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dOct2, a *Drosophila* Oct transcription factor that functions in yeast

(Oct factor/POU domain/trans-activator/embryonic development)

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ABSTRACT **Oct factors are members of the POU family of transcription factors that are shown to play important roles during development in mammals. Here we report the cDNA cloning and expression of a *Drosophila* Oct transcription factor. Whole mount *in situ* hybridization experiments revealed that the spatial expression patterns of this gene during embryonic development have not yet been observed for any other gene. In early embryogenesis, its transcripts are transiently expressed as a wide uniform band from 20% to 40% of the egg length, very similar to that of gap genes. This pattern progressively resolves into a series of narrower stripes followed by expression in 14 stripes. Subsequently, transcripts from this gene are expressed in the central nervous system and the brain. When expressed in the yeast *Saccharomyces cerevisiae*, this *Drosophila* factor functions as a strong, octamer-dependent activator of transcription. Our data strongly suggest possible functions for the Oct factor in pattern formation in *Drosophila* that might transcend the boundaries of genetically defined segmentation genes.**

Classical and molecular genetic analyses in *Drosophila* have postulated that complex networks of developmental regulators form a cascade with a determined hierarchy that guides the transfer of genetic information into embryonic structures. Several genes that encode such regulators have been cloned and many of them have been found to encode proteins that contain a conserved domain called the homeodomain. Homeodomain proteins have been shown to bind to specific DNA sequences and function as transcription regulators *in vitro*, in tissue culture and yeast cells, and in the *Drosophila* embryo (reviewed in refs. 1 and 2). Several homeodomain transcription factors that are potential components of such a regulatory cascade have since been identified in many other organisms, including mammals (reviewed in refs. 3 and 4).

Recently, a family of transcription factors called the POU family has been identified in mammals, in *Caenorhabditis elegans*, and in *Drosophila* that, like the homeodomain proteins, regulates various aspects of cell-fate specification during development. Members of this family share two homologous regions in their DNA binding domain: (*i*) a 60-amino acid homeodomain quite divergent from the classical *Drosophila* homeodomain, called the POU homeodomain, distinguished by the presence of Trp-Phe-Cys (WFC) motif in the C-terminal domain of the DNA recognition helix; (*ii*) a 68- to 70-amino acid region that is unique to this class of transcription factors referred to as the POU-specific domain. These two regions together with a nonconserved spacer region between them constitute the POU domain (5).

The Oct factors are also members of the POU family of transcription factors that bind to the octamer motif, a cis-acting regulatory element found in the promoter and enhancer regions of several genes that are transcribed by both RNA polymerase II and III (reviewed in ref. 6). Several Oct transcription factors that are believed to play critical roles in cellular development and differentiation have been identified in mammals (7–16) and other vertebrates (17, 18). In contrast, little is known about Oct factors from *Drosophila*. Here we report the molecular cloning and spatial expression patterns of a *Drosophila* Oct gene.† We show that the protein encoded by this gene is an octamer-dependent transcription factor that activates transcription in the yeast *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Electrophoretic mobility-shift assays (19) using the consensus octamer oligonucleotide probe (20), expression library screening (21), and whole-mount *in situ* hybridizations (22) were done as described.

Plasmid Constructs. The reporter plasmid pLGO4 was constructed by cloning four copies of the consensus octamer oligonucleotide at the unique *Bgl* II site upstream of the TATA box in the pLGΔBS plasmid (23). To express the dOct1 and dOct2 in yeast, the cDNAs were cloned into the yeast expression plasmid pAD4Δ (24).

Yeast Transformations and β-Galactosidase Assays. The yeast strain SEY6210 (*MATα, leu2-3-112, ura3-52, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, met*) was obtained from Scott Emr (University of California, San Diego). Plasmids were introduced into this strain as described (25) and colonies were selected on minimal plates lacking uracil (to select for pLGO4) or leucine (for pADOct2 or pADOct1) or both. Twenty colonies were pooled from each transformation and grown in minimal medium to an approximate OD_{600} of 1.0. Cultures were collected by centrifugation and resuspended in equal volumes of buffer Z; samples were lysed and assayed as described (26).

RESULTS

Identification of *Drosophila* Oct (dOct) Factors. To identify the presence of a nuclear factor that could specifically interact with the octamer sequence motif, we carried out electrophoretic mobility-shift experiments with crude embryonic nuclear extracts and the consensus octamer oligonucleotide. Fig. 1*A* shows that several protein–DNA complexes were observed when nuclear extracts were incubated with the labeled consensus oligonucleotide (lane 2). When excess unlabeled competitor oligonucleotide was included in the binding reaction mixture together with the labeled probe, all the complexes showed a dose-dependent decrease in binding (lanes 3–7). However, when challenged with a mutant competitor oligonucleotide that carried point mutations in two

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Abbreviation: β-Gal, β-galactosidase.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M93149).

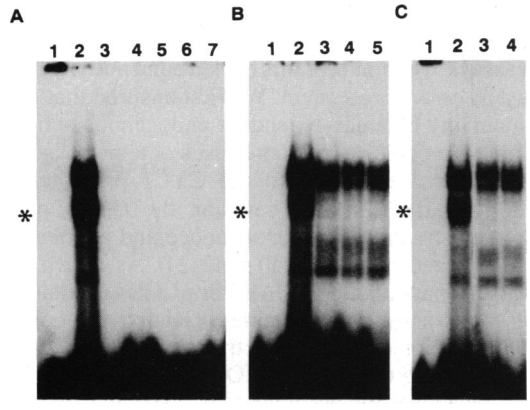

FIG. 1. Electrophoretic mobility-shift assay showing binding of nuclear factors that interact with the consensus octamer binding site. (*A*) Binding of Oct factors to the ^{32}P-labeled wild-type octamer site (lane 2) that can be blocked by competition with 10-, 20-, 30-, 50-, and 100-fold excess of unlabeled wild-type oligonucleotide (lanes 3–7). Lane 1 is the control lane that contained only the probe and no nuclear extract. (*B*) Binding reactions in the presence of 10-, 20-, and 50-fold excess of mutant oligonucleotide (lanes 3–5). (*C*) Binding in the presence of 50-fold excess of oligonucleotides unrelated to the octamer sequence (lanes 3 and 4). Lanes 1 and 2 in *B* and *C* are same as lanes 1 and 2 in *A*. Asterisk indicates a nonspecific complex.

residues within the octamer consensus, only one complex (indicated by an asterisk) disappeared completely even at the lowest concentration of the competitor used (Fig. 1*B*, lanes 3–5). The other complexes remained relatively unaffected, although there seemed to be a general decrease in the band intensities. Similar results were obtained when totally unrelated oligonucleotides of similar sizes were used as competitors (Fig. 1*C*, lanes 3 and 4). We believe that this complex is due to the presence of a nonspecific DNA binding protein not related to the Oct factors. Taken together, these data demonstrate the presence of multiple nuclear factors that can specifically bind to the consensus octamer sequence motif.

Isolation of cDNA Clones Encoding the dOct Genes. Having confirmed the presence of nuclear factors that interact with the octamer motif, we screened a *Drosophila* embryonic (0–20 hr) λgt11 cDNA library using the wild-type octamer oligonucleotide. Two positive clones (of 6×10^5) were identified that interacted with the wild-type octamer recognition site but not with the nonspecific binding sites. Rescreening the same library with a nick-translated cDNA probe yielded several positive clones. Limited sequence analyses of the end regions of the various subcloned cDNA inserts indicated that a majority of these positive clones constituted overlapping sets of different cDNAs. One such set of three overlapping clones represented the dOct1 cDNA of ≈2.1 kilobases (kb). The second set of another three overlapping clones constituted the dOct2 cDNA of ≈2.2 kb. The complete nucleotide sequence of 2190 base pairs of the dOct2 cDNA derived from a composite of the overlapping cDNAs revealed a single open reading frame that begins at position 174 and ends at position 1667 encoding a 498-amino acid polypeptide with a predicted molecular mass of 54 kDa (Fig. 2*a*). However, *in vitro* translation experiments indicated that the dOct2 polypeptide migrates as a 70-kDa species (Fig. 2*b*), presumably due to anomalous mobility in SDS gels. dOct2 synthesized *in vitro* was able to bind specifically to an octamer oligonucleotide probe in a gel-shift assay (data not shown), suggesting that this cDNA indeed encodes a *Drosophila* Oct factor.

Two other groups have recently reported the cDNA cloning of POU domain-containing genes from *Drosophila*. These cDNAs called *pdm-1* and *pdm-2* (28) and dPOU-19 and dPOU-28 (29) are very closely related or identical, respectively, to the dOct1 and dOct2 sequences cloned by us.

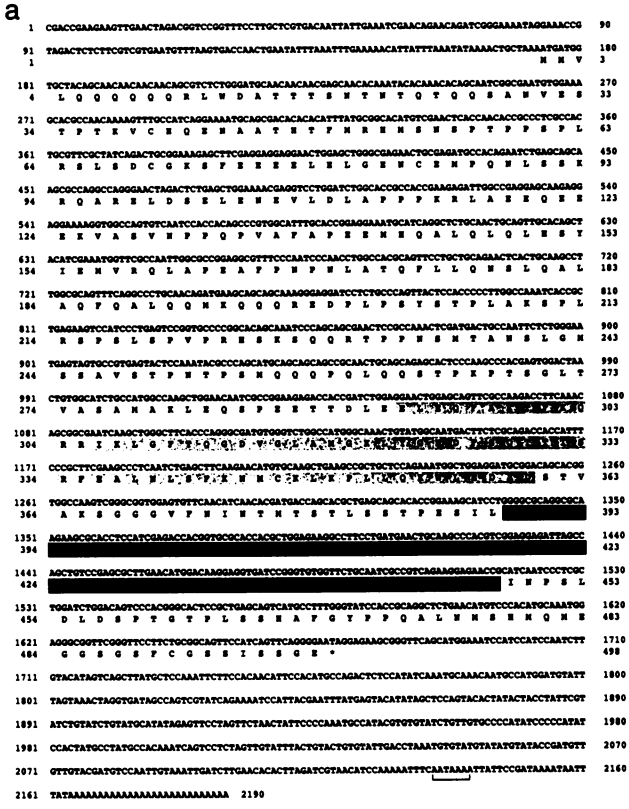

FIG. 2. Nucleotide sequence of the dOct2 cDNA and predicted amino acid sequence of the encoded polypeptide indicated in the one-letter code. A putative polyadenylylation signal in the nucleotide sequence is underlined. The POU-specific and the POU homeodomain are indicated by the shaded and dark boxes, respectively. (*B*) SDS/polyacrylamide gel electrophoresis (27) of the products of the dOct2 mRNA translation. Protein markers are indicated in kDa.

However, no data have been reported concerning the DNA binding or transcription activity of any of the polypeptides encoded by the *pdm* or dPOU cDNAs. The sequences of *pdm-1* and dPOU-19 are identical to each other and to dOct1. Therefore, we do not present any data describing the sequence or the expression patterns of the dOct1 gene. *pdm-2* is identical to the dOct2 sequence from nucleotide position 372 to the end of the cDNA. However, the first 24 nucleotides of the *pdm-2* sequence show no homology to that of dOct2. This deviation of the sequences is very likely due to the presence of intronic sequences in the *pdm-1* cDNA. dPOU-28, on the other hand, is identical to the dOct2 sequence from nucleotide position 1 of the dOct2 sequence but contains 12 additional nucleotides at the 5′ end. However, the encoded protein is presumed to be only 475 amino acids long as compared to 498 amino acids of the presumptive dOct2 polypeptide. While the N termini of the two predicted proteins are

identical, they differ only at their C termini. We believe that this difference in the sequence of the two predicted polypeptides is due to a sequencing error in the dPOU28 cDNA. Both dOct1 and dOct2 (as well as *pdm-1/pdm-2* and dPOU19/dPOU28) map to a similar cytological position at band 33F.

Structure and Sequence Homology of the dOct2 Protein. A search of the protein data base revealed a remarkable degree of sequence identity in the POU domain between dOct2 and several other Oct factors. Based on the extent of sequence homology within this domain, Rosenfeld and coworkers (30, 31) have proposed a classification of the POU proteins into five classes. According to this classification, dOct2 falls in the type II class together with the Oct1 and Oct2 proteins and shares 87–90% sequence identity at the amino acid level over the entire POU domain (Fig. 3). Interestingly, a significantly higher sequence conservation is observed in the POU-specific (91–93%) than in the POU homeosubdomains (81–86%). Within the POU homeodomain, however, the WFC region that lies in the c-terminal one-third of the homeodomain and comprises helix III, the recognition helix, is absolutely identical in every member of this class. Certain amino acid residues at which dOct2 differs from other members of its class are especially noteworthy. Thus, for example, the presence of a Gln residue instead of a Glu at position 26 in the B subdomain of the POU-specific domain appears to be typical to only dOct1 and dOct2. Similarly, the occurrence of a Cys residue in the hinge that connects helix I and II of the POU homeodomain and two Ser residues at positions 5 and 8 in the second helix are unique to dOct2. While either a Thr or an Ala residue is generally encountered at the fifth position in most POU proteins, an Ala residue at position 8 is invariant in every member of the POU family.

Outside the POU domain, no significant homology is observed between dOct2 and any other member of its class, although just preceding and following this domain, only dOct1 and dOct2 share an identical stretch of 9 and 6 amino acid residues, respectively. In the N-terminal region adjacent to the POU domain, dOct2 is particularly rich in Gln (13%), Ser (11%), Pro (11%), and Leu (10%) residues, a common feature shared by several *Drosophila* transcription factors.

dOct2 Is a Transcription Activator. Since mammalian Oct factors have been shown to function as transcription factors (see ref. 6), we tested the possibility that the dOct proteins could function as transcription factors as well. In *Drosophila* tissue culture cells, a high level of Oct gene activity is observed (E. Zandi and C.S.P., unpublished data), which contributes to a significant level of background activity in transient transfection assays. To minimize this background activity, we used the yeast *S. cerevisiae* system. We first ensured that yeast do not contain any octamer-dependent endogenous activity. To do so, multiple copies of the consensus octamer oligonucleotide were cloned upstream to the CYC1 promoter in the plasmid pLGΔBS (23) that contains the CYC1 promoter lacking its upstream activating sequence and is linked to the bacterial β-galactosidase (β-Gal) gene (24). As shown in Table 1, yeast cells that contain pLGΔBS or pLGO4 (containing the octamer motif) show no detectable β-Gal activity, confirming that yeast do not contain an activator that can utilize the octamer motif. To test whether dOct1 and dOct2 could function as trans-activators, we cloned the respective cDNAs into the yeast expression vector pAD4Δ that contains the yeast alcohol dehydrogenase promoter and termination signals (24). Each of these constructs, called pADOct1 and pADOct2, was introduced into the yeast strain harboring pLGO4 and the colonies obtained were assayed for β-Gal activity. Cells that harbor the pADOct2 and the pLGO4 plasmids show 89 units of β-Gal activity (Table 1), suggesting that dOct2 is a strong trans-activator that utilizes the octamer motif for its activity. dOct2 was not able to induce any activity when the octamer motif was not present (in pLGΔBS; see Table 1). To verify that the property to activate the pLGO4 plasmid is unique to dOct2, we transformed yeast cells harboring the pLGO4 with several control plasmids, including the parent plasmid pAD4Δ and plasmids expressing other *Drosophila* transcription factors. None of these controls showed any measurable activity (Table 1; data not shown). Also, dOct2 had no influence on the activity of the intact CYC1 promoter from which the upstream activating sequence (UAS) was not deleted (plasmid pLG699Z; see Table 1). We conclude that dOct2 is an octamer-dependent transcription activator.

dOct1, on the other hand, does not activate the reporter gene efficiently. When expressed together with pLGO4, only 3.8 units of β-Gal activity were measured (Table 1). It is possible that dOct1, like the mammalian Oct1, may require additional coactivators to function efficiently. Mammalian Oct1 functions as a transcriptional activator only in the presence of the viral trans-activator, VP16, and another cellular cofactor (37). Our preliminary data indicate that VP16 does not cooperate with dOct1 in yeast (data not

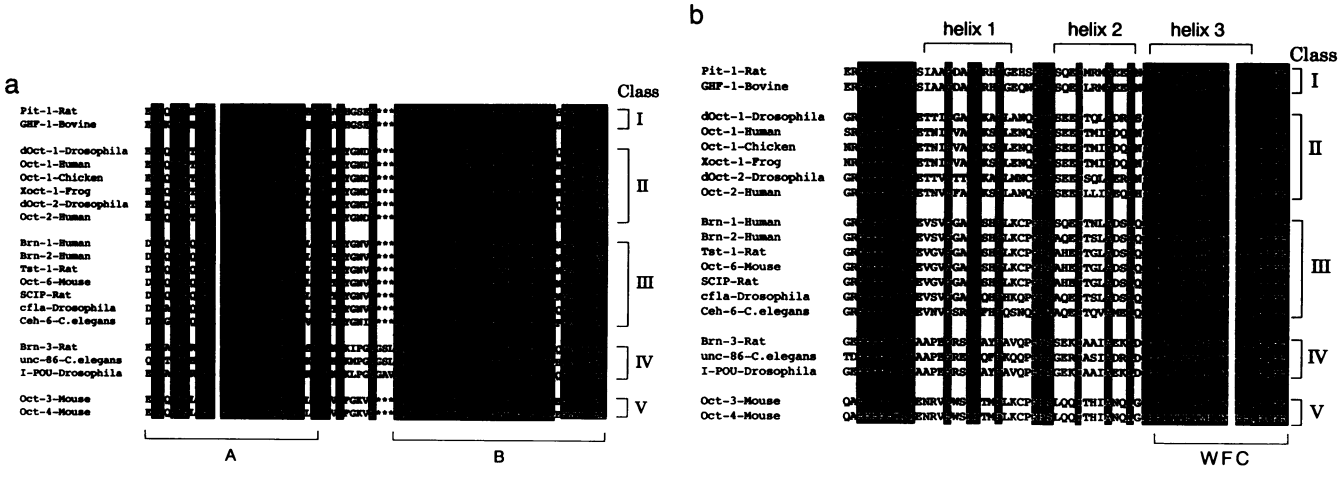

FIG. 3. Comparison of the dOct2 POU domain with those of previously characterized POU domains. (*A* and *B*) Amino acid sequence comparisons of the POU-specific and POU homeodomain regions of the POU proteins and dOct2, respectively. Darkly shaded regions indicate the invariant or one or two conservative substitutions of the amino acid residues. The POU sequences shown here were taken from the following references: human Oct-1 (7); chicken Oct-1 (17); frog Oct-1 (18); *Drosophila* Oct1 (K.P. *et al.*, unpublished data; refs. 28, 29); human Oct-2 (9–11); human Brn-1, human Brn-2, rat Brn-3, rat Tst-1 (30); mouse Oct-6 (12); rat SCIP (13); *Drosophila* cfla (31, 32); *C. elegans* ceh-6 (33); mouse Oct-3 (14, 15); mouse Oct-4 (16); rat Pit-1 (34); bovine GHF-1 (35); *Drosophila* I-POU (31); *C. elegans* unc-86 (36).

Table 1. dOct2 functions as an octamer-dependent transcription factor in yeast

Plasmids in yeast	Cis element	Trans-activator	β-Gal activity*
pLGΔBS + pAD4Δ	CYC1 promoter	—	1.0
pLGΔBS + pADOct2	CYC1 promoter	dOct2	0.5
pLGO4 + pAD4Δ	CYC1 promoter + octamer sites	—	1.2
pLGO4 + pADOct2	CYC1 promoter + octamer sites	dOct2	89
pLG669Z + pAD4Δ	CYC1 promoter and enhancer	—	200
pLG669Z + pAdOct2	CYC1 promoter and enhancer	dOct2	225
pLGΔBS + pADOct1	CYC1 promoter	dOct1	0.5
pLGO4 + pADOct1	CYC1 promoter + octamer sites	dOct1	3.8
pLG669Z + pADOct1	CYC1 promoter and enhancer	dOct1	160

*Units per 10^7 cells. Shown is the average of at least three experiments.

shown), suggesting that there could be different embryonic coactivators that interact with dOct1.

Temporal and Spatial Expression Patterns of the dOct2 Gene. Northern blotting experiments with mRNA from different embryonic stages of development detected a single transcript of 2.3 kb that hybridized with the dOct2 cDNA probe (Fig. 4). While maximum levels of the dOct2 RNA were observed between 4 and 12 hr of development, low levels were detected in 0- to 4-hr embryos and even lower levels were observed in later development (16–24 hr). The size of the dOct2 message detected in the RNA blots is in good agreement with the size of the corresponding cDNA reported here, suggesting that it is close to full length. As a control, the blot was stripped and reprobed with the *Drosophila* transcription factor TFIID cDNA. A single transcript of 1.6 kb was detected that was almost constant in all the lanes (data not shown) as was expected (38).

The spatial distribution of dOct2 mRNA was examined in whole embryos by *in situ* hybridization. During the early cellular blastoderm stage when the RNA first becomes detectable, dOct2 is expressed as a single broad band corresponding to 20–40% of the egg length, very similar to that seen for dOct1 (unpublished data) (Fig. 5*a*). In addition, a dark speck of expression is also observed at the anterior dorsal-most tip of the embryo. Within a short interval, this broad band of expression is lost and, instead, a complicated pattern of a series of stripes appears (Fig. 5 *b* and *c*). While the stripes anterior to the domain that corresponded to the broad band of dOct2 expression appear *de novo*, the more posterior ones are derived from the broad band of expression (Fig. 5*c*). The striped expression is also transient and persists until the onset of gastrulation. In early gastrulating embryos, this pattern is followed by expression in 14 stripes, one in each parasegment, until the extended germ band stage (Fig. 5*d*). Finally, dOct2 is expressed in the brain and in a subset of the neuronal cells of the central nervous system (Fig. 5 *e–g*). The 14-stripe expression pattern and the subsequent neuronal expression of dOct2 message are reminiscent of the dOct1 expression patterns (unpublished data; refs. 28 and 29).

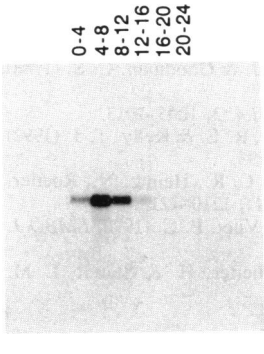

FIG. 4. Developmental Northern blot analysis of the dOct2 transcripts. Poly(A)$^+$ RNA isolated from staged embryos (indicated as hours after egg laying) was hybridized with dOct2 cDNA under standard conditions. The size of the transcript was estimated by using commercial RNA markers.

DISCUSSION

In this paper, we have described the identification and cDNA cloning of an Oct gene from *Drosophila*. This gene is a member of the class II type of POU genes that comprises all of the known Oct1 and Oct2 genes. The remarkable evolutionary conservation and extensive sequence homology in the POU domain with previously known genes that are involved in cell-fate specification suggest that this gene is likely to be a component of one of the gene regulatory networks that operates during *Drosophila* development. Our most important observation is that the spatial patterns of dOct2 expression transcend the domains of expression of the classical *Drosophila* segmentation genes. To date, with the exception of dOct1, we are not aware of any other regulatory gene in *Drosophila* whose spatial expression patterns cover the entire spectrum of expression patterns that are seen for each class of segmentation genes—i.e., the gap, pair rule, and segment polarity genes. Thus, while the initial domain of dOct RNA expression in a broad uniform band is surprisingly similar (i.e., covering several segments), but not identical, to some of the gap gene expression domains [for example, *Kr* and *knirps* (reviewed in refs. 40 and 41)], the striped expression is comparable to a subset of stripes of the pair-rule class [for example, hairy, even-skipped, and *ftz* (reviewed in ref. 42)]. Similarly, whereas the expression of the dOct genes in 14 narrower stripes is clearly reminiscent of the segment-polarity genes [such as engrailed (43)], their expression in the central nervous system parallels those of several of the gap, segmentation, and homeotic genes (44–50). These data point to possible functions for the dOct genes in segmentation

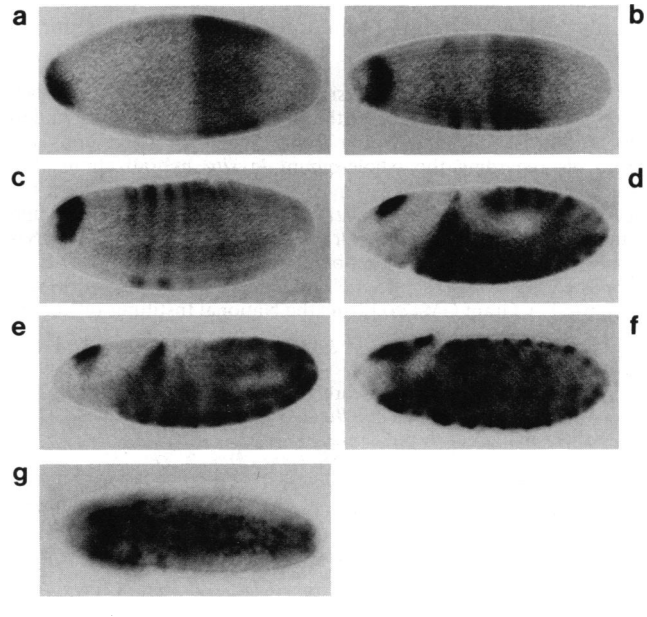

FIG. 5. Spatial distribution of dOct2 RNA during early embryogenesis. *In situ* hybridization of dOct2 cDNA to whole, wild-type embryos was as described (22). Staging of the embryos was done as described by Campos-Ortega and Hartenstein (39). All views are lateral with anterior on the left unless otherwise stated. (*A–C*) Stage 5, cellular blastoderm. (*D* and *E*) Stages 9 and 10, germ band extension. (*F*) Stage 15, germ band retracted. dOct2 expression appears in clusters of cells rather then in stripes. (*G*) Ventral view showing expression in cells of the central nervous system.

and/or in the initial events leading to the commitment of some ectodermal cells to the neurogenic pathway. Given that dOct2 is a transcription factor, it is reasonable to suggest that a consequence of its expression either individually or in combination with as yet unidentified transcription factors contributes to the establishment of a combinatorial code for segmentation and subsequent cellular differentiation.

Transcription activation in yeast has been used successfully to study various aspects of transcription by higher eukaryotic proteins such as DNA recognition and oligomerization, localization of activation domains, isolation of binding sites, and even to detect interacting proteins (see ref. 51 and refs. therein). Thus, the yeast system should provide an ideal opportunity to identify and clone the genes that encode distinct embryonic coactivators that may interact specifically with dOct1. By transforming our yeast strain that harbors the pADOct1 and pLGO4 with an embryonic cDNA library, we hope to clone the gene(s) that encodes a potential cofactor(s). The availability of the cDNA clones should facilitate additional experiments in yeast and provide further insights into the mechanisms of transcriptional regulation in early embryonic development.

Finally, the fact that dOct2 (and presumably dOct1) is a transcription factor does not preclude its possible involvement in DNA replication. Several transcription factors have been shown to regulate DNA replication in viral systems (see ref. 52 and refs. therein). In fact, both mammalian Oct1 and Oct2 can stimulate the replication of adenovirus DNA *in vitro* (53, 54), presumably by facilitating the interaction of the replication machinery with the origin of replication. Furthermore, data from *in vivo* experiments suggest that murine Oct3 and Oct6 may also be involved in DNA replication (13, 55). In light of the highly dynamic expression patterns in early embryonic development and because of their ability to differentially respond to positional cues, it is tempting to speculate that the *Drosophila* Oct factors might also contribute to cell type-specific differences in the regulation of DNA replication. Further genetic and biochemical experiments should help elucidate the functions of the Oct genes during *Drosophila* development.

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