oocyte extract, including C1, were not affected by the immune serum. Identical results were obtained with extracts from cleavage-stage embryos (not shown). We conclude that C4 and C7 from oocytes and early embryos correspond to Oct-60 octamer binding activity because these complexes have the same electrophoretic mobility as complexes formed with synthetic Oct-60, they were specifically recognized by the Oct-60 antisera, and the decline in their abundance during gastrulation coincided with the reduction in Oct-60 mRNA. The residual complexes (primarily C2) that were not recognized by antiserum directed against either Oct-1 or Oct-60 were apparently due to the maternal expression of other octamer-binding proteins.

The Oct-60 antiserum was used to test complexes formed with extracts from gastrula and neurula embryos. The addition of immune serum to extracts from gastrula-stage embryos (stage 11.25) led to a partial reduction in the amount of C3, C5, C6, and C7, as well as the appearance of a new complex that migrated slightly less rapidly than C1, as shown above for the reaction of synthetic Oct-25 with the Oct-60 antiserum. The formation of the supershifted complex suggests that the Oct-25 octamer binding activity was present in gastrula-stage embryos but not in oocytes. This result is supported by the previous observations that complexes in the embryo extracts have the same mobility as those formed with synthetic Oct-25 and their abundance parallels changes in the amount of Oct-25 transcripts during development.

Extracts from stage 18 embryos formed a C4-like complex; however, Oct-60 mRNA was not detectable at this stage. To examine the immunological relationship of Oct-60 to the protein(s) responsible for the complex from neurula-stage embryos, we tested whether this complex could be recognized by the Oct-60 antiserum. Consistent with the absence of Oct-60 transcripts, we found that the immune serum did not affect the formation of complexes in extracts from neurula-stage embryos (Fig. 7B, lanes 4 to 6). More specifically, the C4 complex was not recognized by the Oct-60 antiserum, indicating that the formation of this complex with use of extracts from neurula-stage embryos results in the expression of an octamer-binding protein that is immunologically distinct from Oct-60. The inability of the immune serum to produce a supershifted complex or to partially inhibit the formation of C3 in the neurula-stage extract suggests that this complex does not contain Oct-25, in contrast to the C3 complex from gastrula-stage embryos. Taken together, these results show that complexes with identical electrophoretic mobilities are formed in embryo extracts as a result of the expression of distinct octamer-binding proteins and that multiple sets of octamer-binding proteins are sequentially expressed during development.

DISCUSSION

In this study, we have shown that distinct octamer-binding proteins are differentially expressed during amphibian early development and that several of these proteins are closely related members of the POU-domain family of regulatory proteins. Analysis of the expression of octamer-binding proteins during amphibian early development revealed that a heterogeneous pattern of protein-DNA complexes was formed with use of a consensus octamer site probe and extracts from staged embryos. Several of these complexes were a result of the maternal expression of distinct POU-domain proteins that were present during the midblastula transition and therefore are likely to participate in the transcriptional activation of the embryonic genome. New complexes formed transiently during gastrulation as a consequence of the expression of POU-domain proteins were replaced subsequently by yet other complexes during neurulation. The expression of multiple octamer-binding proteins during embryogenesis appears to be a common phenomenon in vertebrates, since similar complexes were reported in mouse oocytes and 12-day embryos (37). These results expand the list of expressed POU-domain genes and suggest that multiple members of this family of transcriptional regulators participate in regulatory processes during early development.

By screening *Xenopus* cDNA libraries with the DNA

binding domain of Oct-1, novel POU-domain sequences that encode proteins which bind to the consensus octamer motif were isolated. Using antibodies directed against two distinct POU-domain proteins, Oct-1 and Oct-60, we showed that these proteins were present in different complexes that were formed with the octamer site and extracts from oocytes and early embryos. These studies also provided evidence for the involvement of other octamer-binding proteins, including Oct-25, in novel complexes formed with extracts from gastrula- and neurula-stage embryos. Several of the complexes formed with extracts from neurula-stage embryos may result from the expression of previously reported *Xenopus* POU-domain genes (1) that are related to SCIP/Oct-6 and therefore are expected to bind to the octamer motif.

Comparative amino acid sequence analyses suggest that the proteins encoded by the *Xenopus* POU-domain genes described here are structurally related members of the Oct-3 subfamily of POU-domain proteins. This conclusion is supported by the observation that several aspects of the expression of these genes, particularly Oct-60, resemble the expression of Oct-3 during mouse development (31, 36, 38). Both Oct-3 and Oct-60 are expressed in oocytes and early embryos of their respective species. Initially, Oct-3 transcripts are uniformly distributed in mouse oocytes and early blastocysts. Oct-3 expression subsequently declines in trophectoderm-derived cells and becomes progressively restricted: first to the inner cell mass and then to ectoderm, neural ectoderm, and primordial germ cells. By day 11, Oct-3 transcripts are present only in primordial germ cells, and Oct-3 expression in the adult is restricted to gonadal cells. Similarly, Oct-60 expression was primarily restricted to the female germ line and early embryos. Although maternal Oct-60 transcripts were localized to the animal pole of oocytes and eggs, this region gives rise to ectodermal and neuroectodermal cell types analogous to those that transiently express Oct-3 in mouse embryos. Oct-60 transcripts were most abundant in oocytes, were barely present in kidney, and were not detectable in a wide range of adult tissues, including brain and testis. Expression of the two other POU-domain genes, Oct-25 and Oct-91, during gastrulation coincides temporally with the period of restricted Oct-3 expression in mouse embryos. The expression of Oct-25 and Oct-91 was also highly restricted in adult somatic tissues and was observed only in kidney and brain, respectively.

Despite similarities in their structure and overall patterns of expression, there are several differences between the amphibian POU-domain genes reported here and the mammalian Oct-3 gene. The POU domains of the proteins encoded by each of the amphibian genes are most related to Oct-3; however, there is very little structural similarity outside of the DNA binding domain, indicating that none of these amphibian POU genes are precisely homologous to Oct-3. By comparison, the amphibian Oct-1 protein shares approximately 88% amino acid identities with its human homolog over their entire lengths (19, 40). A notable apparent difference is that frog oocytes and embryos express multiple POU-domain genes instead of a single Oct-3 gene, as reported in mammals (25, 31, 38). It may be relevant that the amphibian POU-domain genes were isolated from oocyte and embryo cDNA libraries, whereas Oct-3 was isolated from cDNA libraries prepared from cultured EC cells. Because nuclear extracts prepared from mouse embryos form a more complicated pattern of protein-DNA complexes with the octamer motif than do extracts from EC cells (36), this finding suggests that additional Oct-3-like proteins might be expressed during mammalian early development, perhaps in distinct cells or at different times. Alternatively, it is possible that because of differences between amphibian and mammalian early development, amphibian Oct-3-like proteins may have specialized activities that correspond to subsets of the functions performed by the presumably unique mammalian Oct-3 and, furthermore, that one or more of the amphibian proteins might have activities not performed by Oct-3.

Important questions that are not directly addressed by these studies concern the function of the amphibian Oct-3-like proteins. Given the role of POU-domain proteins in other systems, the amphibian POU-domain proteins described here are likely to be involved in transcriptional regulation and possibly the control of cell proliferation. Many POU-domain proteins are positive transcriptional regulators, and this may be the case with the amphibian POU-domain proteins. Presumably the maternally expressed Oct-1 and Oct-60 might activate transcription of specific genes during oogenesis or at the midblastula transition. However, it is clear that the octamer motif can be the target for transcriptional repression by POU-domain proteins, including Oct-3 and SCIP/Oct-6 (20, 23). In this regard, one or more of the amphibian POU-domain proteins might function as a negative transcriptional regulator.

Additional clues to the functions of these genes come from analysis of their expression. The germ line-specific expression of Oct-60 raises the possibility of its involvement in the determination of germ cell precursors. The formation of germ cells in vertebrate embryos is due to cytoplasmic determinants that are sensitive to UV irradiation. Irradiation of fertilized amphibian eggs with UV light prevents the formation of germ cells and results in the production of sterile adults (3, 41). The sensitivity of germ cell determinants to UV irradiation suggests that they contain nucleic acid, presumably mRNA. The localization of these determinants to the vegetal poles of fertilized eggs was shown by the preferential sensitivity of the vegetal region to UV irradiation, compared with irradiation of the animal pole, and by the observation that cytoplasm from the vegetal region of normal zygotes rescued the formation of germ cells when injected into irradiated embryos, whereas the cytoplasm from the animal region did not rescue germ cell formation. The localization of maternal Oct-60 transcripts to the animal pole in oocytes argues that most of this mRNA is not part of the germ plasm and therefore not involved in germ cell formation. However, we cannot presently exclude the possibility that the fraction of Oct-60 transcripts present in the vegetal region has a function distinct from that localized in the animal pole.

The other two amphibian POU-domain genes were expressed sequentially in gastrula- and neurula-stage embryos. Because these genes were not expressed at significant levels in somatic tissues, it is unlikely that they play a positive role in the maintenance of a differentiated state. Other workers have shown that Oct-91 expression in embryos is cell autonomous and not activated by growth factors, indicating that expression of Oct-91 is regulated independently of mesoderm formation (10, 16). Oct-91 transcripts do not appear to be localized in dissected embryos at stages 8 to 9, but they become highly localized to ventroposterior endoderm by stages 14 to 15. Although the distribution of Oct-25 and Oct-60 transcripts in embryos is not known, they are clearly expressed at different times. The present data suggest that the Oct-3-like POU-domain proteins are expressed in distinct cell types at different times in development. If this is

true, it is unlikely that these proteins serve redundant or overlapping functions. It will clearly be important to define the domains of expression of these genes more precisely. We anticipate that the production of immunological reagents will be useful to further define the expression of these genes and to obtain insights into their functions.

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