The Alcohol Dehydrogenase Gene Is Nested in the outspread Locus of Drosophila melanogaster

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ABSTRACT

This report describes the structure and expression of the outspread (osp) gene of Drosophila melanogaster. Previous work showed that chromosomal breakpoints associated with mutations of the osp locus map to both sides of the alcohol dehydrogenase gene (Adh), suggesting that Adh and the adjacent gene Adh' are nested in osp. We extended a chromosomal walk and mapped additional osp mutations to define the maximum molecular limit of osp as 119 kb. We identified a 6-kb transcript that hybridizes to osp region DNA and is altered or absent in osp mutants. Accumulation of this RNA peaks during embryonic and pupal periods. The osp cDNAs comprise two distinct classes based on alternative splicing patterns. The 5' end of the longest cDNA was extended by PCR amplification. When hybridized to the osp walk, the 5' extension verifies that Adh and Adh' are nested in osp and shows that osp has a transcription unit of ~74 kb. In situ hybridization shows that osp is expressed both maternally and zygotically. In the ovary, osp is transcribed in nurse cells and localized in the oocyte. In embryos, expression is most abundant in the developing visceral and somatic musculature.

The outspread (osp) locus of Drosophila melanogaster was identified initially by an EMS-induced recessive mutation, osp', which resulted in the wings of adult flies being spread out laterally from the long axis of the body (E. H. Grell, unpublished data). It was first referred to in a publication by Aaron (1979) as a mutation associated with some mutations of the alcohol dehydrogenase (Adh) gene. Additional osp mutations have been induced by a variety of mutagens, 14 by EMS, 31 by X- or gamma ray, one by diepoxycyanate and two by formaldehyde (O'Donnell et al. 1977; Woodruff and Ashburner 1979; Ashburner et al. 1982; Chia et al. 1985). All fall into a simple genetic complementation group and result in essentially the same wing phenotype as osp'. There is some allele-specific variation in the angle at which the wings are held out from the body and whether or not the wings curve back toward the body, or tent. Complete loss of osp function (e.g., heteroallelic deficiency) results in a strong osp phenotype; however, the flies still are viable and fertile in both sexes.

Although deletions of osp are often mutant for Adh, the two loci can be mutated separately. osp was mapped just distal to Adh on the basis of recombination between osp76n and Adh132, both presumed point mutations. However, Chia et al. (1985) molecularly mapped five osp breakpoints distal to and two proximal to Adh. By this criterion, Adh maps within osp. In addition Adh', a gene of unknown function but related to Adh in sequence and structure, maps within osp by the same criterion (referred to as Adh-dup by Schaeffer and Aquadro 1987; Kreitman and Hudson 1991).

Working from the hypothesis that Adh is nested within the osp locus and that osp is a large gene, we undertook molecular studies to identify the limits and organization of the osp locus. We extended a chromosomal walk in the region and mapped additional osp breakpoints to it. We localized a genomic fragment that encoded a portion of the osp transcript and used that fragment to characterize osp transcription and to isolate osp cDNAs. The longest cDNA, extended by PCR and hybridized back to the chromosomal walk, confirmed that Adh and Adh' are indeed nested in the osp locus. This also demonstrated that osp is a large gene with a minimum of four exons spanning at least 74 kb.

The finding that Adh and Adh' are nested within osp raised the issue of whether this organization has an effect on the regulation of transcription of the nested genes. To address this issue, the temporal expression and tissue distribution of the osp transcript was characterized. The osp gene produces a polyadenylated transcript of ~6 kb that is transcribed during all developmental stages with peaks of expression during embryonic and pupal periods. In the embryo, osp is present as a maternal product that is anteriorly localized. Its dynamic zygotic expression shows early elements of pair rule control and later is most abundant in the visceral and somatic musculature. The peaks of osp expression do not coincide with those of Adh nor are the two expressed in the same tissues in embryos.
<table>
<thead>
<tr>
<th>Aberration</th>
<th>Previous name</th>
<th>Breakpoints</th>
<th>Molecular&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td><strong>A. On b Adh&lt;sup&gt;+&lt;/sup&gt; cn bw</strong></td>
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<tr>
<td>Df(2L)A47</td>
<td>34E1;35B2</td>
<td>Proximal to +108&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Df(2L)A48</td>
<td>35B2-3;35D5-7</td>
<td>0 to +0.7'</td>
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<td>Df(2L)A63</td>
<td>Not visible</td>
<td>−13.1 to −18.2'; proximal to +108&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Df(2L)A72</td>
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<td>+0.7 to +1.3'</td>
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<tr>
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<td>−110.8 to −113'; +76.5 to +78.5&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Df(2L)A217</td>
<td>34F5;35B3</td>
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<tr>
<td>Df(2L)A245</td>
<td>34A4;35B2</td>
<td>−137.8 to −134.6&lt;sup&gt;c&lt;/sup&gt;; proximal to +90&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Df(2L)A260</td>
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<td>−144.4&lt;sup&gt;c&lt;/sup&gt;; proximal to +90&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Df(2L)A379</td>
<td>35B1;35B3;40-41;57A8-10&lt;sup&gt;*&lt;/sup&gt;</td>
<td>−61.8 to −66.2'; +84.5&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>−73'; proximal to +90&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Df(2L)fn52</td>
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<td>Df(2L)nuc10</td>
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<td>Proximal to +90&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Df(2L)nuc13</td>
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<td><strong>B. On Adh&lt;sup&gt;+&lt;/sup&gt; pr pk cn sp</strong></td>
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<td>Proximal to +108&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Df(2L)bn84a4</td>
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<td>−37.5 to −42.5'</td>
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<tr>
<td>Df(2L)bn84a7</td>
<td>34C1-2;35B1-2</td>
<td>Not determined</td>
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<td>Df(2L)bn84a8</td>
<td>34D3;35B1-2</td>
<td>−37'</td>
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</tr>
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<td><strong>C. On In(2LR)O, Cy dp&lt;sup&gt;+&lt;/sup&gt; pr cn</strong></td>
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<tr>
<td>Df(2L)nuc20</td>
<td>34F1-2;35B2</td>
<td>+11.8 to +13.7'</td>
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<td><strong>D. On al dp b nuc&lt;sup&gt;21530&lt;/sup&gt; pr 1(2)pan cn f</strong></td>
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<td>Df(2L)TE35B-8</td>
<td>Df(2L)TE146(Z)GW8</td>
<td>34E4-5;35A2-4</td>
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<td>Df(2L)TE146(Z)GW9</td>
<td>34F1-2;35B2</td>
<td>Proximal to +108&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Df(2L)TE35B-11</td>
<td>Df(2L)TE146(Z)GW11</td>
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<td>−107.5 to proximal to +50'</td>
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<td>Df(2L)TE35B-15</td>
<td>Df(2L)TE146(Z)GW15</td>
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<td>Df(2L)TE146(Z)SR48</td>
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<td>+51.8 to +57.3&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Df(2L)TE35B-54</td>
<td>Df(2L)TE146(Z)SR54</td>
<td>35B1-2;35C1</td>
<td>−107.5; +11.7 to +30.0'</td>
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<td>34F3;35B2</td>
<td>−11.2 to −13.3&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td><strong>E. On Adh&lt;sup&gt;+&lt;/sup&gt; pr cn</strong></td>
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<tr>
<td>Df(2L)fn2</td>
<td>35A3;35B2</td>
<td>Proximal to +108&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Df(2L)fn7</td>
<td>34E1-2;35B3-5</td>
<td>Proximal to +108&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Proximal to +108&lt;sup&gt;b&lt;/sup&gt;</td>
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<td><strong>F. On Adh&lt;sup&gt;+&lt;/sup&gt; pr cn</strong></td>
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<td>Tp(2;3)osp&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35B3-4;36Cl1-D1;98E2-3&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>Df(2L)osp18</td>
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<td>−37.5&lt;sup&gt;'&lt;/sup&gt;</td>
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<td>In(2L)osp&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35B3;38D3-5</td>
<td>−1.7 to −4.1&lt;sup&gt;'&lt;/sup&gt;</td>
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<tr>
<td>Df(2L)osp29</td>
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<td>+36.9&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>In(2L)osp&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35B2-3;38B3-6</td>
<td>−1.7 to −4.1&lt;sup&gt;'&lt;/sup&gt;</td>
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<tr>
<td>T(2;3)osp&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35B3-4;89B9-11</td>
<td>+14 to +18&lt;sup&gt;'&lt;/sup&gt;</td>
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<tr>
<td>Df(2L)osp144</td>
<td>Not visible</td>
<td>−67.1 to −68.3; −8.3&lt;sup&gt;'&lt;/sup&gt;</td>
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<tr>
<td>Df(2L)osp201</td>
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<td>−8.3 to −18.2 (1-kb deletion)&lt;sup&gt;'&lt;/sup&gt;</td>
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<tr>
<td><strong>G. On nuc&lt;sup&gt;21530&lt;/sup&gt; pr pk cn sp</strong></td>
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<tr>
<td>Df(2L)TE35BG-4</td>
<td>Df(2L)TE36(R)GW4</td>
<td>35B3-4;35C3</td>
<td>Proximal to +108&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Df(2L)TE35BG-28</td>
<td>Df(2L)TE36(R)GW28</td>
<td>35B2;35B7</td>
<td>Proximal to +108&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Df(2L)TE35BG-35</td>
<td>Df(2L)TE36(R)GW35</td>
<td>35B2;35D4</td>
<td>Proximal to +108&lt;sup&gt;b&lt;/sup&gt;</td>
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</tbody>
</table>
However, both osp and Adh transcripts are expressed in the adult female ovary and are maternally transmitted to oocytes.

The nested relationship between osp and Adh may be significant with respect to the evolution of the two genes. A comparative analysis of known nested genes suggests that the introns of large genes may provide attractive sites in which small intronic genes can become established, be expressed, and evolve. The extensive research that has been done on Adh evolution can now be extended to a new framework, the evolution of nested genes.

**Materials and Methods**

**Drosophila stocks:** Stocks used in this study are listed in Table 1. The A series of deficiency stocks [e.g., Df(2L)A47, Df(2L)A48] are X-ray-induced Adh null mutations (Aaron 1979; Ashburner et al. 1982). The f3 series are formaldehyde-induced Adh null mutations: Df(2L)fn52 was originally obtained from W. Soper; the rest were described by O'Donnell et al. (1977) and Ashburner et al. (1982). Df(2L)ncl10 and Df(2L)ncl13 were isolated by G. Harrington and M. Ashburner (Flybase). The b series of deficiencies were described by Chia et al. (1985), except for Df(2L)j84a7 (Gubb et al. 1988). The Df(2L)TE35B series was described by Gubb et al. (1985, 1986). The Df(2L)TE35BC series was described by Gubb et al. (1984). The osp aberrations were described in Chia et al. (1985). Df(2L)el77 and Df(2L)W were described by Woodruff and Ashburner (1979), In(2L)C158 by Ashburner and Lemeunier (1976), T(2;3)H47 by Hilliker and Truss-Coutler (1987), T(2;3)Mpe by Hughes and Shelton (1980) and T(2;3)p6 by Kaufman (1978). Genetic analysis of the mutations is shown in Figure 1.

**Screening of genomic libraries, mapping breakpoints by Southern analysis and in situ hybridization to chromosomes:** These procedures are described in Davis et al. (1990) with the following exceptions. In this study the genomic libraries used were: a Canton S library (Maniatis et al. 1978), Canton S and Oregon R libraries in AEMBL4 (provided by M. Goldberg), a library constructed from a stock carrying markers y;cn bw sp and isogenic for all four chromosomes in AEMBL5 (made by J. Tamkun and J. Kennison; provided by J. Tamkun), and a library made from the stock Df(2L)DC1712/(In(2L)R)O, Cy dpbp1 pr cn2 (CyO) (a gift from D. Gubb).

To simplify the mapping of breakpoints by restriction enzymes to a single homologue, many chromosomes were used in heterozygous combinations with Df(2L)A48, a deletion with its distal breakpoint at 0 to +0.3 on the chromosomal walk (Chia et al. 1985) and its proximal breakpoint at 19 loci proximal to the Adh locus (Ashburner et al. 1982). Lethal derivatives of TE35BC (Gubb et al. 1984), Tp(2;3)osp1, Tp(2;3)p6 and In(2L)C158 were balanced over In(2L)R/CyO, Cy dpbp1 pr cn2. Other aberrations were induced on known progenitor chromosomes (Table 1), thus changes in hybridization pattern between aberrations and their precursors were judged by direct comparison.

The position of all breakpoints that are not deletions have been confirmed by in situ hybridization to polytene chromosomes.

**RNA isolation and Northern analysis:** Total RNA was isolated from staged collections from a Canton S wild-type strain. RNA was extracted using guanidinium thiocyanate (Chomcynski and Sacchi 1987), denatured and run in formaldehyde agarose gels (Maniatis et al. 1982), then transferred to nylon filters (Amersham) following the manufacturer's instructions. For the analysis of osp mutants, RNA was treated in the same fashion except that it was blotted onto nylon-reinforced nitrocellulose (Schleicher and Schuell) using the Amersham protocol for transfer to nitrocellulose.

Filters were probed with randomly primed DNA fragments

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**Table 1**

<table>
<thead>
<tr>
<th>Aberration</th>
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<th>Breakpoints</th>
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<td>Df(ZL)TE35BG3</td>
<td>Df(ZL)TE36(R)GV²</td>
<td>Df(2L)35B5;35D1-2</td>
<td>Proximal to +108⁴</td>
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<tr>
<td>Df(2L)TE35BG3</td>
<td>Df(2L)TE36(R)GV²</td>
<td>+T(2;3)35B;81</td>
<td>Proximal to +108⁴</td>
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<tr>
<td>Df(2L)el77</td>
<td>Df(2L)el77</td>
<td>35A1-3;35B3</td>
<td>+44.2 to +51.4⁴</td>
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<td>Df(2L)el77</td>
<td>Df(2L)el77</td>
<td>35A2-3;35B3-5</td>
<td>Proximal to +108⁴</td>
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<td>In(2L)C158</td>
<td>26D1-2;35B5c</td>
<td>+100.4 to +105.4⁴</td>
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<td>T(2;3)H47</td>
<td>35AB;65F</td>
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<td>T(2;3)p6</td>
<td>T(2;3)p6</td>
<td>T(2;3)35B3;83E2-8 +</td>
<td>+36.7 to +40.7⁴</td>
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</table>

*Previously ck⁶TE35BGSp pk cn sp⁵ (continued) |

²Molecular limits of breakpoints in the Adh region according to the coordinates of Chia et al. (1985).

¹This study.

²Chia et al. (1985).

³Davis et al. (1990).

⁴New order 21-35B3/57A8.10-49.41/35B1.34-40/57A8.10-60F.

⁵Previously ab dp b nocTE35BG² pr l(2)pm1 cn.

⁶Gubb et al. (1990).

⁷Previously ck⁶TE35BGSp pk cn sp.

⁸New order 21A-35B3/83E2-8;89A9-10/83E2-8;89A9-10/50C14-60F;61A-80/50C14-35B3/89A9.10-100F.
(FEINBERG and VOGELSTEIN 1983) from portions of the genomic walk that correspond to osp lesions (see above) or were identified by reverse Northern analysis as possible transcription units (G. AL-ATIA; B. MENG, personal communications). These were: hob2-3.8, hob1.12-3.8, hob1.12-4.7, AgAC3-2.4, AW2-10 and AW5.02-2.9 and -4.6 kb fragments (Figure 2).

Polyadenylated RNA was selected on oligo-dT cellulose columns (Collaborative Research) as described in MANIATIS et al. (1982).

osp mutant chromosomes were normally maintained over balancer chromosomes wild type for osp. To analyze the osp RNA encoded by the mutant chromosomes, mutant alleles were recovered as heterozygotes with Df(ZL)A1?8, which is deleted for most of the osp region and is null for osp expression (Figure 6). RNA was extracted from heterozygous flies scored as adults for the osp wing phenotype. Blots of total RNA, 20 µg per lane, were probed with the 4.7-kb EcoRI fragment from λob1.12 (referred to subsequently as ob1.4.7).

Isolation and characterization of cDNAs: Aliquots of a 0–3-hr embryonic cDNA library in λgt10 (POOLE et al. 1985) was screened using standard techniques (MANIATIS et al. 1982). An aliquot of the 8–12-hr embryonic cDNA library in the plasmid vector pNB40 (BROWN and KAFASTOS 1988) was screened as described in that paper using ob1-4.7 as a probe.

cDNAs were verified as corresponding to the osp transcript by hybridization to the expected 6-kb RNA on Northern blots (as above) and by hybridization to Southern blots of the walk phage DNA. cDNAs were first mapped by hybridization to bidirectional Southern transfers (SMITH and SUMMERS 1980) of EcoRI-digested DNA from phage which spanned the entire walk region. The phage that hybridized were mapped in greater detail using Southern blots of doubly digested phage DNA.

Partial sequences of the cDNAs were obtained by double strand sequencing using a US Biochemicals sequencing kit,
essentially as described by the manufacturer. To prepare the plasmids, 2-ml overnight cultures were prepared by the lysozyme boiling technique (HOLMES and QUIGLEY 1981) with these alterations. After boiling and pelleting, supernatants were extracted twice with equal volumes of phenol/chloroform, then ethanol precipitated in 0.3 M sodium acetate, pH 5.2. Samples were treated with RNase A and denatured before use in sequencing reactions.

**PCR amplification beyond the 5' end of the longest osp cDNA:** A 12–24-hr embryonic cDNA plasmid library, which contains directed inserts (BROWN and KAFATOS 1988), was used as a template for PCR amplification to obtain the 5' portion of the osp cDNA. The 3' primer was the reverse complement of sequences at the 5' end of 9114, the osp cDNA that thus was used for all subsequent experiments. Several clones, including posp5'-319, were partially sequenced and verified that the contents of the PCR band were homogeneous and overlapped 9114. Clone posp5'-319 was also used to probe chromosomes in situ to demonstrate that it mapped to its site of origin at 35B.

**In situ hybridization to whole mounts:** Whole embryos aged 0–24 hr and adult ovaries were collected and prepared as described by TAUTZ and PFEIFLE (1989), except that ovaries were fixed in the absence of heptane and, after staining, preparations were dehydrated and mounted in Permount (BDH Chemicals). cDNAs 9114, 7131 and 7111 were each cloned into pBluescript KS+ (Stratagene) for further analysis. Several clones, including posp5'-319, were partially sequenced and verified that the contents of the PCR band were homogeneous and overlapped 9114. Clone posp5'-319 was also used to probe chromosomes in situ to demonstrate that it mapped to its site of origin at 35B.

**RESULTS**

The molecular map of the outspread region: This study extends the work of CHIA et al. (1985) on the...
molecular mapping of the Adh region of the D. melanogaster genome. CHIA et al. (1985) cloned the distal portion of the osp locus as part of a 164-kb genomic walk in overlapping phage, ending 40 kb proximal to the Adh locus with AW4.04. By Southern mapping genomic DNA from osp mutants, they found five osp lesions distal to and two proximal to Adh, in addition to 21 aberrations that spanned the Adh region. In this study, we have extended the walk to 110 kb proximal to Adh (Figure 2) and have mapped additional osp mutations and chromosome variants to define the outer limits of the osp locus.

CHIA et al. (1985) defined the distal limit of osp as lying between -11.2 and -37 kb on the molecular map. This limit was based on their mapping of the proximal breakpoints of the noc osp" deficiency Df(2L)b84a8 at -37 kb and the noc- osp" deficiency TE35B-8, which ends in the 4.7-kb EcoRI fragment from λobl.12, at -11.2 to -15.9. GUBB et al. (1990) redefined the distal end of osp by mapping a new noc-osp" deficiency, Df(2L)GT4, which breaks between -11.2 and -13.3 and extends distally (Figure 3). The distal limit is now defined as lying between -11.2 and -13.5 kb.

Four osp deficiencies were mapped to the interval +40.0 to +105.4 kb: Df(2L)e77, Df(2L)TE35B-48, Df(2L)A178 and Df(2L)A379. In(2Lr)A379. Genomic DNA from each line was digested with EcoRI and hybridized with the following probes. Df(2L)e77/Df(2L)A48 DNA, probed with the 7.2-kb parental XhoI fragment and a fusion fragment of 9.7 kb (Figure 4, lane 1). Thus Df(2L)e77 is broken in the 7.2-kb EcoRI fragment between +44.2 and +51.4. When DNA from Df(2L)TE35B-48/Df(2L)A48 was probed with the 7.7-kb XhoI fragment of λW7.11, parental EcoRI fragments of 0.8, 1.4 and 4.9 kb were seen (Figure 4, lanes 2, 3). The 6.4-kb fragment was replaced by a fusion fragment of 5.5 kb (Figure 4, lane 3). From this we conclude that Df(2L)TE35B-48 is broken between +51.8 and +57.3, close to the EcoRI site at +57.3. When probed with λW9.06, DNA from Df(2L)A178/Df(2L)A48 lacked the parental fragment of 17.7 kb (Figure 4, lane 4) and instead had a fusion fragment of 15.0 kb (Figure 4, lane 5). Therefore the proximal limit of Df(2L)A178 is in the 17.0-kb EcoRI genomic fragment at +76.5 to +78.5. Together with data from CHIA et al. (1985), this defines Df(2L)A178 as

![Figure 3](image-url)

FIGURE 3.—Summary: molecular map of osp mutations. The molecular extent of the osp locus, as determined by Southern mapping of osp mutations, is indicated by the thick bar at the top. The outer limits of osp are defined on the distal side by the noc osp" mutation Df(2L)GT4 and noc- osp" Df(2L)TE35B-8. The aberrations that define the proximal side of osp are the osp" mutations, Tp(2;3)osp" and Tp(2;3)osp' and Tp(2;3)H47, and osp" In(2L)c158. Mutations were mapped to EcoRI fragments; error bars indicate the uncertainty of the location of the breakpoint within the EcoRI fragment. There is a large error bar for the proximal side of osp due to the lack of diagnostic mutations between Tp(2;3)osp" and Tp(2;3)osp' and Tp(2;3)H47, and In(2L)c158. Interstitial deficiencies and fragments within which inversions break are represented by dashed bars. Deficiencies that extend beyond the region shown here are indicated by lines ending in arrowheads. Df(2L)A379 is a deficiency associated with an inversion, and is represented by a horizontal deficiency line and a vertical arrow for the inversion breakpoint. Aberrations mapped by others are (a) CHIA et al. (1985) and (b) GUBB et al. (1990). Tp(2;3)H47 was diagnosed by in situ hybridization of λW5.02 to chromosomes. Adh and Adh' are indicated by arrows, which show their transcriptional orientation.
a deletion of ~190 kb. DNA from Df(2L)A379; In(2-LR)A379/Df(2L)A48, probed with λW9.06, yielded only a 5.0-kb fragment (Figure 4, lane 6). The absence of a fusion fragment suggests that this aberration is broken close to the EcoRI site at +84.5.

Three additional aberrations, two osp; and one osp', have been mapped to this region. DNA from Tp(2;3)-osp'/CyO, digested with EcoRI and probed with the 4.6-kb EcoRI fragment of AW5.02 (Figure 2), yielded four fragments (Figures 4, lane 8). Fragments of 3.3 and 1.3 kb are from the CyO balancer chromosome (Figure 4, lane 8) and are due to a polymorphism at +42.6 (Figure 2). The 4.6-kb band from the parental chromosome (Figure 4, lane 8) has been replaced by 4.8- and 1.9-kb fusion fragments, thus Tp(2;3)-osp' appears to be broken in the AW5.02-4.6-kb EcoRI fragment, between +39.6 and +44.2. This result was confirmed by in situ hybridization to Tp(2;3)-osp' chromosomes. When AW5.02 was used as probe, the mutant second and third chromosomes hybridized, in addition to the CyO balancer chromosome.

DNA from T(2;3)ph3/CyO, digested with HindIII and probed with the 2.9- and 4.6-kb EcoRI fragments of AW5.02, yielded parental fragments of 4.0 and 3.5 kb (Figure 4, lane 9). In addition, fusion fragments of 10.5 and 1.9 kb were found. The intensity of signal from the parental 4.0-kb fragment is weak, consistent with the translocation being broken within this fragment, between +36.7 and +40.7. This was confirmed by in situ hybridization to T(2;3)ph3 chromosomes.

The breakpoint of T(2;3)H47 was shown to lie within the DNA contained in AW5.02, between +34.4 and +50.6, by in situ hybridization. Genomic DNA from AW5.02 hybridized to 35B and also to a site on the third chromosome.

The osp' inversion In(2L)C158 defines the proximal outer limit of osp. In(2L)C158, found in a natural population (ASHBURNER AND LEMEUINER 1976), breaks between osp and the locus l(2)35Bb. DNA from In(2L)-C158/CyO was digested with XhoI and probed with the proximal 5.0 kb of phage AW11.01. In addition to the 15.0-kb parental fragment present in Canton S (Figure 4, lane 10), two novel fragments of 16.0 and 7.1 kb were obtained (Figure 4, lane 11).
that this inversion is broken between +100.4 and +105.4 and confirmed it by hybridizing AW11.01 in situ to In(2L)C158 chromosomes (data not shown). These data demonstrate that the proximal end of osp lies to the left of the In(2L)C158 proximal breakpoint, i.e., left of +105.4 on the molecular map.

The remaining aberrations have proximal limits beyond AW11.01. Locations of the osp mutations are summarized in Figure 3.

The mapping of mutants defines the proximal end of osp as lying between +39.6, as defined by Tp(2;3)osp+1; T(2;3)pb+1 and T(2;3)H147 and +105.4, as defined by In(2L)C158. Taken together with previous work (GUBB et al. 1990), we conclude that the osp locus lies between −11.2 to −13.3 and +39.6 to +105.4 on the walk map, and is 52.8 to 118.7 kb in length.

Identification of the osp transcript and its developmental expression: Fragments of cloned genomic DNA to which osp lesions mapped were used to probe developmental Northern blots. Although six different fragments were used as probes (see MATERIALS AND METHODS), only the EcoRI 4.7-kb fragment from the proximal end of Aobl.12 (Figure 2) was successful in detecting RNA. It hybridized a major band of ~6 kb. The transcript is most abundantly expressed in embryonic and pupal stages of development, although it is detected at all stages (Figure 5). The control reprobing of the blot with an actin 5A clone showed that similar amounts of RNA were present in each lane (data not shown). This 6-kb RNA is detected in polyadenylated RNA fractions from pupae (data not shown).

The 6-kb transcript is absent in osp mutants: The evidence that the 6-kb band represents the osp transcript is that this transcript is absent in 14 different osp mutants and defective in one, but present in stocks that are not mutant for osp. Adult flies that were heterozygous for one of 15 osp alleles over Df(2L)A178 were tested for accumulation of the 6-kb osp transcript (Figure 6). Flies with deficiencies distal to osp, e.g., G74, b84a4 and b84a8 (CHIA et al. 1985), produce the 6-kb transcript. In contrast, osp mutants lack the 6-kb transcript. These include deficiencies such as A220, osp18, osp29, A63, A72, A48 and A379; inversions osp9′ and osp23; translocations pb+ and osp9; and transpositions ospb and H147. The inversion proximal to the osp region, C158, which is osp+ but inverted between osp and the adjacent gene l(2)35Bb, does not disrupt production of the 6-kb transcript.

For Df(2L)osp44, novel transcripts of 4.6, 3.1, 2.4, 2.0 and 1.3 kb were detected. This deficiency extends from approximately −10 to −70 (CHIA et al. 1985) and likely removes part of the osp 3′ exon (described in further detail later). The origin of the osp44 transcripts is not known. However, osp44 has a strong osp phenotype, which indicates that these short RNAs are not sufficient for osp function.

Characterization of the osp cDNAs: One cDNA homologous to ob1-4.7, 211, was obtained from the 0–3-hr embryonic cDNA library (POOLE et al. 1985). Two cDNAs, 7111 and 9114, were isolated from the 12–24-hr embryonic cDNA library (BROWN and KAFATOS 1988). cDNA 9114 contained the longest cDNA insert of 4.2 kb, short of the 6 kb required for a complete osp cDNA. When used to probe Southern blots of the walk region, the cDNAs all mapped to the left of Adh (summarized in Figure 7).

The structures of the osp cDNAs provide evidence of alternative splicing of osp transcripts. When mapped to Southern blots of osp region phage DNA digested with EcoRI (summarized in Figure 7), cDNA 211 hybridized only to the 4.7-kb fragment of Aobl.12, at −15.8 to −11.2; 7111 hybridized to the 4.7-kb Aobl.12 fragment, the 2.4-kb lgAC3 fragment, and the 2.0-kb fragment of lgAC2 (which overlaps completely the 2.4-kb fragment of lgAC3). cDNA 9114 hybridized the regions covered by 7111 plus an additional portion of lgAC3. These results suggest that there are at least two osp RNA splice variants that share a common 3′ segment.
Some of the variation observed between the cDNAs may be due to incomplete splicing, however, the cDNAs all detect a 6-kb band on Northern blots. The 6-kb band must represent a set of transcripts of approximately the same length. The possibility of some of the variants arising from an osprelated gene is unlikely. Reduced stringency genomic Southerns probed with 9114 yielded no bands aside from those representing the osp walk region (data not shown).

The PCR 5’ extension of the longest osp cDNA hybridizes to restriction fragments both proximal and distal to Adh. The cDNAs failed to provide direct molecular evidence that Adh was nested in osp. Since the cDNAs were not full length, it was possible that by extending them further, such evidence would be obtained. Thus, the region 5’ to the longest osp cDNA, 9114, was extended by PCR. Since osp is abundant in embryos and an excellent embryonic cDNA library was available, we amplified the 5’ extension from the pNB40 12–24 hr embryonic cDNA library (BROWN and KAFATOS 1988). The primers con-

![Figure 6](image_url)

**Figure 6.**—Northern analysis of osp mutants. Total RNA from adults was probed with the 4.7-kb EcoRI fragment from λob1.12 (top) or rhp9 (bottom). All osp mutants were tested in heteroallelic combination with Df(2L)A178. Lanes (1) Canton S, (2) Df(2L)A220, (3) parental strain Adh' Deltapren, (4) Df(2L); osp29, (5) Df(2L); osp7, (6) T(2;3)osp29, (7) In(2L)osp7, (8) Df(2L); osp7, (9) Df(2L);ospH44, (10) Df(2L);ospH48, (11) Df(2L);A63, (12) Df(2L);A72, (13) Df(2L);A379, (14) In(2L)C158, (15) Canton S, (16) T(2;3)H47, (17) Df(2L);A48, (18) T(2;3)osp7, (19) In(2L);osp7, (20) Df(2L);GT4, (21) Df(2L);b84a4, (22) Df(2L);b84a8. Sizes of the bands are indicated to the left.

![Figure 7](image_url)

**Figure 7.**—Summary of the structure of the osp cDNAs. cDNAs were hybridized to osp walk phage DNA digested with either EcoRI or EcoRI in combination with a second enzyme. Walk fragments that were hybridized are represented by lines corresponding to the different cDNAs above the genomic map. The names of the cDNAs are to the right of the lines and their actual lengths to the left. Adh and Adh' are indicated by arrows just above the genomic map. The walk phage and EcoRI fragments hybridized are shown below.
sisted of a 3' primer internal to the 5' end of 9114 and a 5' primer internal to the pNB40 vector.

The amplification yielded a major band of 1.5 kb, bringing the total osp cDNA length up to 5.5 kb, close to full length. The identity of this band was verified by hybridization to an internal probe. It was then cloned to make posp5'-319 and used to probe DNA from the osp walk phage (Figure 8). As expected, it hybridized to the EcoRI 2.4-kb fragment of λgAC3 and to λgAC2-EcoRI 2.0 kb, from which the primer originated, distal to Adh. In addition, it hybridized to fragments proximal to Adh, to AW3-EcoRI 15 kb and to the EcoRI 2.7-kb fragment shared by λW7.11 and λW8.061 at +63.0 to +65.7 kb (summarized in Figure 9). The 5' extension identifies two new exons, indicating that osp is composed of at least four exons.

To characterize the 5' extension further, posp5'-319 was used to probe Northern blots and polytene chromosomes. As a Northern probe, it detected only the 6-kb transcript. The only site of chromosomal hybridization was SB2-3, where osp and Adh are located. In summary, hybridization to a predicted internal probe, to its site of origin on the walk, to the osp RNA, and to the chromosomal location of osp confirms that the 1.5-kb PCR amplification product corresponds to a portion of the osp gene product. The 5' extension represents at least two new exons proximal to Adh and Adh'. The cDNAs extend from −15.8 to −11.2 to +63.0 to +65.7 kb, demonstrating that the osp transcription unit is ≥74 kb in length.

Characterization of maternal and zygotic expression of osp: The presence of osp transcripts in 0–3-hour embryos (Figure 5) indicates that osp is expressed in ovaries. Expression in ovaries of wild-type flies was examined further by in situ hybridization (Figure 10). The osp transcript is present in multiple nurse cells in the gerarium. By stage 3, it is localized principally to the presumptive oocyte where it continues to be detected through stage 10. The osp transcript is present in a broad band in the anterior end of late stage oocytes. The anterior localization is retained until after cellular blastoderm (Figure 10A). In addition, osp appears to be expressed by follicle cells (Figure 10B). Follicle cells may contribute to an accumulation of osp transcript around the periphery of the oocyte (Figure 10A, stage 10), however, most of the transcript detected at the periphery is internal to the oocyte (Figure 10B). In ovaries from heteroallelic osp−/osp− females Df(2L)A63/Df(2L)A178, no transcription was detected.

In wild-type embryos, osp displays a highly dynamic pattern early in embryogenesis. From the earliest stages of embryogenesis through early syncytial blastoderm, maternal osp transcript is localized at the anterior end of the embryo (Figure 11A). As the ventral furrow forms, osp is expressed at low levels lateral to the furrow. Later it is expressed in several prominent stripes (Figure 11B) that extend across the entire germ band, visible only briefly as the germ band elongates. At stage 7, osp is expressed strongly in the maxillary and labial segments and appears to be expressed in faint segmental stripes (Figure 11C). By stage 8, osp expression in the mandibular and maxillary segments is intensified (Figure 11D). In addition, its segmental expression resembles pairs of spheres within the mesodermal layer (Figure 11E). Segmental staining in areas of mesodermally derived tissues is prominent throughout the period of germ band extension. As the germ band retracts, the pattern along the ventral surface is refined and a few cells along the ventral midline are prominent (Figures 11F) in a pattern similar to aspects of achaete-scute (CABRERA et al. 1987) and nautilus (MICHELSON et al. 1990) expression. Subsequently, the staining parallels the shape of the somatic and visceral musculature (M. BATE, personal communication; Figure 11, G–L). Some tissues that are not of mesodermal origin, including the salivary glands (Figure 11, F–J) and later the malphigian tubules (Figure 11, K and L) also stain intensely. osp transcripts are detected through stage 17. In control embryos produced by heterozygous osp parents Df(2L)−A63/Df(2L)A178, no staining was detected at any stage.

DISCUSSION

Adh and Adh' are nested in the osp locus: osp is a large gene of up to 119 kb, with a minimum transcription unit
Drosophila Adh Nests in outspread

of 74 kb that encodes a set of 6-kb transcripts. It appears, on the basis of the cDNAs recovered, to possess at least four introns and to encode at least two classes of RNAs distinguishable as splice variants. We conclude that Adh and Adh' are nested within osp, since they are encompassed by osp cDNAs (summarized in Figure 9).

In addition to Adh', the open reading frame (ORF) 3' of Adh, a 5' ORF was previously identified 4.2 kb upstream from the Adh adult (distal) promoter (KREITMAN and AGUADE 1986). This 5' ORF lies within a restriction fragment which we have shown contains an osp exon. The 5' ends of cDNAs 9114 and 7111 overlap this 5' ORF. The known sequence of 9114 is virtually identical to the 5' ORF throughout its length of 247 bp; thus this ORF is almost certainly part of the osp gene product. The conceptual translation of the 5' ORF does not yield a protein with significant homology to any known protein. The region between -660 and -5000 bp 5' of the Adh adult promoter, in which this ORF is located, contains an enhancer for larval Adh (POSAKONY et al. 1985; CORBIN and MANIATIS 1989). It remains to be determined if alterations to this portion of the osp locus have an effect on expression of larval Adh.

Characteristics of nested genes: In addition to the osp-Adh nested locus, six nested gene sets have now been described. In Drosophila, these are the gene for guanine-adenosine ribosyl transferase (GART) and intronic pupal cuticle gene (Pep) (HENIKOFF et al. 1986a), the dunce gene and intronic genes Sgs-4, PIG-1, ng-1, ng-2, ng-3 and ng-4 (CHEN et al. 1987; FURIA et al. 1990, 1991, 1993), the R1 locus of cAMP dependent kinase (Pka-R1) and two intronic genes of unknown function (KALDERON and RUBIN 1988), and the doublesex (dsx) locus and the intronic adult male specific transcription unit (CHEN 1995). Two sets of nested genes have been identified in humans: the neurofibromatosis type 1 gene (NF1) and intronic oligodendrocyte myelin glycoprotein gene (OMGP), EVI2A and EVI2B (XU et al. 1990), and factor VIII and an intronic gene of unknown function (LEVINSON et al. 1990). NF1 also has a nested structure in mice (CAWHON et al. 1990). A few generalizations can be drawn from these cases.

1. Nested genes are broadly distributed but they may not be stable over extended periods of evolutionary time. The presence of nested genes in the human, mouse and Drosophila genomes suggests that they may be distributed throughout the Metazoa. For GART, the nested arrangement is present in D. pseudoobscura (HENIKOFF and EGHTEVARZADEH 1987), separated from D. melanogaster by 5 million years, but absent in Chironomus tentans, a dipteran separated from D. melanogaster by 50 million years (CLARK and HENIKOFF 1992). For factor VIII, the mouse gene possesses no intron and the homologue of the intronic gene is located distantly, unlike the human locus (LEVINSON et al. 1990).

2. Intronic genes are small genes located in large genes. Intronic genes range in size from the 0.6-kb
ng-1 (Furia et al. 1993) to the 2.7-kb OMGP (Xu et al. 1990). However, intronic genes may possess introns, as in the cases of Adh (Benyajati et al. 1983), Pep (Henikoff et al. 1986a) and EVI2A and EVI2B (Cawthon et al. 1990).

Nest genes range from the 10-kb GART (Henikoff et al. 1986a) to the 240 kb NF1 (Xu et al. 1990; Goldberg and Collins 1991). Large genes with sizeable introns appear to provide big targets or residences for intronic genes. With the exception of dsx, all nest genes encode enzymes. Known nested genes are associated with genetic disorders or mutations, and since large genes are more easily mutated than are short ones, they are probably overrepresented in this survey.

3. The number of genes per intron and per locus, and their transcriptional orientations, are not constrained. GART (Henikoff et al. 1986a) and factor VIII (Levinson et al. 1990) have only one intronic gene each, osp and Pha-R1 (Kalderon and Rubin 1988) each have two, NF1 contains three (Xu et al. 1990). dunce, with the largest number of intronic genes at a single locus, has at least six: four in one intron and two in another (Furia et al. 1993). Intronic genes may be in the same or opposite orientation to the nest gene and to each other. The dunce locus includes examples of both (Furia et al. 1993).

4. A number of intronic genes share sequence similarity or tissue specificity with other intronic genes at the same locus. Nested in osp, Adh and Adh' are similar in sequence and appear to be related by an ancient gene duplication (Schaeffner and Aquadro 1987; Kreitman and Hudson 1991). The EVI2A and EVI2B genes that nest in NF1 are unrelated in sequence but are both expressed in peripheral blood mononuclear cells (Cawthon et al. 1990, 1991). Of the six intronic genes of dunce, four are expressed in third instar larval salivary glands, although they differ somewhat in temporal specificity. Two reside in each of the two introns (Furia et al. 1993). The nest genes do not share sequence similarity with their intronic genes, and in only two cases, those of Adh and osp and NF1 and OMGP (Xu et al. 1990), is there a suggestion of overlapping tissue specificity.

The presence of neighboring intronic genes with shared origins or transcriptional regulation suggests that the introns of nest genes can be accommodating environments in which other genes can
survive, can be expressed and can evolve. It is important to know what features distinguish nest genes and their introns as permissive residences. One feature may be an open chromatin conformation. Three of the five nested genes found in Drosophila, 
\textit{dunce}, \textit{GART} and \textit{dsx}, are located at chromosomal regions that undergo puffing in the salivary gland chromosomes (ASHBURNER 1967, 1969). The other two, \textit{osp} and \textit{Pla-R1}, are located in regions composed of interbands and faint bands. These cytological features are consistent with local decondensation of chromatin.

\textbf{Transcriptional interference between \textit{osp} and \textit{Adh}:} For nested genes, the question of transcriptional interference between the genes arises. Transcriptional interference can be inferred through competing patterns of transcription. At the cellular level, \textit{osp} and \textit{Adh} have been examined most closely in embryos, where expression of the two genes does not overlap. \textit{osp} is expressed primarily in mesodermal tissues, but also in the salivary glands and Malpighian tubules (but not other gut-derived tissues). \textit{Adh}, on the other hand, is expressed in fat body and in gut derivatives not including the Malpighian tubules (LOCKETT and ASHBURNER 1989). However, in adult females \textit{Adh} and \textit{osp} are both expressed in the ovary, thus defining a tissue and a time when transcriptional interference may occur.

\textbf{The nature of the \textit{osp} gene product:} The maternal \textit{osp} transcript is expressed in nurse cells of developing egg chambers in the ovary and is packaged into the oocyte, where it is localized at the anterior end. It is also expressed in the follicle cells.

The zygotic \textit{osp} transcript is detected very early in embryogenesis, by $\sim$3.7 hr into development. In general, zygotic transcription in the embryo begins at $\sim$2
hr of development. Since the rate of transcription in Drosophila is 1.1–1.4 kb/min (THUMMEL et al. 1990; SHERMOEN and O’FARRELL 1991; IRVINE et al. 1991) and the probe used here detects portions of the osp RNA ≥60 kb from its transcriptional start site, osp transcription must begin at ~3 hr of development, just after the embryo completes cellularization. osp transcripts are detected throughout the embryonic period. osp is strongly expressed in a segmental pattern as the germ band elongates, extends and retracts, predominantly in tissues of mesodermal origin. The expression pattern of osp suggests that, while it may have a complex role in development, it may be particularly important in development of muscle. Since its sequence, its protein localization, and its interactions are not lethal, the mutants are fertile and no defects have been observed among the null mutations of osp. Particularly important in development of muscle, since it may have a complex role in development, it may be functioning as an enhancer of the Drosophila Deltex gene.}

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