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The *Drosophila* SRF homolog is expressed in a subset of tracheal cells and maps within a genomic region required for tracheal development

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SUMMARY

The *Drosophila* homolog of the vertebrate serum response factor (SRF) was isolated by low stringency hybridization. Nucleotide sequence analysis revealed that the *Drosophila* SRF homolog (DSRF) codes for a protein that displays 93% sequence identity with human SRF in the MADS domain, the region required for DNA binding, dimerization and interaction with accessory factors. The DSRF gene is expressed during several phases of embryonic development. In the egg, both the RNA and the protein are maternal in origin and slowly decrease in amount during gastrulation. After germ band retraction, high levels of zygotic expression are observed in a distinct subset of peripheral tracheal cells distributed throughout the embryo. Many of these cells are at the tip of tracheal branches and are in direct contact with the target tissues. The DSRF gene was mapped to position 60C on the second chromosome, and overlapping deficiencies which remove the gene were identified. Analysis of tracheal development in embryos carrying these deletions revealed a degeneration of most of the major branches of the tracheal system. Although the initial migration of tracheal cells was not affected in those deficient embryos, many tracheal cells appeared not to maintain their correct position and continued to migrate. Thus, the DSRF gene might play a role in the proper formation and maintenance of the trachea.

Key words: serum response factor, cell migration, tracheal development

INTRODUCTION

Stimulation of cultured cells by serum, mitogens or purified growth factors results in the transient transcriptional activation of a large family of genes, including the proto-oncogenes c-*fos,* c-*jun,* c-*myc* and c-*rel*, without the need of prior protein synthesis. Many of these 'immediate early' genes code for transcription regulatory proteins the activity of which leads to subsequent changes in gene expression patterns. These events ultimately determine the response of cells to growth factor stimulation.

A common serum regulatory element (SRE) is found in the promoter regions of many growth-factor-stimulated immediate early genes (Treisman, 1992; Treisman and Ammerer, 1992). The SRE is both required and sufficient for a rapid and transient increase in the initiation rate of the associated transcription unit, following stimulation of cells with many growth factors. Activation of the SRE involves both protein kinase C-dependent and -independent signal transduction (Graham and Gilman, 1991), and is linked to the pathways involving *ras* and *raf* gene products (for references, see Marais et al., 1993). The mechanism of SRE function is thus of great interest both from the point of view of transcription regulation and with respect to nuclear interpretation of signal transduction cascades.

The vertebrate SRF is a DNA-binding protein that recognizes the SRE as a dimer and plays a major role in mediating signal transduction through this sequence element. SRF is a member of a novel class of transcription regulatory proteins and shares homology in its DNA-binding domain with the yeast MCM1 and ARG80 proteins (Norman et al., 1988). At SRE sites, SRF functionally cooperates with accessory proteins in a ternary complex, the activity of which appears to be modulated via growth factor-regulated phosphorylation (Marais et al., 1993; Hill et al., 1993; Zinck et al., 1993).

Despite the wealth of information available on the function of SRF in cell culture, relatively little is known about the role of SRF and SRF-related gene products during the development of an organism. In *Xenopus laevis*, an SRF homolog has been found to be expressed in the unfertilized egg and subsequently, at increased levels, during gastrulation (Mohun et al., 1991). In addition, the expression of two SRF-related genes (RSRFs) appeared to be restricted to the somitic mesoderm of the early embryo and subsequently to the body muscle of the tadpole (Chambers et al., 1992). As SRF-binding sites are present in many muscle-specific promoters and are required for their activity (Treisman, 1992; Treisman and Ammerer, 1992), the function of these *Xenopus* SRF-related genes might be the regulation of embryonic, muscle-specific transcription. In

contrast, both RSRF genes have been found to be expressed ubiquitously in the adult frog and might therefore play additional roles during this stage of the life cycle.

To investigate the role of possible SRF homologs during the development of *Drosophila melanogaster*, we have isolated SRF cross-hybridizing sequences from *Drosophila* genomic and cDNA libraries. The cDNA we characterized in detail can encode a protein that shows a high degree of sequence similarity to that of human SRF. The gene we studied may therefore represent the *Drosophila* homolog of SRF (DSRF). In addition to low levels of expression in all somatic muscles, the DSRF gene is turned on, upon germ band retraction, at high levels in a subset of cells of the peripheral tracheal system. The DSRF gene was mapped to position 60C on the second chromosome and two overlapping deficiencies removing several genes including DSRF were identified. Embryos carrying these deficiencies show distinct alterations in the tracheal system. DSRF might thus be important for the proper establishment of the respiratory system of *Drosophila melanogaster*. The identification of a SRF homolog in *Drosophila* should now help to elucidate the role of SRF in cell-cell interaction and signal transduction during development.

MATERIALS AND METHODS

General methods

Isolation of DNA from lambda phages and plasmids, restriction endonuclease digestions, gel electrophoresis and blotting of DNA onto nitrocellulose membranes were performed as described by Maniatis et al. (1982). Several genomic clones hybridizing to a 1090 bp *Eco*RI-*Bgl*II fragment of a human SRF cDNA clone pG3.5, a gift of Richard Treisman (Norman et al., 1988), were isolated from a genomic *Drosophila* library in lambda DASH II. Screening was performed under low stringency conditions (McGinnis et al., 1984). Using subcloned genomic DNA fragments as probes, two cDNA clones were isolated from a 3- to 12-hour embryonic cDNA library kindly provided by L. Kauvar. cDNA fragments were subcloned into Bluescript vectors (Stratagene) or m13 vectors. DNA sequences were determined by the procedure of Sanger et al. (1977) using nested deletions generated from a plasmid subclone.

Preparation of a DSRF-specific antiserum

A 666 bp *Bam*HI fragment of the DSRF cDNA (positions 447-1113, Fig. 1) was subcloned into the *Bam*HI site of pGEX1 (Smith and Johnson, 1988), a glutathione-S-transferase fusion vector. The resulting plasmid encodes a fusion protein of glutathione-S-transferase and DSRF sequences (amino acid positions 25-246). The fusion protein was purified according to the methods of Smith and Johnson (1988). Rats were immunized intracutaneously at multiple sites with 100 μg of fusion protein in incomplete Freud's adjuvants every 3 weeks.

Immunostainings, in situ hybridizations and microscopy

Embryos were immunostained according to standard procedures (Ashburner, 1989), followed by a secondary antibody conjugated with biotin or with alkaline phosphatase. Biotinylated secondary antibodies were revealed using the horseradish peroxidase ABC kit (Vectastain). In the case of double stainings, horseradish peroxidase stainings were performed after the alkaline phosphatase staining. The tracheal-specific antibody (55, identical to 84, see Klämbt et al., 1992) was kindly provided by Benny Shilo. The anti-crumbs monoclonal antibody (Tepass et al., 1990) was kindly provided by E. Knust. To visualize β-galactosidase expression, a monoclonal antibody (Promega) or a rabbit polyclonal antibody (Cappel) was used. For light microscopy, immunostained material was viewed in a Zeiss Axiophot compound microscope using differential interference contrast optics. For documentation, images were photographed on EKTAR 100 (Kodak) film. Embryos double stained with fluorescent secondary antibodies were analyzed by confocal laser microscopy with a Zeiss Axioplan inverted microscope incorporated on a Noran Odyssey videospeed confocal laser scanning microscope (clsm). Multiple optical sections from a Z-Series were combined to form composite images, which were stored in rewriteable magneto optical disks prior to artificial color processing with Adobe Photoshop software. In situ hybridizations to whole-mount embryos was performed as described by Tautz and Pfeifle (1989) with minor modifications (see Affolter et al., 1993). The entire DSRF cDNA was used as a probe.

Fly strains

The following fly strains were used for the analysis: Df(2R)*Px*, Df(2R)*Px*^{2} (here referred to as *Px* and *Px*^{2}) (Bloomington); H82 and LG19 (Klämbt et al., 1992).

Identification of mutant embryos

Unambiguous identification of mutant embryos was essential, especially to study the early effects of deficiencies on tracheal development. To identify homozygous *Px*^{2} embryos, we have used a β3-tubulin-specific antiserum kindly provided by R. Renkawitz-Pohl (see Gasch et al., 1988). *Px*^{2} removes the *β3-tubulin*-coding sequences and therefore does not react with the antiserum. For this and other mutations on the second chromosome, we also made use of a *CyO* chromosome that contained a hindgut/anal pad-specific *lac-Z* fusion construct (see Affolter et al., 1993).

Genomic Southern analysis

Genomic DNA for Southern blot analysis was prepared as described by Wilson et al., (1989). Control DNA was extracted from 200 *Sco/CyO* adult flies. To obtain homozygous *Px*^{2} genomic DNA we collected homozygous embryos as follows: *Px*^{2}/*CyO* virgins were crossed to OreR males, and the male heterozygous *Px*^{2} progeny were subsequently crossed to *Px*^{2}/*CyO* virgins. The progeny of this cross are viable except for the *Px*^{2} homozyous embryos, which do not hatch. Eggs were collected for 3 hours on grape juice plates containing a ring of yeast at the periphery and subsequently aged at 25°C for 24 hours. After hatching, the live larvae went into the yeast ring, which was discarded, and the unhatched embryos (*Px*^{2} homozygous in majority) were selected again with yeast on a new grape juice plate. Unhatched embryos were dechorinated before genomic DNA extraction.

For genomic Southern analysis, 5 μg of each DNA batch was digested with the indicated restriction enzyme and fractionated by agarose gel electrophoresis. Blots were prepared as described by Maniatis et al. (1982) and were prehybridized, hybridized and washed at high stringency, according to McGinnis et al. (1984). Probes were prepared using a random primed DNA-labeling kit (Amersham). The DSRF probe consisted of a 310 bp *Bgl*I DNA fragment located in the 5′ end of the cDNA (see Fig. 1). The *pdm-1* (Affolter et al., 1993) and *β3-tubulin* (pc60-1; Gasch et al., 1988) probes were prepared from linearized plasmid DNA.

RESULTS

Isolation and sequence of DSRF

To identify *Drosophila* genes that share sequence similarity to human SRF, we have screened *Drosophila* genomic and cDNA libraries with a DNA fragment of the previously isolated

human SRF cDNA under low stringency conditions (see Materials and Methods). As judged by restriction mapping of eight individual genomic phage inserts, only one class of phage was recovered in this screen. A DNA fragment containing the region of cross-hybridization between the human SRF cDNA and the *Drosophila* genomic DNA was subsequently used to screen a 3- to 12-hour embryonic cDNA library. Two cDNA clones with inserts of similar length and similar restriction maps were recovered. The slightly longer insert was subcloned and sequenced (Fig. 1). This cDNA clone can be translated into a single long open reading frame of 450 amino acids, starting with an ATG at position 376 and ending with a stop codon at position 1726.

Sequence comparison between the conceptually translated DSRF open reading frame and the amino acid sequence of human SRF revealed quite a striking degree of similarity. Within the conserved 55 amino acid 'MADS-box' motif (derived from MCM1-ARG80-Agamous-Deficiens-SRF; see Treisman and Ammerer, 1992), only two conserved amino acid replacements are found between human SRF and the

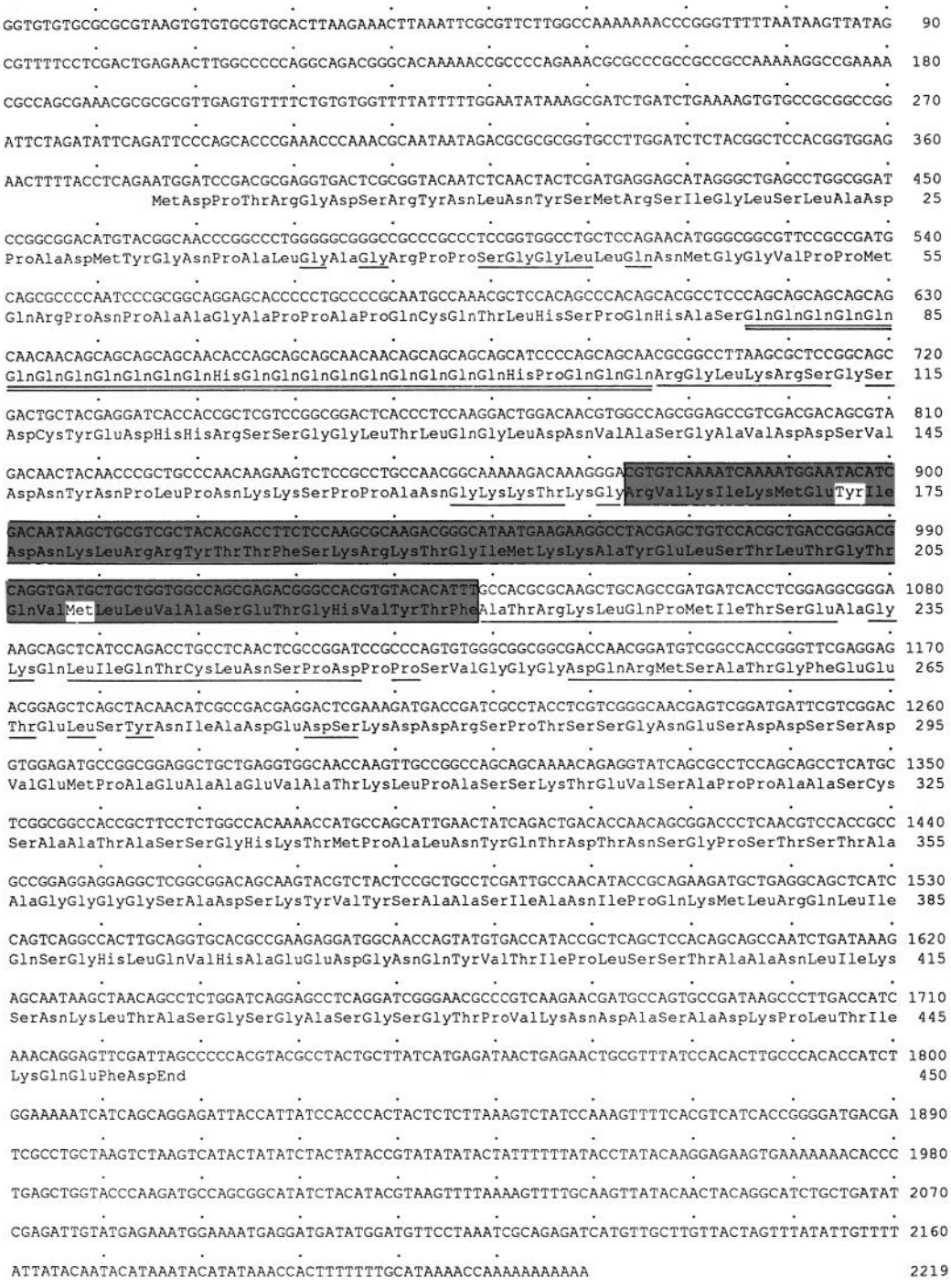
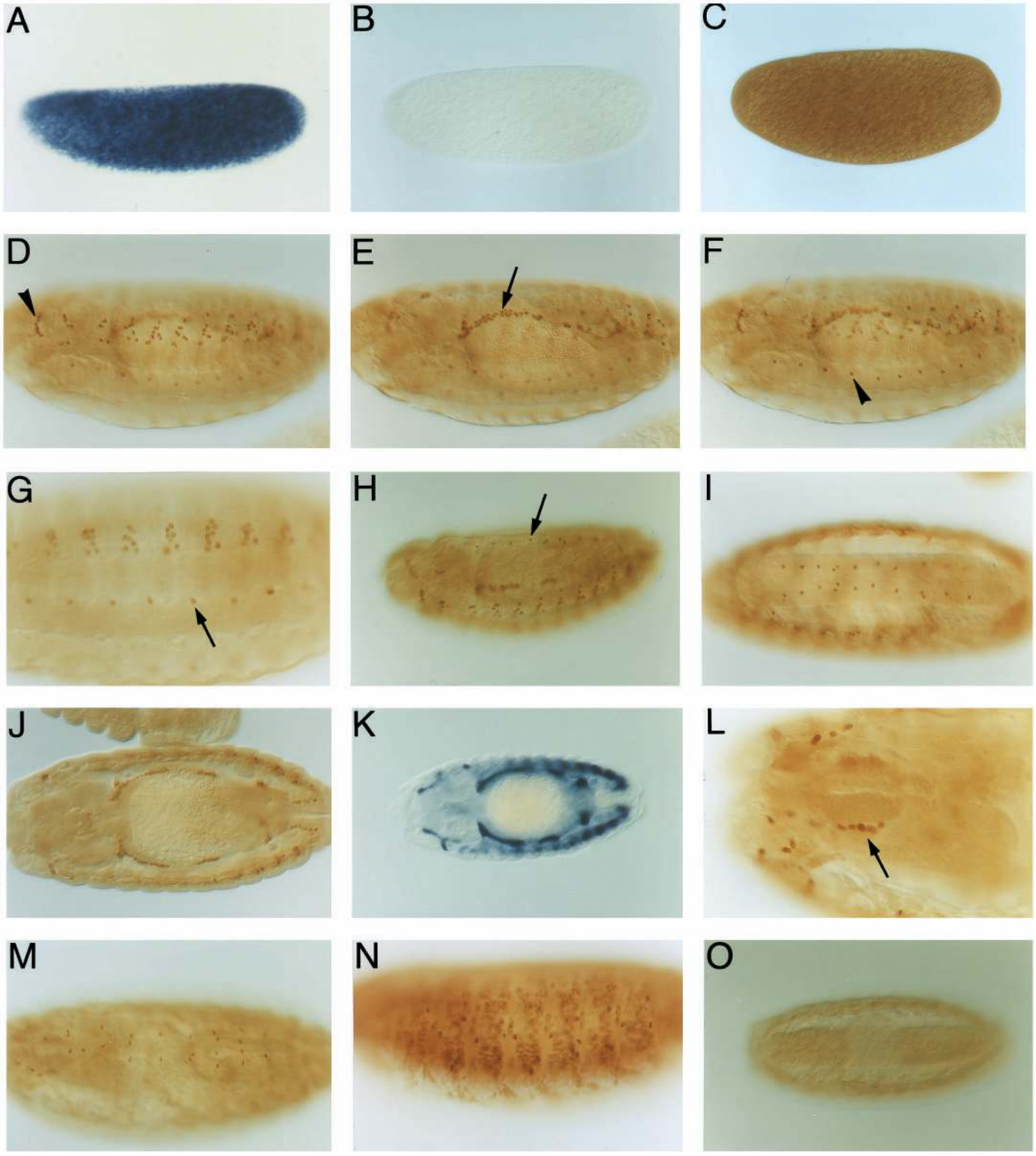


Fig. 1. Nucleotide and deduced amino acid sequence of DSRF. The nucleotide sequence of the cDNA clone lacking the *Eco*RI linkers is shown. At the 5′ end, the cDNA contains 13 C residues between the *Eco*RI site and the first nucleotide shown. The cDNA can only be translated into a single long open reading frame, the amino acids of which are shown below the nucleotide sequence. The region of the MADS box is shaded in grey; the two amino acids that differ between DSRF and human SRF in this box are not shaded. Amino acids that are identical in DSRF and human SRF in conserved regions outside the MADS box are underlined. A stretch of amino acids highly enriched in glutamine residues in DSRF is doubly underlined. A glutamine rich region also occurs in human SRF but is located C-terminal to the MADS domain, between amino acids 353 and 368 (Norman et al., 1988). At the amino terminus, DSRF and human SRF are identical at three out of four positions; the second amino acid is a leucine in human SRF. DSRF sequence data have been submitted to EMBL/GenBank under accession number X77532.

Drosophila cDNA we isolated (Fig. 1). C-terminal to the MADS-box, only three amino acid replacements are found in a stretch of 28 amino acids in a region of sequence similarity between human SRF, MCM1, ARG80 and our clone. In addition to this domain of 93% amino acid identity, the cDNA clone we isolated encodes a protein that shares additional smaller regions of similarity with human SRF (see Fig. 1 and legend).

There are a number of RSRF genes in vertebrate genomes which all differ substantially from the single copy SRF gene (Pollock and Treisman, 1991; Chambers et al., 1992). No invertebrate gene has thus far been isolated that shares as much



amino acid sequence similarity with human SRF as the *Drosophila* transcript described herein. Importantly, most of the biochemical and in vivo regulatory functions inherent to SRF and MCM1 (DNA binding, homodimerization, ternary complex formation with distinct accessory proteins) have been mapped to the region of high sequence similarity between SRF and the 450 amino acid *Drosophila* protein (Christ and Tye, 1991; Mueller and Nordheim, 1991; Primig et al., 1991; Bruhn et al., 1992). Considering these observations, we think that we have isolated the *Drosophila* homolog of the human SRF gene and therefore refer to the gene we characterized, thereafter, as DSRF.

Expression of DSRF

The embryonic expression pattern of DSRF was analysed using both in situ hybridization to whole-mount embryos (Tautz and Pfeifle, 1989) and immunolocalization with a DSRF-specific antiserum (see Materials and Methods). DSRF expression at the RNA and protein level are virtually indistinguishable (Fig. 2 and data not shown). Because it is easier to analyze and identify individual cells or cell types that express DSRF using the nuclear staining of the antiserum, in the following we primarily describe the protein expression pattern as revealed with the DSRF-specific antiserum (see also legend Fig. 2).

In unfertilized eggs, both DSRF RNA (Fig. 2A) and protein (Fig. 2C) can be readily detected and are uniformly distributed throughout the egg. This maternally provided pool of RNA and protein slowly disappears during cellularization and germ band elongation (data not shown). Strong zygotic expression of DSRF was found to resume only after the germ band had almost fully retracted. High levels of protein were detected in nuclei in a rather complex pattern of distribution throughout the embryo. Ten clusters of 6-9 cells were observed on the lateral anterior side of each hemisegment between the second thoracic and the eighth abdominal segment (Fig. 2D,G,H); only 3-5 cells were stained in the anterior part of the second thoracic segment). In addition, in each of the hemisegments T1 through A7, single DSRF-expressing cells were identified ventrally in the vicinity of the developing CNS (Fig. 2G) and dorsally in the vicinity of the amnioserosa (Fig. 2H; no dorsal cell was found in T1 and A7). The DSRF-expressing cells that are in close proximity to the condensing CNS in stage 13 embryos appear to be integrated into the CNS at later stages (Fig. 2I). In some hemisegments of the CNS, two nuclei expressed DSRF in these later stages. In stage 15 embryos, the dorsal-most DSRF antiserum-positive cells come to lie close to the dorsal midline (Fig. 2M).

Besides the relatively stereotypical pattern of DSRF expression in each hemisegment from T1/T2 to A7/A8 described above, additional cells in the embryo accumulate high levels of DSRF protein after germ band retraction. A row of cells approximately two cells wide was detected along the fused midgut (Fig. 2E,H,J). These cells are loosely attached to the visceral mesoderm (data not shown) and possibly represent cells of the visceral tracheal system (see below). Additional accumulation of nuclear DSRF was found in single cells of late stage 13 embryos (upon germ band retraction; Campos-Ortega and Hartenstein, 1985) in the head region (Fig. 2D,L), and in rows of nuclei along the pharyngeal muscles (Fig. 2L) and along the hindgut in the posterior end of the embryos (Fig. 2J).

In the embryonic stages 15 and 16, additional weak but consistent reactivity with the DSRF antiserum was detected in the nuclei of all somatic muscle cells (Fig. 2N). To ascertain that this staining did not represent cross-reactivity with other potential SRF-related antigens but reflected low levels of DSRF expression in somatic muscles, we have stained embryos in which the DSRF gene is deleted (see below). In those embryos, none of the aspects of the strong staining pattern revealed by the DSRF-specific antiserum was retained and the somatic muscles did not stain over background levels (Fig. 2O). We therefore conclude that DSRF is expressed strongly in a subset of cells throughout the developing embryo and weakly in all the somatic muscle nuclei.

Strong DSRF expression in a subset of tracheal cells

To determine the tissue type of those cells that accumulate high levels of DSRF, we have carried out a series of double labeling experiments using DSRF antiserum and antibodies that recognize known structures in the *Drosophila* embryo. The weak DSRF expression seen in all somatic muscles at late stages of embryogenesis prompted us to investigate whether certain of the strongly expressing cells represented a subset of muscle cells or precursors for the adult musculature (see, for example, Bate et al., 1991). Using antisera against β3-tubulin, muscle myosin and twist, we found that none of the cells containing high amounts of DSRF overlapped with cells recognized by these antisera (data not shown). The strongly DSRF antiserum-positive nuclei are therefore not located in a subset of somatic muscles or muscle precursors.

Fig. 2. DSRF is expressed in a complex pattern during embryogenesis. The embryonic expression pattern of DSRF has been analyzed using either whole-mount in situ hybridization with a DIG-labelled DSRF cDNA probe (A,K) or a DSRF-specific antiserum (C-O). The preimmune serum of the rabbit used to prepare the DSRF antiserum was used in the egg in B. Either unfertilized eggs (A-C) or wild-type embryos (D-O) were used for analysis. Both DSRF RNA (A) and protein (C) can readily be detected in unfertilized eggs. RNA and protein fade away during germ band extension and zygotic expression is only detectable upon retraction of the germ band. All the aspects of the strong pattern of DSRF expression appear at approximately the same time of development (data not shown). In D, E and F, the same stage 14 embryo is shown in three different focal planes. Just beneath the epidermis, a cluster of approximately 6-9 nuclei is visible in the most anterior portion of each hemisegment from T2 to A8 (D). Expression is also seen in the head (arrowhead in D). Along the midgut, a row of two adjacent nuclei expresses high levels of DSRF protein (arrow in E). In the same embryo, single positive nuclei (in some cases two nuclei) are observed in 10 hemisegments of the central nervous system (CNS) (arrowhead in F). These nuclei are located at the edge of the CNS in younger embryos (arrow in G) and appear to migrate into the CNS during the process of its condensation (I). One or two DSRF-expressing nuclei are also visible in the dorsal part of each hemisegment from T2 to A6 (arrow in H; M, in a stage 16 embryo). Along the pharyngeal muscle in the head, a row of approximately 6-8 nuclei express DSRF (arrow in L). During all embryonic stages, the distribution of DSRF protein closely matches the RNA expression pattern (compare J with K). Longer staining of stage 15 or older embryos reacted with the DSRF antiserum revealed a second pattern of expression of the DSRF gene. Staining is found in all the nuclei of the somatic musculature (N). In Px^2 homozygous embryos that lack the DSRF gene, all aspects of the described expression patterns (including expression in the somatic musculature) were absent (O).

As mentioned above, the DSRF-expressing cells along the midgut seen in Fig. 2 appeared to be associated with or to be part of the visceral tracheal system. To test this hypothesis, we have used a polyclonal antiserum that recognizes components secreted into the lumen of the tracheal tubes (antiserum 55; kindly provided by B. Shilo). We found that not only the cells along the midgut (Fig. 3A), but actually **all** the cells that accumulate DSRF to high levels are tightly associated with the tracheal system (Fig. 3A-F; see Manning and Krasnow (1993) for a comprehensive review on tracheal development). All the DSRF-positive nuclei in the CNS are in close proximity to the tracheal lumen, and ganglionic branches of the trachea only enter the CNS in places where DSRF-expressing cells occur (Fig. 3B). Each of the DSRF-positive nuclei in the head region was found to be intimately linked to the tracheal lumen as well (Fig. 3D-E) and the same holds true for all the cells in the posterior part of the embryo (data not shown). It therefore appears that all the cells that contain high levels of DSRF transcript and protein are part of the peripheral tracheal system. Intriguingly, however, none of the tracheal cells that compose the dorsal trunk accumulate DSRF (see Fig. 3A,C).

Recently, Hartenstein and Jan (1992) as well as Manning and Krasnow (1993) have argued that the embryonic tracheal system contains, as evidenced by differentially expressed enhancer trap lines, a number of different cell types; other cells have been found to be tightly associated with tracheal cells (so-called peritracheal cells; Hartenstein and Jan, 1992). Two enhancer trap lines (6-81a and H82) were identified (Bier et al., 1989) that showed *lacZ* expression at a high level in all the cells of the tracheal system, but not in peritracheal cells. In these enhancer trap lines, the P element had inserted into the gene coding for the *Drosophila* homolog of the fibroblast growth factor receptor (DFGF-R), which was independently found to be expressed in all the tracheal cells and to be required for tracheal cell migration (Glazer and Shilo, 1991; Klämbt et al., 1992).

To investigate whether the cells that express DSRF at high levels are indeed tracheal cells, or whether they represent peritracheal cells or cells of neighboring target tissues, we performed double labeling experiments using a rabbit anti-β-galactosidase antiserum (to identify the tracheal cells in H82 embryos) and the DSRF-specific rat antiserum. Using confocal microscopy, we found that all the DSRF-positive nuclei also accumulated β-galactosidase protein. The tracheal cell that invades the central nervous system (revealed by its reactivity with a fluorescein-coupled anti-β-galactosidase antibody, Fig. 4A) also expresses DSRF as demonstrated by the overlap of the fluorescein and the rhodamine labels (Fig. 4C). The other tracheal cells that make up the ganglionic branch do not express detectable levels of DSRF (Fig. 4B). All the DSRF-expressing cells located on the lateral side of the developing embryo (Fig. 4B) also express β-galactosidase and therefore are part of the tracheal system (Fig.

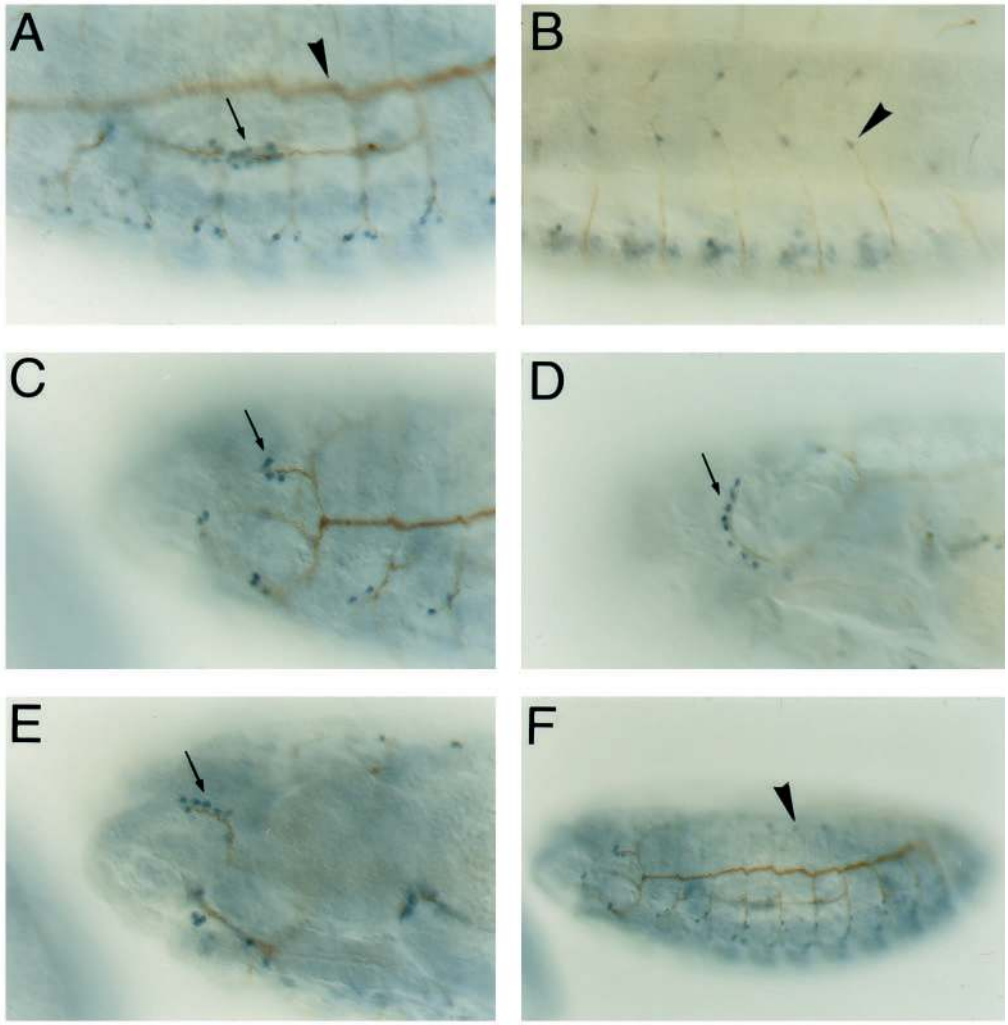


Fig. 3. DSRF is expressed in a subset of cells associated with the tracheal lumen. The tracheal system (brown) was visualized using a lumen-specific antiserum (see Materials and Methods). DSRF-expressing nuclei (blue) were revealed with the DSRF-specific antiserum and are clearly closely associated with the peripheral tracheal system (A-F). This is shown for some of the nuclei along the anterior midgut (arrow in A), for the nuclei in the CNS (arrowhead in B) and for the DSRF-expressing nuclei in the head (C-E). In C, the arrow points to the dorsal cephalic branch, in D to the ventral cephalic branch, and in E to the pharyngeal branch (for nomenclature used, see Manning and Krasnow, 1993). No association of DSRF antiserum-positive nuclei is seen along the dorsal trunk (arrowhead in A), and only the distal-most cell of the ganglionic branch (arrowhead in B) and of the dorsal branch (arrowhead in F and data not shown) accumulate high levels of DSRF.

4B,C). Inspection of other regions of the embryo revealed that the entire array of cells expressing high levels of DSRF overlapped with *lacZ*-expressing tracheal cells (data not shown). Therefore, the DSRF gene is indeed expressed in the subset of tracheal cells that build the embryonic respiratory system.

Cytogenetic location of the DSRF locus

To map the DSRF gene, biotinylated probes were prepared from genomic and cDNA clones and hybridized to *Drosophila* salivary gland polytene chromosomes. In each case, hybridization was observed at the distal tip of the right arm of the second chromosome at position 60C (Fig. 5A). To confirm the cytogenetic location of DSRF and to identify a deficiency which removes the DSRF gene, we examined the in situ hybridization pattern Px^2, in which chromosomal bands 60C5 to 60D10 are deleted (see Kimble et al., 1990). We found that in larvae heterozygous for this deficiency, only one of the parental chromosomes hybridized with DSRF-specific probes, whereas the corresponding part on the other chromosome was deleted (data not shown). To confirm that Px^2 indeed deletes the DSRF transcription unit (or at least part of it), embryos enriched in deficiency homocygotes were collected (see Materials and Methods) and the DNA used for Southern hybridizations. Genomic DNA isolated from Px^2 has a significantly reduced DSRF hybridization signal when compared to DNA isolated from control flies (Fig. 5D, compare lanes a and b). Px^2 also deletes the *β3-tubulin* gene (Kimble et al., 1990) and we found a decrease in the *β3-tubulin* hybridization signal similar to that observed with the DSRF-specific probe (Fig. 5C). The residual signal is presumably due to contamination of the embryo collection with non-deficiency embryos. In contrast, control DNA and Px^2 DNA contained similar amounts of DNA sequences complementary to *pdm-1*, a POU-box gene located at 33F on the second chromosome (Affolter et al., 1993). These studies confirm that Px^2 deletes both the *β3-tubulin* and the DSRF gene.

To further narrow down the chromosomal region harboring DSRF, we have analyzed a second partially overlapping deficiency mutant, *Px*, which deletes the 60C region. Using the DSRF-specific antiserum and marked *CyO* balancer chromosomes, we found that both Px^2 and *Px* homozygous embryos and the transheterozygous Px/Px^2 embryos did not express DSRF protein in either the tracheal system or in the somatic muscles (data not shown; see Fig. 2). This localizes the DSRF gene to the overlap between these two deficiencies and therefore to the region between 60C5/6 and 60D1/2 (see Lindsley and Zimm, 1992).

Deficiencies in the 60C region lead to disruption of major tracheal branches

The tracheal system develops from ten small bulges, the tracheal placodes, which form in lateral positions in segments T2 to A8 in stage 10 embryos (for a detailed descriptions of the development of the trachea, see Campos-Ortega and Hartenstein, 1985; Manning and Krasnow, 1993). During stages 10 and 11, the approximately 20 tracheal precursor cells undergo their second and third post-blastoderm mitoses, and then invaginate into the underlying mesoderm. By the end of stage 11, branches begin to bud. Without further cell divisions, the complex branching pattern of the tracheal system is established, mainly during stages 12 and 13 via cell migration and extension. It is only during the very late stages of the establishment of the basic pattern of the system that we found DSRF to be turned on zygotically (see Fig. 2D,E).

Tracheal defects in late stage 17 embryos lacking the chromosomal region 60C5 to 60D1 have been reported in a previous study (Kimble et al., 1990). To visualize the developing tracheal system in wild-type or mutant embryos of earlier stages, we have used both a lumen-specific antiserum (no. 55) and the anti-crumbs monoclonal antibody (Tepass et al., 1990; Klämbt et al., 1992; see Material and Methods). The branching pattern of a wild-type stage 14 embryo as visualized by the anti-crumbs antibody is shown in Fig. 6A and C. A schematic representation of three hemisegments of the tracheal system of a stage 14 embryo including a description of the nomenclature used is depicted in Fig. 7. Px^2 homozygous embryos already revealed striking alterations in the tracheal system of stage 14 embryos (Fig. 6B,D). The lumen of most of the dorsal branches (large arrows) and of all of the ganglionic branches (thin arrows) appears to be disrupted and residual *crumbs* expression (or antiserum 55 expression; data not shown) was detected only in the region of the tracheal cells expressing DSRF in wild-type embryos. Distinct defects were also seen in the transverse connectives and the lateral trunk,

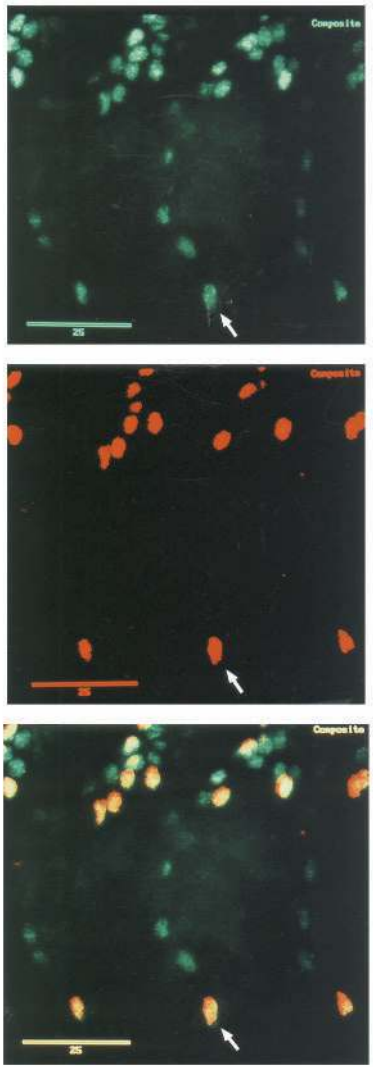


Fig. 4. DSRF is expressed in a subset of tracheal cells. H82 embryos were simultaneously incubated with a β-galactosidase antibody (A; green, revealed with fluorescein-coupled secondary antibodies) and the DSRF antiserum (B; red, revealed with rhodamine-coupled antibodies). Superimposition of the fluorescein and the rhodamine patterns revealed that all the DSRF-expressing cells also express β-galactosidase and therefore are part of the tracheal system; however, only a fraction of all the tracheal cells express DSRF. In the ganglionic branches, only the tip cells express DSRF (arrows in A-C). The embryo shown is in stage 14 of embryonic development.

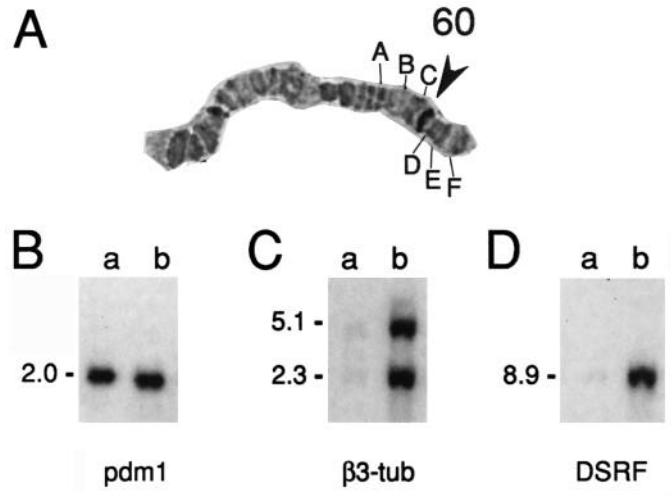


Fig. 5. Chromosomal location of the DSRF gene. (A) The single strong signal obtained after hybridization with the DSRF cDNA clone is located at the tip of the right arm of chromosome 2 at position 60C. (B-D) Southern blots of genomic DNA digested with *Eco*RI and probed either with a *pdm-1*, a *β3-tubulin* or a DSRF cDNA (see Materials and Methods). DNA was isolated from Px^2 homozygous embryos (lane a) or from *Sco/CyO* flies (lane b). B and D represent the same filter, which has first been probed with the DSRF cDNA and subsequently with the *pdm-1* cDNA.

resulting in a complete lack of connections between the dorsal and the lateral trunk, and the failure to build a lateral trunk. Defects were also detected in the head, where none of the terminal branches of the tracheal system were properly established in the mutant embryos; the tracheal branches along the gut were similarly disrupted (data not shown). In contrast to these dramatic defects, the dorsal trunk remained intact in embryos carrying these deficiencies (Fig. 6B). The tracheal phenotype described above was also seen in embryos transheterozygous for *Px* and Px^2 (data not shown) and is thus caused by the absence of a gene(s) located in the region between 60C5/6 and 60D1/2.

Deficiencies in the 60C region lead to defects in tracheal cell migration

To investigate whether and how tracheal cell migration is affected in the deficiency mutants, we crossed a chromosome, which harbors an enhancer trap insertion in the *Drosophila* FGF receptor (DFGF-R) gene, into the DSRF-deficient Px^2 background. DFGF-R is expressed in all cells of the tracheal system (Glazer and Shilo, 1991; Klämbt et al., 1992) and embryos containing the DFGF-R enhancer trap insertion chromosome show early and persistent accumulation of β-galactosidase immunoreactivity in all the tracheal nuclei. This provides a histochemical tag for following the migration pattern of tracheal cells during development in mutant embryos and for comparing it to the wild-type situation. Due to the complex pattern of the tracheal system, we have mainly focused on defects observed in the thoracic and abdominal region, in particular the dorsal and the ganglionic branches of the tracheal system.

Tracheal development appeared normal in mutant embryos until germ band retraction (stage 13), the time when zygotic DSRF expression is first detectable in the tracheal system (data not shown). In more than 90% of stage 14/15 mutant embryos, the 5-8 tracheal cells that presumably would have formed the dorsal branch in the wild-type situation, have migrated to positions close to the dorsal midline in the mutants (Fig. 6H). This abnormal migration behaviour is consistent with the tracheal phenotype seen with the crumbs antibody, which revealed the apparent absence of a dorsal branch except for an accumulation of crumbs in the dorsal portion of each hemisegment (see Fig. 6B). Similar accumulation of β-galactosidase-expressing cells in clusters were identified in the vicinity of the CNS (Fig. 6F,G), leading to the observed disruption of the ganglionic branch in embryos lacking DSRF expression (see Fig. 6D). The same phenotype with respect to the expression of the enhancer detector P element inserted into the DFGF-R gene was observed in Px/Px^2 transheterozygous embryos (data not shown). Again, and as already observed by the analysis of the mutants with the crumbs antiserum, cells of the dorsal trunk, the most anterior ventral anastomosis and the first dorsal branch were not affected (data not shown).

From the analysis of tracheal development in mutants lacking the chromosomal region 60C5/6 to 60D1/2, we conclude that this region contains one or several genes that are required for normal tracheal development. The requirement for genetic functions essential to the establishment of the tracheal network becomes apparent only upon completion of the main migration events involving tracheal cells (i.e. after the retraction of the germ band). It is manifested by a decay of a rather normal looking tracheal network because (at least in the case of the dorsal and the ganglionic branch) cells do not stop migration, but instead seem to follow the cell leading the tip of the outgrowing branch. Although the DSRF gene is the only gene characterized to date in this genomic region that is expressed in a specific subset of tracheal cells, we do not know yet whether its removal in *Px* and Px^2 is responsible for or contributes to the tracheal phenotypes observed in deficiency embryos (see Discussion). The analysis of DSRF-specific mutations will be required to answer this question definitively.

DISCUSSION

We have reported the isolation of a *Drosophila* gene that represents an apparent homolog of the human SRF gene. The putative DSRF protein is virtually identical in amino acid sequence to its vertebrate counterpart in the region which is required for most of its known biochemical functions. Human SRF and DSRF might thus serve in similar biochemical pathways in both vertebrates and invertebrates. Our analysis of DSRF represents a first step towards the elucidation of the developmental role of this highly conserved transcriptional regulator.

DSRF is expressed in cells of the peripheral tracheal system

The strong zygotic accumulation of DSRF in the nuclei of peripheral tracheal cells provides a novel and intriguing expression pattern. DSRF is turned on quite late during tracheal development, at a time when the main phase of tracheal cell migration towards target tissues nears completion. Many of the DSRF accumulating nuclei belong to those

tracheal cells that are closest to and contact directly the target tissues tracheated by particular branches. This is particularly evident for DSRF-positive cells in the CNS, as well as those found along the dorsal vessel, the pharynx and the mid- and hindgut. In the visceral branches, for example, only those cells that are in direct contact with the visceral mesoderm accumulate high levels of DSRF; the cells that make up for the connection between the visceral branches and the transverse connectives do not express DSRF (see Figs 2E,J, 6).

The localization of the lateral DSRF-expressing tracheal cells with respect to the tracheal lumen is shown schematically in Fig. 6. Many of these cells do not seem to be tightly associated with the transverse connectives or with the lateral trunk, but appear in positions in which finer branches form during development (the so-called E, F, G and H branches; see Manning and Krasnow, 1993). These branches are associated with single tracheal cells and also seem to be in direct contact with target tissues. Therefore, many of the DSRF-expressing cells seem to have in common their direct interaction with target tissues. It is thus conceivable that these cells receive information upon contacting the target tissues which is then reflected in the activation of the DSRF gene.

Potential role of DSRF in tracheal development

The striking expression pattern of DSRF in the tracheal system suggests a role for DSRF in the proper development of the respiratory system. Indeed, chromosomal deficiencies that remove the gene profoundly alter tracheal development leading to a degeneration of the tracheal system in stage 14 embryos. We cannot rule out the possibility that another gene(s) in the Px^2 deficiency is responsible for the tracheal phenotype we observed in the mutant embryos. However, there are certain striking correlations between the temporal and spatial expression pattern of DSRF in wild-type embryos and the tracheal defects observed in the deletion mutants. Defects in the tracheal system of deficiency embryos are first detectable upon germ band retraction, at a time point which coincides with the onset of strong DSRF expression in the tracheal system. None of the cells that form the dorsal trunk expressed DSRF and no

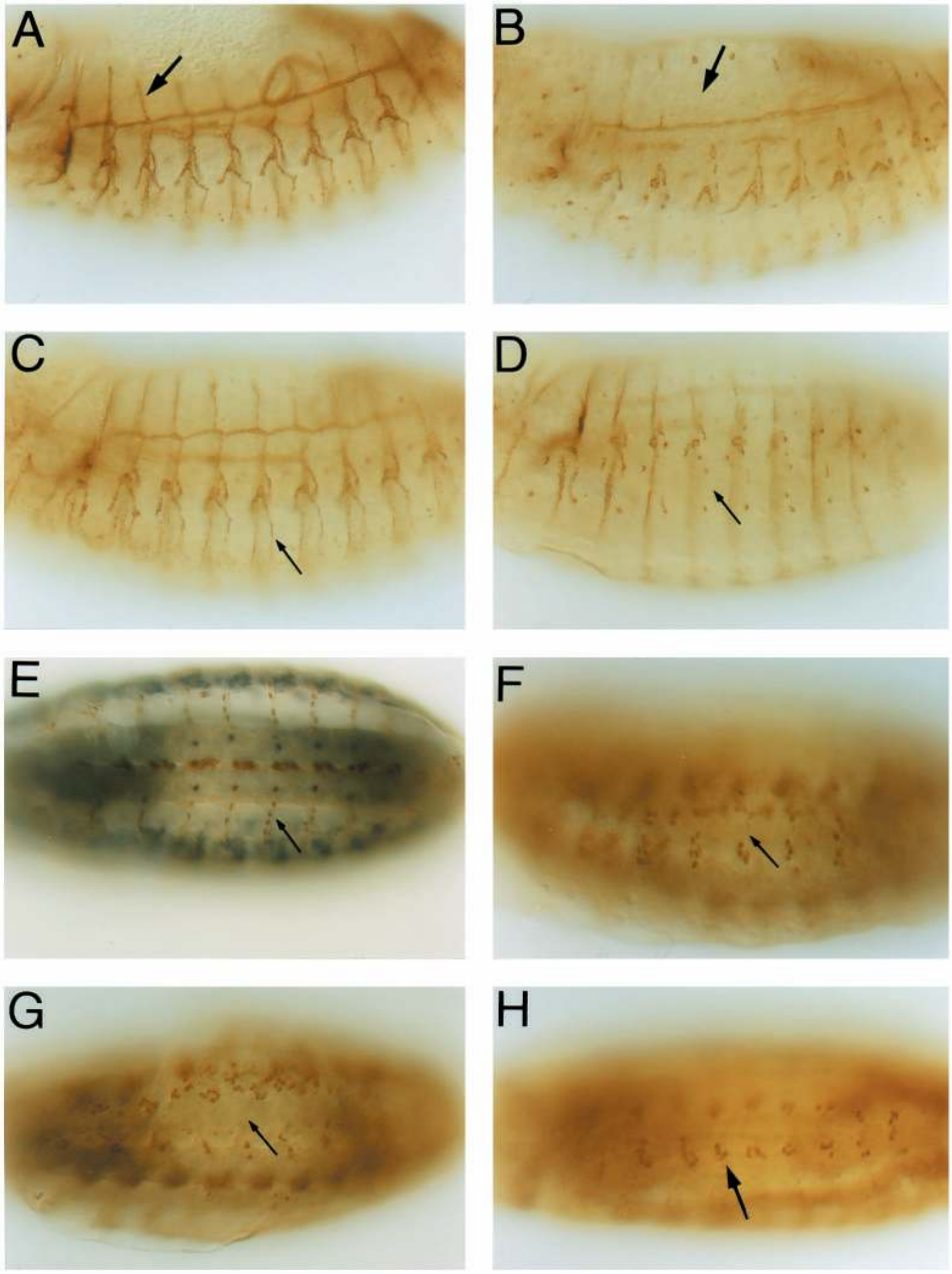


Fig. 6. Px^2 homozygous embryos show alterations in tracheal development. In panels A-D, the tracheal system of late stage 13, early stage 14 embryos is revealed with the crumbs monoclonal antibody. (A,C) Wild-type embryos; (B,D) Px^2 homozygous embryos. Clearly, the dorsal branch (fat arrow) and the ganglionic branch (thin arrow) are disrupted in the deficiency mutants. In E-H, the tracheal system of heterozygous H82 embryos is revealed with the anti β-galactosidase antibody (brown). In E, a control embryo is also stained for DSRF-expressing cells (blue). As shown in Fig. 4, all the DSRF-expressing nuclei overlap with β-galactosidase-expressing nuclei. The Px^2 homozygous embryos shown in F-H have also been incubated with the DSRF-specific antiserum and stained to reveal immunoreactivity, but lack DSRF expression due to the chromosomal deficiency. The ganglionic branch is disrupted in Px^2 homozygous embryos (thin arrow) and 3-5 cells cluster near the CNS (F,G). A dorsal view of a Px^2 homozygous embryo reveals the absence of cells lining up along the dorsal-ventral axis to form a dorsal branch (thick arrow in H). Instead, 4-6 tracheal nuclei cluster in close proximity to the dorsal midline.

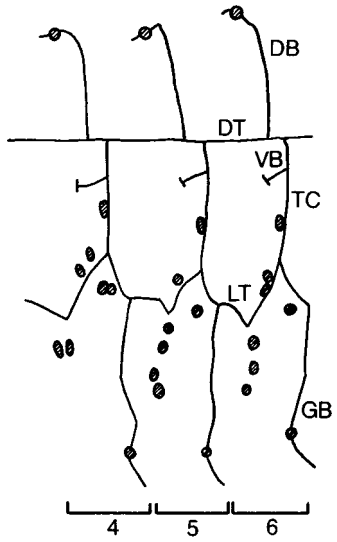


Fig. 7. Location of the lateral cluster of DSRF-expressing nuclei in three embryonic metameres. DSRF-positive nuclei are shown as hatched round areas. The drawing represents a stage 14 embryo stained with the lumen-specific antibody 55 and the DSRF-specific antiserum. The visceral branches (VB) are truncated. DB, dorsal branch; DT, dorsal trunk; TC, transverse connective; LT, lateral trunk; GB, ganglionic branch. Tracheal metameres 4 to 6 are shown. In addition to the lateral cluster, the single DSRF-positive cells in the tip of the ganglionic and the dorsal branch are also shown.

defects were observed in this prominent tracheal structure in the deficiency mutants we analysed (Fig. 2O,Q). No DSRF expression is seen in the most anterior ventral anastomosis as well as in the tip cell of the first dorsal branch, structures which are also not affected in the deficiency mutants (data not shown). In contrast, all the tracheal branches that contained DSRF-expressing cells were severely disrupted in the deficiency mutants, a situation most clearly evident in the dorsal and the ganglionic branches. However, both the lateral trunk and the transverse connections show defects, but most (or all) of the cells building these structures do not accumulate DSRF in their nuclei.

The analysis of the tracheal system in Px^2 mutants using a DFGF-R enhancer detector fly strain revealed that the tracheal cells **neighboring** DSRF-expressing cells in the wild-type situation did not properly stop their migration at the appropriate developmental stage, but rather appeared to follow the leading cell of the branch and eventually clustered in close proximity to the target tissue. It is conceivable that tip cells leading outgrowing branches receive specific information upon contacting the target tissue and communicate it to the following cells in order to arrest their migration. The phenotypes we observe in the deficiency embryos suggest that DSRF might be required to receive and/or transmit this information. Consistent with such a mechanism, DSRF is not turned on in the absence of tracheal cell migration towards target tissues, a situation found in embryos carrying a mutations in the DFGF-R or *pointed* genes (data not shown). It is of course possible that Px/Px^2 transheterozygotes remove another genetic function(s) which is required for tracheal development or that only a deletion of several loci results in the striking defects we observed. There exist several smaller deficiencies in the chromosomal region deleted in Px/Px^2 transheterozygous (e.g. C470; see Kimble et al., 1990) but none of those we have tested affected DSRF expression or showed tracheal defects (data not shown).

Irrespective of whether DSRF and/or other genes are responsible for the observed alterations, the tracheal phenotype itself is very intriguing and reveals something about the complexity of the genetic regulatory network essential for the establishment and the maintenance of the complex cellular network of the trachea. The two best-characterized genes required for tracheal development code for the DFGF receptor and for an ETS-domain containing putative transcriptional regulator *(pointed)* and play essential roles very early during tracheal development. In DFGF-R null mutations, tracheal placode formation is normal but no outgrowth of the major branches is observed (Klämbt et al., 1992). In *pointed* mutants, the outgrowth of certain branches is incomplete (Klämbt,1993). Both genes are expressed early in the tracheal placodes and are required (directly or indirectly) for some aspects of cell migration. In contrast, the chromosomal region 60C5 to 60D2 is required later during tracheal development and seems to provide functions essential for the maintenance of the previously established branching pattern. Functions required for the arrest in cell migration should be uncovered by a genetic dissection of this region. The striking expression pattern of DSRF suggests a role of the latter in this process, but further studies are required to investigate this matter.

Other possible functions of DSRF during *Drosophila* development

DSRF gene products, both RNA and protein, are present in the unfertilized egg and DSRF might thus play a role during the early development of the *Drosophila* embryo. Potential SRF target sequences have been identified in the promoter region of the *Drosophila* actin 5C gene, which presumably codes for a cytoplasmatic form of actin and might be involved in cell movements associated with gastrulation (Bond-Matthews and Davidson, 1988). DSRF is also weakly expressed in all somatic muscles of the embryo, but we do not know whether it plays a role in the function of this tissue. We could not detect any abnormalities in the somatic muscle pattern of Px^2 homozygous embryos (data not shown; see also Kimble et al., 1990).

In addition to the embryonic expression, DSRF RNA and protein accumulate in a striking pattern during late larval stages in the developing wing discs. Gene products are mainly found in those regions of the wing discs which, according to the fate map, would give rise to the wing blade per se; little or no DSRF expression is found in cells that correspond well with the known location of cells that will become wing veins and margin of the wing (Felix Loosli and M.A., data not shown). Clonal analysis using DSRF gene specific mutants will be required to elucidate the function of the gene in the establishment of the wing.

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