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Spatiotemporal relationships between a novel *Drosophila* stripe expressing gene and known segmentation genes by simultaneous visualization of transcript patterns

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Abstract. We describe the cloning of the *Drosophila* transcription unit *odd-striped* (*ost*), which maps close to the P-element insertion site of the enhancer-trap line 05279. In order rapidly to gain information on its chromosomal localization, and to determine the spatial and temporal expression patterns of *ost* in whole-mount embryos, we established a double-label in situ hybridization protocol to localize two different DNA or RNA sequences simultaneously. The double-label in situ hybridization method involves digoxigenin- and biotin-labeled DNA probes that are processed to result in blue alkaline phosphatase and brown peroxidase reaction products, respectively. Using reference probes as internal standards, we show that the *ost* transcription unit is located within the cytogenetic band interval 89A1,2 on the right arm of the third chromosome, and that it exerts diagnostic segmentation gene expression patterns in the embryo. The *ost* transcripts are initially expressed in an anterior cap at the blastoderm stage, followed by a transient pair-rule gene expression pattern, which eventually changes into a segmental polarity gene pattern at gastrulation. Our results establish detailed spatial and temporal relationships between *ost* expression and the known patterns of the segmentation genes *fushi tarazu* and *wingless*, and demonstrate the potential of the double-label method with differently tagged DNA probes.

Introduction

Much of our current knowledge of the processes underlying the phenomenon of biological pattern formation derives from a detailed examination of *Drosophila* segmentation (reviewed by St Johnston and Nüsslein-Volhard 1992; Pankratz and Jäckle 1993). This ongoing study was initiated by mutagenesis screens designed to identify the genes required for both setting up the polarity in the embryo and establishing the segmental pattern of the larval body (Nüsslein-Volhard and Wieschaus 1980; Nüsslein-Volhard et al. 1984, Jürgens et al. 1984; Wieschaus et al. 1984). The pattern defects associated with the various mutant phenotypes suggested that the polarity of the developing embryo rests on a limited number of maternal components that were deposited in the egg, and that zygotically active segmentation genes are necessary to subdivide the embryo into increasingly smaller units along the anterior-posterior axis, i.e., gap genes function in broad and overlapping regions of the embryo as reflected by the absence of adjacent segments in gap mutants, pair-rule genes are necessary in alternating segment equivalents, which are deleted in pair-rule mutant embryos, and segment polarity genes are required to establish the polarity within each of the segments (reviewed by Akam 1987; Ingham 1988). The subsequent molecular analysis of genes that act at each level of the segmentation gene cascade in conjunction with in situ hybridization techniques, which were initially used to locate distinct DNA sequences on polytene salivary chromosomes (e.g. Langer-Safer et al. 1982), demonstrated that the segmentation genes are expressed in the segment anlagen that are affected in the respective mutants (reviewed by Pankratz and Jäckle 1993).

The finding that *Drosophila* segmentation genes are molecularly conserved across species opened up the possibility of isolating developmental genes in organisms less favorable for a genetic approach than *Drosophila* (e.g., McGinnis et al. 1984; Noll 1993). The adaptation of in situ hybridization techniques for the detection of transcript expression patterns in vertebrate embryos suggested that the molecular conservation might extend into a functional one, since corresponding genes were found to be expressed in corresponding tissues of various species (e.g., reviewed by McGinnis and Krumlauf 1992; Patel 1994; Tautz and Sommer 1995). Once reverse genetics techniques became available to establish gene functions by, for example, "gene knock-outs" in mice, the functional significance of expression patterns could be established. Along with this progress, which eventu-

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ally enabled the connection between homologous gene functions in various animal species (e.g., Quiring et al. 1994), the increasing refinement of in situ hybridization techniques for establishing spatial and temporal relationships between expression patterns of the isolated genes had proven valuable. Now, in situ hybridization to whole-mount embryos is an essential tool to assess rapidly the transcript pattern in entire organisms. In addition, antibody staining techniques were employed to visualize the patterns of two gene products simultaneously and thus, the spatial and temporal relationships between the expression patterns of different genes can be compared within the same specimen. Corresponding double-label in situ hybridization techniques are as yet comparatively time consuming, since they were established for the use of RNA probes exclusively.

Here we report the cloning of a new *Drosophila* gene, termed *odd-striped* (*ost*), which maps close to the integration site of the P-element based enhancer-trap 05279. *ost* exerts segmentation gene-like expression patterns during early embryogenesis. For the chromosomal localization of the *ost* gene and to determine the spatial and temporal relationships between the *ost* expression pattern and the expression patterns of the pair-rule gene *fushi tarazu* (*ftz*) (Hafen et al. 1984) and the segment polarity gene (*wingless*) (*wg*) (Baker 1987), respectively, we established a simple and rapid double-label in situ hybridization protocol that involves biotin- and digoxigenin-tagged (DIG-tagged) DNA probes.

Materials and methods

Preparation of labeled DNA probes for in situ hybridization. DNA probes were prepared from *ftz* (Kuroiwa et al. 1984), *wg* (Baker 1987), *en* (Kornberg et al. 1985) and *ost* cDNAs, and from a lambda phage clone containing the tropomyosin gene (Karlik et al. 1984), respectively. In order to isolate the recombinant DNA inserts, purified DNA was cleaved with the appropriate restriction enzyme, the resulting DNA fragments were separated by agarose gel electrophoresis (Sambrook et al. 1989) and the insert DNA was eluted from the gel using the QuiaEx extraction kit according to the protocol provided by the manufacturer (Quiagen, Hilden). Labeling reactions with purified insert DNA were done in a 1.5 ml Eppendorf vial.

For biotin labeling, 1–2 µg of insert DNA were dissolved in 14 µl distilled water, denatured by boiling (10 min) and snap cooled in liquid nitrogen. After thawing on ice, 2 µl $pd(N)_6$ mixture (10 mg/ml; Pharmacia) and 4 µl biotin high prime kit (Boehringer Mannheim) were added and the reaction mix was incubated for 12–16 h at room temperature. For DIG-labeling, 0.2–0.5 µg insert DNA were solved in 9 µl distilled water, denatured by boiling (10 min) and snap cooled in liquid nitrogen. After thawing on ice, 2 µl 10× labeling buffer (950 mM Pipes, pH 6.6, 50 mM $MgCl_2$, 0.66% β-mercaptoethanol), 6 µl $pd(N)_6$ mixture (10 mg/ml; Pharmacia), 2 µl 10× DIG dUTP, dNTP mixture (Boehringer Mannheim) and 1 µl Klenow enzyme (5 U(µl; Boehringer Mannheim) were added while the reaction was kept on ice. The reaction mix was incubated at 15° C for 1 h followed by a 3 h incubation at room temperature.

After incubation, DNA was precipitated by adding 1 µl glycogen (20 mg/ml; Boehringer Mannheim), 2 µl 5 M NaCl and distilled water to adjust the final volume to 100 µl. After mixing, 300 µl of 100% ethanol (–20° C) was added with thorough mixing. The reactions were incubated for 15 min at –20° C and the precipitates were collected by high-speed centrifugation in an Eppendorf centrifuge (30 min at 4° C) The pellets were washed in 70% ethanol, dried in a speed-vac and dissolved in 50 µl hybridization solution [HybS, 50% formamide, 5× standard saline citrate (SSC, 1× is 150 mM NaCl, 15 mM sodium citrate), 50 µg/ml heparin, 0.1% Tween 20, 100 µg/ml sonicated and boiled salmon sperm DNA]. Probes were stored for up to several weeks at –20° C.

Double-label in situ hybridization to polytene chromosomes. Polytene chromosomes were prepared from an Oregon R *Drosophila* wild-type strain raised under standard conditions. Larvae were grown up to third instar larval stage at 18° C using food supplemented with extra yeast. Larvae were washed in phosphate buffered saline (PBS, 140 mM NaCl, 7 mM Na_2HPO_4, 3 mM KH_2PO_4) and dissected on a slide in 45% acetic acid. Salivary glands were washed twice with 45% acetic acid, and squashes were prepared according to a standard protocol (Ashburner 1989). After dehydration in 100% ethanol, the slides were air dried and stored dry. Prior to hybridization, the slides were incubated in 2× SSC at 68° C (30 min), in 2× SSC (2 min; room temperature), in 70% ethanol (10 min) and in 100% ethanol (5 min) and air dried. Subsequently, the chromosomes were denatured by placing the slides into freshly prepared 0.07 N NaOH, pH 12.5 (3 min; room temperature). Slides were washed in 2× SSC (three times 5 min; room temperature), dehydrated (see above) and air dried. 2 µl of the DIG-labeled probe and 4 µl of the biotin-labeled probe (see above) were mixed in 20 µl HybS (total volume per slide), denatured by boiling (10 min; 100° C), chilled on ice and subsequently added to the chromosome preparation. The hybridization solution was covered with a coverslip (22 mm^2) and incubated for 12–16 h at 60° C in a moist chamber. Coverslips were removed and the slides were washed twice in 2× SSC (60° C, 15 min) and three times in PBT (PBS, 0.1% Tween 20) (10 min; room temperature). 100 µl antibody mixture [anti-DIG antibodies coupled to alkaline phosphatase (dilution 1:200 in PBT) and anti-biotin antibodies coupled to peroxidase (1:100 diluted in PBT)] was added to the chromosome preparations, which were covered with a coverslip (22×40 mm) for the incubation at room temperature (1 h in a moist chamber). Slides were washed three times for 3 min in PBT. The peroxidase reaction was initiated by adding 100 µl of a mixture of 25 µl diaminobenzidine (10 mg/ml) and 1 µl H_2O_2 solution (1:100 diluted) in 1 ml PBT. After 5–15 min at room temperature and visual control under the microscope, the peroxidase reaction was stopped by washing slides first three times for 3 min in PBT and then three times for 3 min in alkaline phosphatase buffer (AP buffer, 100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). Subsequently, the alkaline phosphatase reaction was initiated by incubating the chromosome preparations in 100–200 µl of substrate solution [4.5 µl nitroblue tetrazolium salt (NBT) and 3.5 µl 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (X-phosphate) per milliliter AP buffer] under a coverslip (22×40 mm) for 15 min up to several hours, depending on the desired signal strength, which was controlled under the microscope. The alkaline phosphatase reaction was stopped by washing the slides in excess water (three times 5 min each) and chromosomes were counterstained with Giemsa (Sigma; 1:20 diluted in deionized water or phosphate buffer, pH 6.8) for 100 s up to 3 min. Chromosomes were destained for 2 min under running deionized water. After air drying, chromosomes were embedded in 30 µl Permout (Fisher Scientific) under a coverslip (22 mm^2). Antibodies and chemicals for the staining reactions were ordered from Boehringer Mannheim.

Double-label in situ hybridization to whole mount embryos. Embryos were collected and fixed as described previously (Tautz and Pfeiffle 1989), with the following modification: 8 ml heptan, 1.5 ml fixing solution and 0.5 ml 37% formaldehyde were used for fixing. Fixed embryos were transferred into a 1.5 ml Eppendorf vial and washed three times for 5 min each in about 1 ml PBT. After washing, embryos were incubated 2–3 min in 1 ml PBT containing 30–50 µg/ml proteinase K. After removal of the proteinase K solution, embryos were incubated in 1 ml glycine solution (2 mg/ml in PBT) for 2 min, washed twice for 5 min each in 1 ml

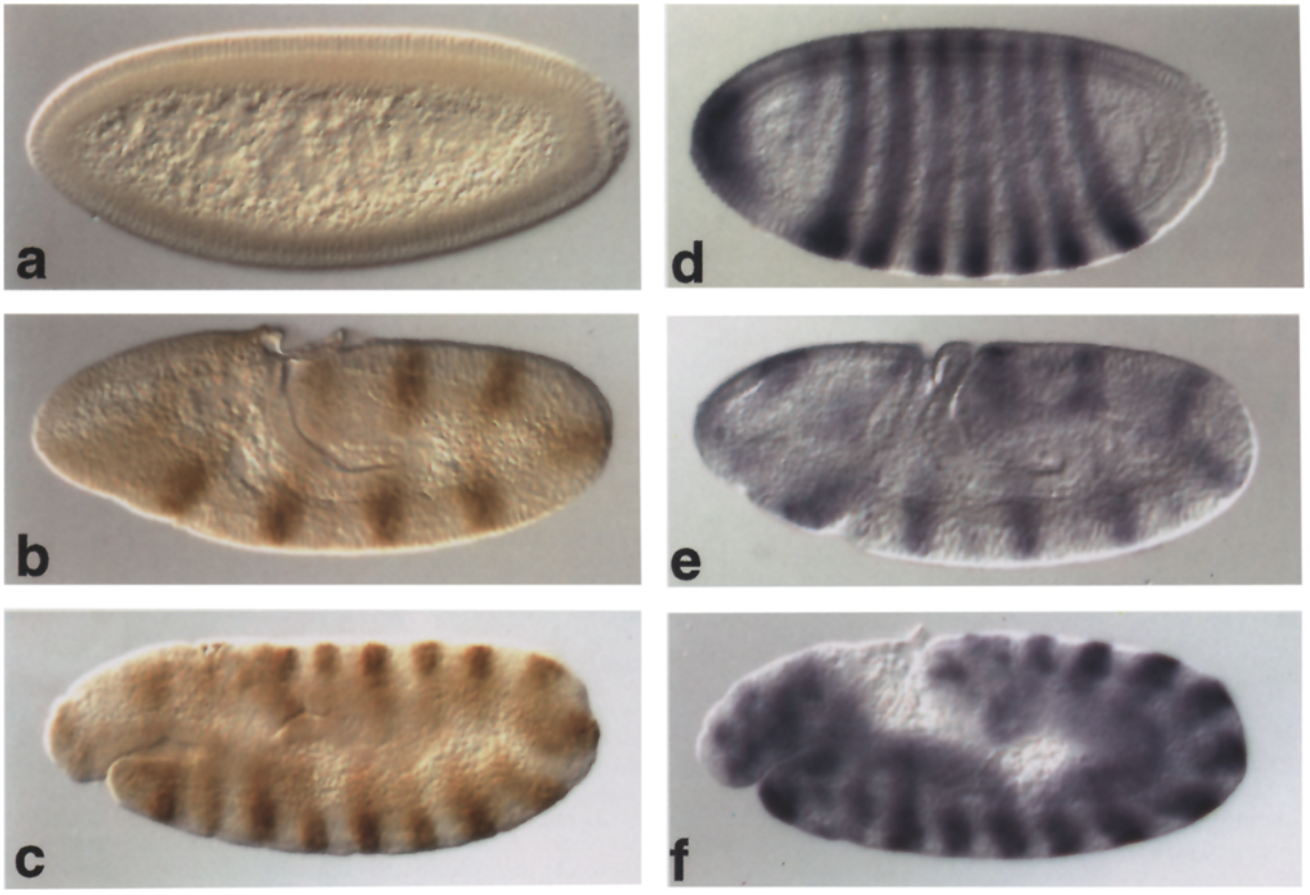

Fig. 1a–f. Enhancer-trap-dependent β-galactosidase and corresponding *ost* transcript expression patterns during cellular blastoderm and early gastrulation of *Drosophila* embryogenesis. **a–c** β-galastosidase expression of the 05279 enhancer-trap line visualized by anti-β-galatosidase antibody staining. Note the lack of β-galactosidase staining at the cellular blastoderm stage (**a**), the seven stripe pair-rule-type expression pattern at stage 9 (**b**) and the segment polarity-type expression pattern at stage 11 (**c**). **d–f** *ost* Expression visualized by in situ hybridization employing a digoxigenin-labeled (DIG-labeled) *ost* cDNA probe. Note an anterior cap expression domain and the pair-rule-type expression at cellular blastoderm stage (**d**), which extends into stage 9 (**e**), and the segment polarity-type expression pattern at stage 12 (**f**). Staging of embryos was according to Campos-Ortega and Hartenstein (1985); orientation of embryos is anterior to the left and dorsal side up

PBT and re-fixed in 1 ml of 4% formaldehyde, PBS solution for 20 min. Afterward, embryos were washed five times for 5 min each in 1 ml PBT, once for 10 min in 1 ml PBT:HybS (1:1) and once for 10 min in 1 ml HybS. After prehybridization (1 ml HybS, 60 min, 45° C in a waterbath), the prehybridization solution was replaced by 40 µl HybS containing 0.1 vol. DIG-labeled probe and 0.2 vol. biotin-labeled probe (see above), which were denatured by boiling (10 min). After hybridization (overnight at 45° C in the waterbath) embryos were washed once for 10 min with 1 ml HybS at 45° C, followed by washes in HybS, HybS:PBT (1:1) and five washes in PBT (1 ml each, 15 min, room temperature). After the washes, pre-adsorbed anti-DIG antibodies coupled to alkaline phosphatase (dilution 1:2000) and anti-biotin antibodies coupled to peroxidase (dilution 1:1000) in 500 µl PBT were added and embryos were incubated for 2 h at room temperature followed by five washed in PBT (1 ml for 10 min each). The peroxidase reaction was developed for 30–60 min by incubating embryos in 1 ml PBT containing 0.25 mg/ml diaminobenzidine in a staining dish after adding 5 µl of H_2O_2 solution (1:100 in PBT). The peroxidase reaction was stopped by washing the embryos twice for 5 min and four times for 10 min in 1 ml PBT after transferring them back into an Eppendorf vial. Before the alkaline phosphatase reaction was initiated, embryos were washed three times for 10 min in freshly prepared alkaline phosphatase buffer (AP_e buffer, 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris, pH 9.5, 1 mM levamisol, 0.1% Tween 20). After the last washing step, embryos were transferred into a staining dish in 1 ml AP_e buffer and the alkaline phosphatase reaction was started by adding 4.5 µl NBT and 3.5 µl X-phosphate (Boehringer Mannheim) to the embryos. The reaction was stopped by washing the embryos three times for 5 min with PBT, followed by dehydration in an ethanol series (twice for 10 min in 70% ethanol, twice for 10 min in 100% ethanol). Embryos were mounted in GMM (Lawrence et al. 1986).

Antibody staining. Antibody staining of whole mount embryos of the P-insertion line 05279 were carried out as described recently (Macdonald and Struhl 1986) using the Vectastain ABC Elite horseradish peroxidase system.

Results and discussion

Reporter gene expression of the 05279 enhancer trap and cloning of the endogenous transcription unit nearby

The P-element based enhancer-trap line 05279 of *Drosophila* originates from the Spradling collection (Karpen

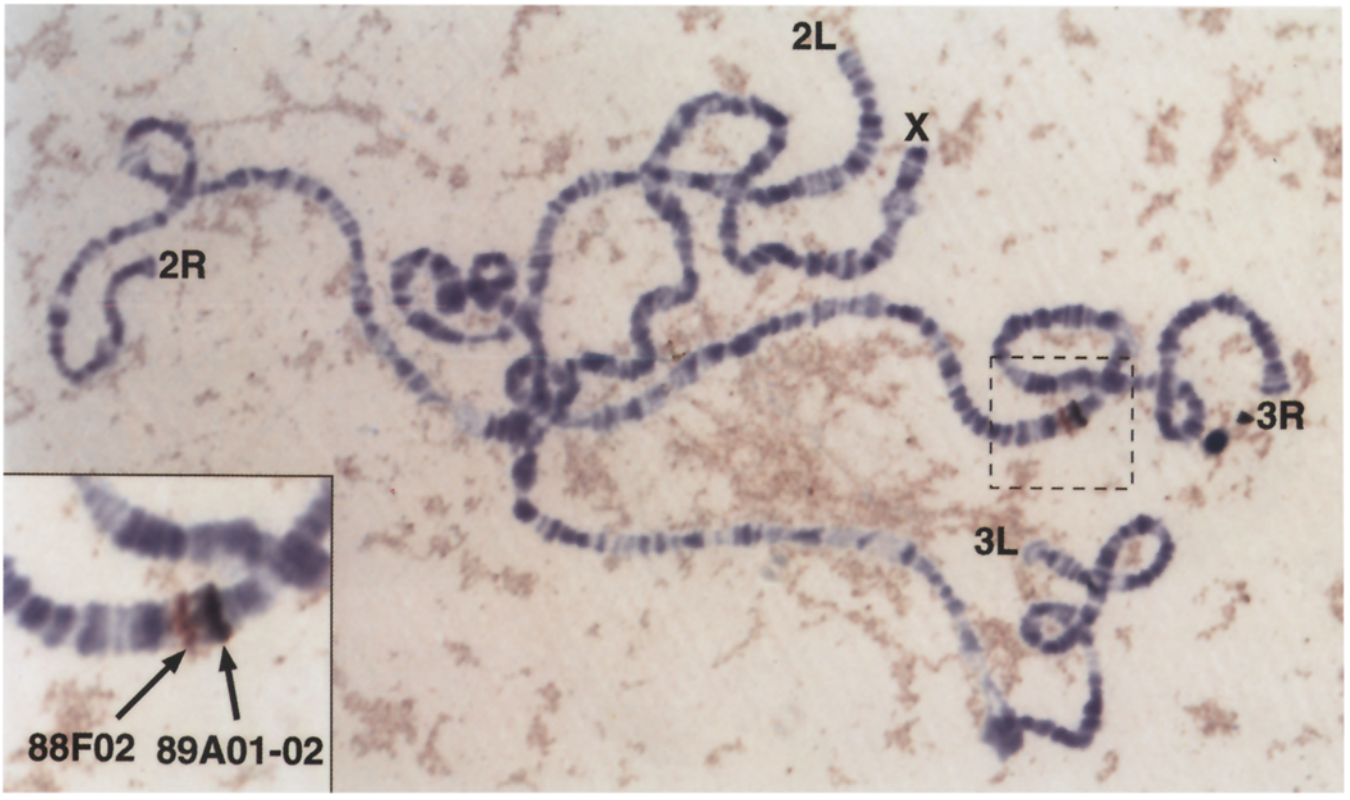

Fig. 2. Double-label in situ hybridization with *ost* and tropomyosin DNA probes on squashes of *Drosophila* polytene chromosomes. The *ost* DNA probe was labeled with biotin, and the tropomyosin DNA probe was labeled with DIG; enzymatic reactions were carried out (Materials and methods) to yield brown and blue reaction products, respectively. After embedding in Permount, the colors change from brown to black and from blue to purple. The telomeric regions of giemsa-counterstained chromosomes are labeled to designate the different chromosome arms (*X*, *2R* and *2L*, *3R* and *3L*; chromosome *4* is attached to the centromere, bridging the X chromosome and chromosome arm *3R*). The *inset* shows an enlarged region of the chromosome corresponding to the *dotted square*, indicating the position of the tropomyosin gene previously mapped to the 88F2 band (Karlik et al. 1984; purple signal) and the location of the *ost* transcription unit at position 89A1-2 (black signal)

and Spradling 1992). In order to visualize the expression pattern of the *lacZ* reporter gene inherent to enhancer-trap lines, we stained whole mount embryos with anti-β-galactosidase antibodies during various stages of development. The β-galactosidase expression pattern observed in embryos suggests that the enhancer trap participates in the the *cis*-acting control of an endogenous gene that is initially expressed during gastrulation (Fig. 1a, b). The spatial aspects of the expression pattern, which include a switch from 7 to 14 stripes along the anterior-posterior axis, suggest a nearby endogenous gene that is transcribed in a pattern suggestive of pair-rule/segment polarity genes. In order to clone this endogenous gene and to analyze further the spatial and temporal aspects of its expression patterns in the developing embryo, we performed plasmid rescue cloning to harvest DNA sequences adjacent to the enhancer-trap insertion site (Montell et al. 1992). As will be described elsewhere, we identified the corresponding DNA fragment, which served as an entry point to the identification of a transcription unit, which was termed *ost* on the basis of its expression pattern (see below). *ost* codes for a primary transcript ot at least 22 kb and for at least two differentially spliced mRNAs of about 3.5 and 6.3 kb. The sequence of the smaller transcript has been established (C.H., unpublished result), but the putative 525 amino acid protein shares no significant sequence similarity with known proteins and lacks diagnostic protein motifs. Thus, we were unable to group the putative gene function with respect to a biochemical function.

In situ localization on polytene chromosomes involving an internal position marker as reference

The 05279 enhancer-trap line contains a single P-element insertion, which was tentatively mapped to the 89A region on the right arm of the third chromosome (not shown). In order to confirm that the DNA of *ost* originates from the corresponding chromosomal region, we performed double-label in situ hybridization experiments using a DIG-tagged DNA probe of the *ost* transcription unit and a biotin-labeled DNA probe of the tropomyosin gene (for details see Materials and methods), which was localized in the 88F1,2 chromosome region (Karlik et al. 1984). After hybridization to squashes of polytene chromosomes, the DIG- and biotin-labeled probes were processed, resulting in purple alkaline phosphatase and

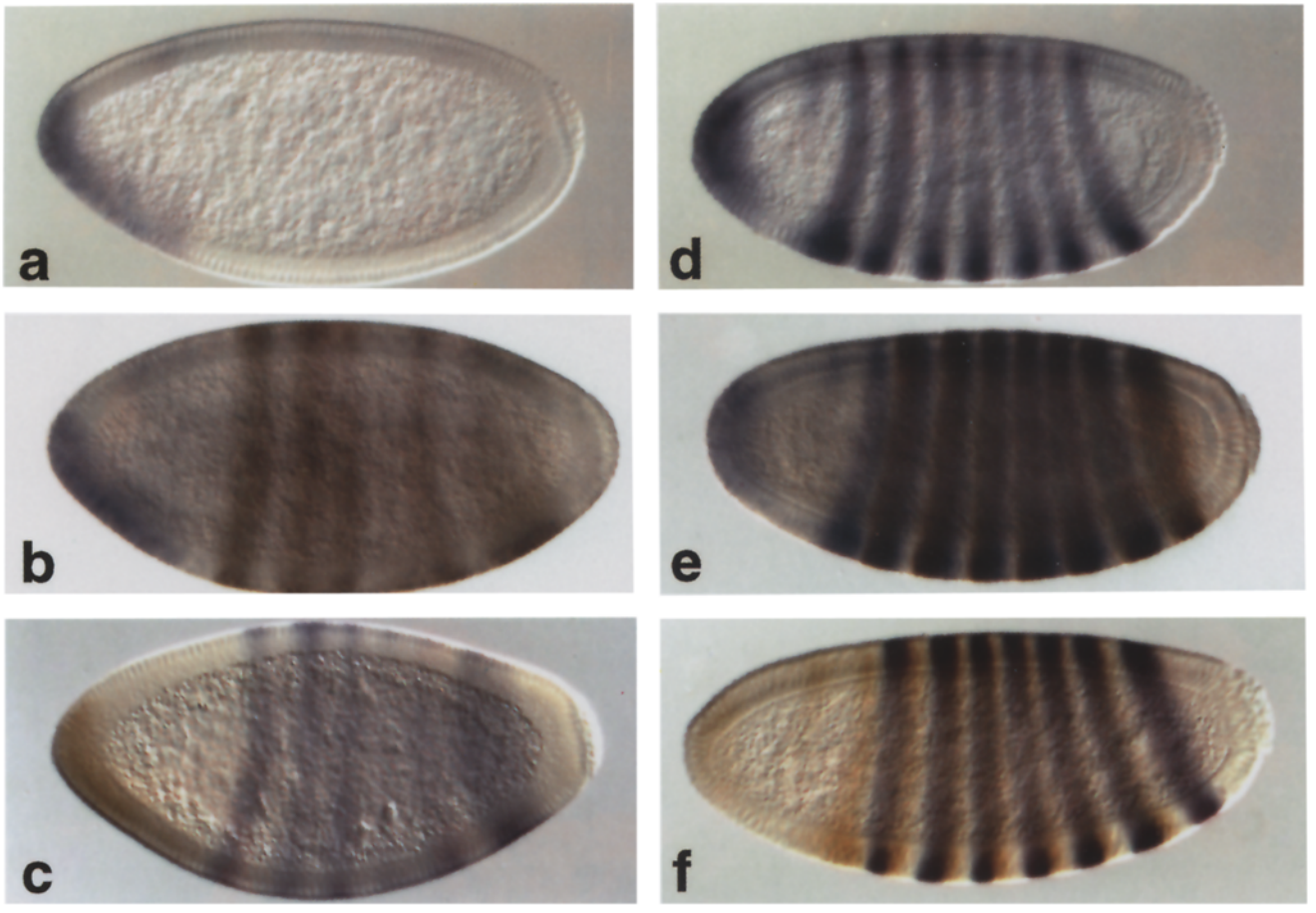

Fig. 3a–f. Simultaneous visualization of *ost* and *ftz* expression patterns during early and late blastoderm stages by whole-mount double-label in situ hybridization. **a–c** Early blastoderm stage (13th zygotic nuclear division cycle). Initial *ost* expression as visualized by a DIG-labeled *ost* cDNA probe is found in an anterior cap expression domain (blue signals in **a**, **b**) when the *ftz* transcripts have already formed stripes 1–3, 5 and fused stripes 6, 7 (brown signals due to the biotin-tagged *ftz* cDNA probe detection in **b**). Note the same expression patterns when the *ost* cDNA probe was labeled with biotin and the *ftz* cDNA probe with DIG (**c**). **d–f** Late blastoderm stage (after 14th zygotic nuclear division cycle). Note the anteriorly restricted anterior cap domain of *ost*, and its vague expression in an anterior "half-stripe" which is in front of the first of the seven pair-rule-type stripes of *ost* expression (**d**). Simultaneous visualization of *ost* and *ftz* expression (probes were labeled as in **b**) shows that the apparently evently spaced stripes of *ost* expression assumed on the basis of single probe in situ hybridization are in fact not evenly spaced as described in the text (**e**). Note the same expression patterns when the probe labeling was reversed as in (**c**), but the peroxidase reaction was reduced (**f**). For details on the spatial and temporal differences between *ost* and *ftz* expression see Fig. 4). Staging of embryos was according to Campos-Ortega and Hartenstein(1985); orientation of embryos is anterior to the left and dorsal side up

black peroxidase reaction products, respectively. Note that the color changes from normally blue alkaline phosphatase and brown peroxidase reaction products are due to the embedding medium Permount used to seal the chromosome squashes prior to the photomicrographs shown in Fig. 2 being taken. This color difference was found to be optimal for distinguishing signals within adjacent chromosome bands. Figure 2 shows that *ost* and the reference gene tropomyosin (Karlik et al. 1984) can be clearly distinguished by the color code and that *ost* is located within the band interval 89A1,2.

Embryonic expression patterns of ost transcripts

As shown in Fig. 1d, e, the expression patterns of *ost*, as revealed by in situ hybridization with a DIG-labeled cDNA probe, differ from the β-galactosidase staining pattern of the 05279 enhancer-trap line (Fig. 1a–c), since *ost* transcripts can already be observed at cellular blastoderm. This difference in the temporal appearance of *ost* transcripts and reporter gene-derived β-galactosidase might be attributed to the fact that transcripts accumulate faster to detectable levels than the protein product β-galactosidase and/of to the different sensitivities of the two techniques applied. In addition, the anterior cap domain of *ost* expression has no correspondence in the β-galatosidase expression patterns, suggesting that the latter does not receive regulation by the respective *ost cis*-acting element. Furthermore, the additional sites of *ost* expression in the head region of the gastrulating embryo (Fig. 1f), which are also not clearly discernible in the enhancer-trap expression pattern (Fig. 1c), suggest that *ost* may encode a novel segment polarity gene. However,

since the enhancer-trap insertion did not generate an embryonic lethal mutation or alterations in the segment pattern of homozygous embryos (C.H., unpublished result) and no segmentation gene has been localized within the 89A1,2 region of the third chromosome (Jürgens et al. 1984; Lindsley and Zimm 1992), the functional significance of the *ost* expression patterns in the embryo remains to be shown.

Spatiotemporal relationships between ost and ftz expression as revealed by double-label in situ hybridization

In order to examine the spatial and temporal relationships between *ost* expression and the known patterns of the pair-rule gene *ftz* and the segment polarity gene *wg*, respectively, we used double-label in situ hybridization with DIG- and biotin-tagged cDNA probes following the protocol described in Materials and methods. As shown in Figs. 3 and 4, this method allows the simultaneous visualization of two expression patterns in different colors in the same specimen, and although both probes reveal cytoplasmic staining patterns, the method appears sensitive enough to distinguish between two patterns including those occurring in overlapping regions.

As revealed by the DIG-labeled *ost* probe, initial expression of the transcript occurs in the anterior cap region after the 13th zygotic nuclear division cycle (Fig. 3a). At this stage of development, when the plasma membranes begin to separate the syncytial nuclei at the periphery of the embryo (Foe and Alberts 1983; Campos-Ortega and Hartenstein 1985), *ftz* stripe expression (Hafen et al. 1984) is already seen as indicated by simultaneous detection of *ftz* expression with biotin-labeled cDNA (Fig. 3b). In order to ensure that the temporal difference between *ost* and *ftz* stripe expression is not due to differences in the sensitivity of the two differently tagged cDNA probes, we repeated the staining, employing probes containing the reverse label with virtually the same result (Fig. 3c).

Once the anterior cap retracts at its posterior margin, the seven stripes of *ost* expression become apparent during the 14th nuclear division stage (Fig. 3d). Based on the double-label in situ hybridization patterns shown in Fig. 3e and f, which show embryos at corresponding stages stained with *ftz* and *ost* cDNA probes tagged either way with DIG or biotin, it became apparent that the spacing of the *ost* stripes is not as regular as that of the *ftz* stripes. This means that the anterior-most first *ost* stripe is located two cells in front of the first *ftz* stripe, while the second stripe is directly adjacent to the first *ftz* stripe. In contrast, *ost* stripes 3 through 7 overlap with their anterior borders by one cell with the posterior borders of the corresponding *ftz* stripes. In order to document this overlap unambiguously, we examined double-stained embryos at an early stage of zygotic nuclear cycle 14. At this stage, *ost* is just at an initial phase of being transcribed. Due to the comparatively long size of the *ost* primary transcript of at least 22 kb, the transcript staining of *ost* is seen in a punctate pattern in the nuclei

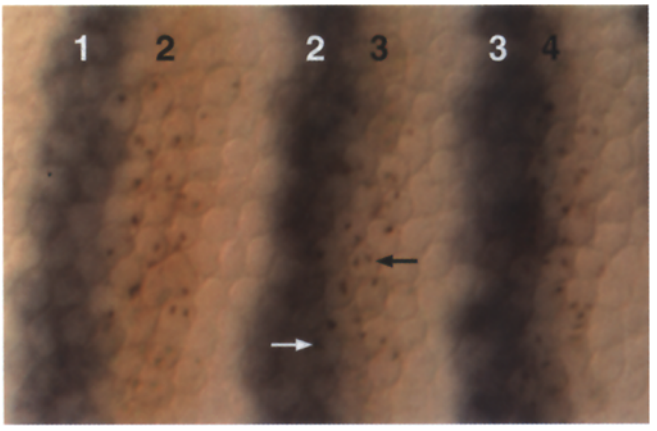

Fig. 4. Simultaneous visualization of *ost* and *ftz* expression at the early 14th nuclear division at the blastoderm stage. *ftz* expression is visualized by a DIG-labeled *ftz* cDNA probe (blue signal); *ost* expression corresponds to brown signals owing to use of a biotin-tagged *ost* cDNA probe. *ftz* stripes are designated by *white numbers* and *ost* stripes by *black numbers*. Note that the biotin-labeled probe is sensitive enough to detect nascent *ost* transcripts in the nuclei. Examples where the two chromosomal sites of *ost* transcription can be seen in the same focal plain are indicated by a *black arrow*. Furthermore, due to nuclear *ost* and cytoplasmic *ftz* staining (for reasons described in the text), the borders of *ftz* and *ost* expression can be unambiguously identified as indicated by *white arrows* marking the anterior-most *ost* expressing cell row of *ost* stripe 3. Orientation of the embryo is anterior to the left and dorsal side up

while the short 2 kb transcript of *ftz* (Kuroiwa et al. 1984) already accumulates in the cytoplasm. Figure 4 shows that double-label in situ hybridization is sensitive enough to discriminate between nuclear and cytoplasmic staining patterns, which confirmed the positioning of *ost* expression domains relative to the *ftz* stripe pattern.

Spatial relationships between ost and wg expression

In order to establish the spatial relationships of the *ost* segment polarity-like stripe pattern, we first examined whether the double-labeling protocol is suitable for distinguishing between the known segment polarity gene expression patterns of *en* and *wg*. Figure 5a–c shows the simultaneous visualization of *en* and *wg* expressing cells on the ventral side of stage 9 and stage 12 embryos (embryonic stages according to Campos-Ortega and Hartenstein 1985, respectively. At stage 9, the two genes are expressed in adjacent rows of cells, with *wg* in the anterior and *en* in the posterior row as has been described earlier (Baker 1987; Ingham et al. 1985; Kornberg et al. 1985). While the posterior row of *en* expressing cells labels the posterior border of the newly formed segments at stage 12, *wg* restricts its expression to become prominent in a group of ventrolateral cells that include part of the developing imaginal discs in the thoracic region (reviewed by Cohen 1993) (Fig. 5b, c). *ost* expression at stage 9 occurs in a broader and more diffuse ring of cells that gradually fades from the position of the *wg* expressing cells anteriorly (Fig. 5d), and it demarcates the ante-

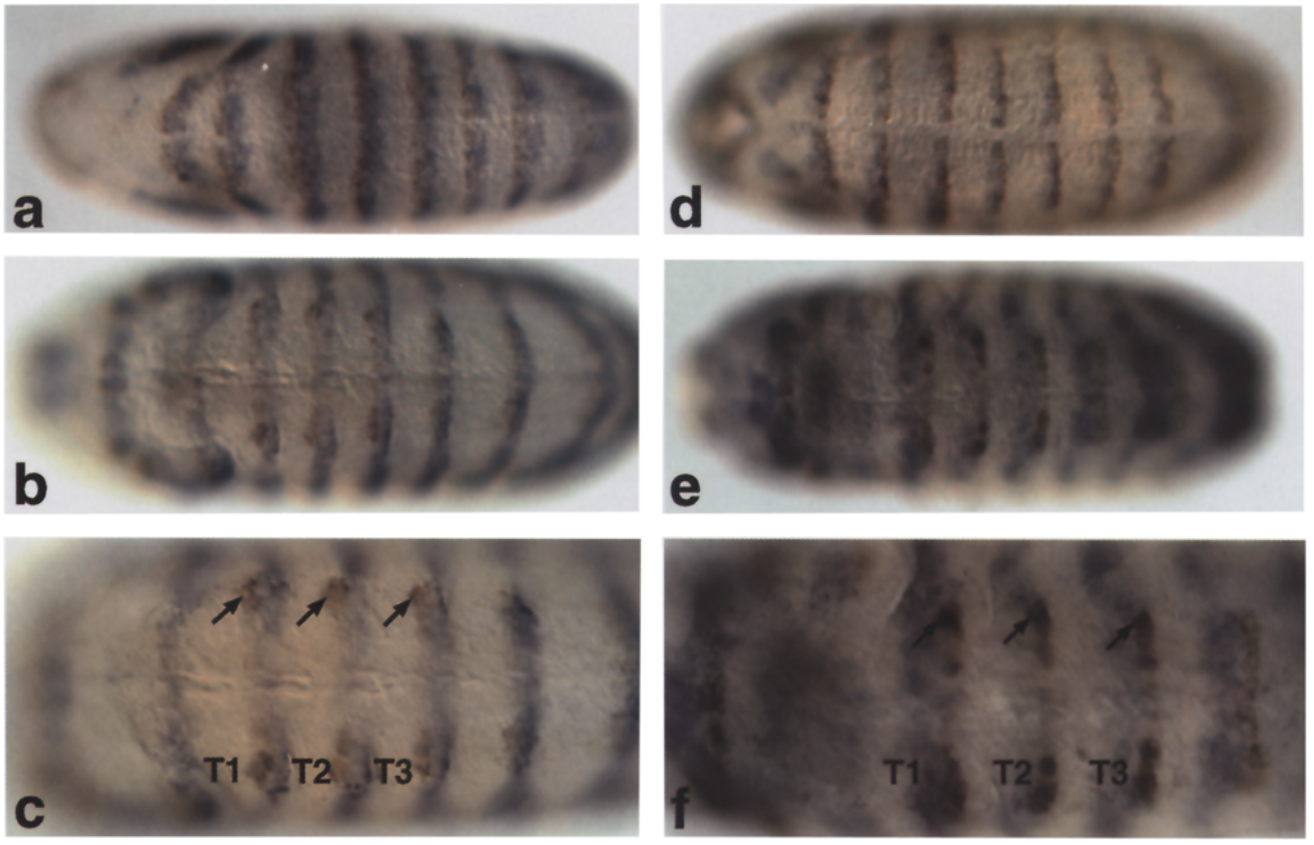

Fig. 5a–f. Simultaneous visualization of *wg* and *en* expression (**a–c**) and *ost* and *wg* expression (**d–f**) during early gastrulation. **a–c** *wg* staining in brown, and *en* expression in blue; **d–f** *wg* staining in brown, and *ost* expression in blue. Stage 10 (**a**, **d**) and stage 12 (**b–f**). Note that the spatial relationship between *wg* and *en* expression (*wg* is anteriorly adjacent to *en* expression) are as previously described (Martinez Arias et al. 1988), and that *ost* expression expands from the *wg* expression domain to the anterior margin of each segment as judged by morphological criteria (**e**, **f**). Restriction of *wg* expression and the overlap of *ost* and *wg* expressing cells in the thoracic imaginal disc anlagen is shown in the enlarged stage 12 embryos in **c** and **f** (*arrows*). T1-3 refer to thoracic segments 1–3. Staging of embryos was according to Campos-Ortega and Hartenstein (1985). Ventral view of embryos; anterior pole is the left

rior border of each segment at stage 12(Fig. 5e, f). At this stage, *wg* and *ost* expressing cells overlap in cells that eventually give rise to the imaginal discs of the thorax, while the *ost* and *wg* expressing cells are adjacent to each other in the abdominal segments (Fig. 5f).

Conclusions

We provide evidence that the double-label in situ hybridization protocol described here is suitable for localizing two distinct DNA sequences in chromosomes and visualizing the spatiotemporal patterns of two different RNA species simultaneously in the same specimen. Applying this method to whole-mount *Drosophila* embryos, we were able to demonstrate that a newly identified transcription unit with an as yet unknown molecular and genetic function exerts expression patterns during early embryogenesis. The results suggest that the *ost* gene product may therefore be required to function as a pair-rule or segment polarity gene, a proposal that needs to be confirmed by future functional studies. The detection of punctate patterns in the nuclei, which were recently shown to represent nascent transcripts (Shermoen and O'Farrell 1991; Rothe et al. 1992), suggests that the sensitivity of biotin-tagged DNA probes is sufficiently high for the in situ detection of minor transcripts.

The enzymatic reactions used to detect the biotin (peroxidase) and DIG (alkaline phosphatase) labeled probes are developed sequentially. Thus, it should be possible to adjust the relative intensity of the second reaction product so that the signals can be distinguished from the signals of the first staining reaction. In extreme cases, where two transcripts differ with respect to their relative abundance, it appears best to synthesize the biotin-labeled probe with the more abundant transcript and/or to vary the amount of each probe used to balance the signal from the abundant transcript with that of the rare one. We have not yet compared the sensitivity of the protocol described here with recently published double-label in situ hybridization with DIG-, fluorescein- and biotin-labeld RNA probes (Jowett and Lettice 1994; Hauptmann and Gester 1994; O'Neill and Bier 1994), but we expect them to be more sensitive. On the other hand,

the protocol described here is faster once the hybridization probes have been prepared, and they can be used in parallel for chromosome and whole mount in situ hybridization as well as for filter hybridization.

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