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

Markus Affolter*, Jacques Montagne, Uwe Walldorf, Jay Groppe, Urs Kloter, Marilina LaRosa and Walter J. Gehring

Department of Cell Biology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland

*Author for correspondence

**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 EcoRI-BglII 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 antisera

A 666 bp BamHI fragment of the DSRF cDNA (positions 447-1113, Fig. 1) was subcloned into the BamHI 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 Freund’s adjuvants every 3 weeks.

Immunostainings, in situ hybridizations and microscopy

Embryos were immunostained according to standard procedures (Asbahrner, 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-crumps 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 Axioshot 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 Axiosplan 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)Px2 (here referred to as Px and Px2) (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 Px2 embryos, we have used a β-tubulin-specific antisera kindly provided by R. Renkawitz-Pohl (see Gasch et al., 1988). Px2 removes the β-tubulin-coding sequences and therefore does not react with the antisera. 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 Px2 genomic DNA we collected homozygous embryos as follows: Px2/Cyo virgins were crossed to OreR males, and the male heterozygous Pm2 progeny were subsequently crossed to Pm2/Cyo virgins. The progeny of this cross are viable except for the Px2 homozygous 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 (Px2 homozygous in majority) were selected again with yeast on a new grape juice plate. Unhatched embryos were dechoronated 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 BglII DNA fragment located in the 5′ end of the cDNA (see Fig. 1). The pdm-1 (Affolter et al., 1993) and β-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 DNA 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-AARG80-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 EcoRI linkers is shown. At the 5' end, the cDNA contains 13 C residues between the EcoRI 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
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.
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 (DFGFR), which was independently found to be expressed in all the tracheal cells and to be required for tracheal cell migration (Glazer and Shilo, 1991; Klambt 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. 4F). 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.
and we found a decrease in the distribution, mainly during stages 12 and 13 via cell migration and the complex branching pattern of the tracheal system is established, mainly during stages 12 and 13 via cell migration and

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 Px², in which chromosomal bands 60C5 to 60D10 are deleted (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 Px² indeed deletes the DSRF transcription unit (or at least part of it), embryos enriched in deficiency homocigotes were collected (see Materials and Methods) and the DNA used for Southern hybridizations. Genomic DNA isolated from Px² has a significantly reduced DSRF hybridization signal when compared to DNA isolated from control flies (Fig. 5D, compare lanes a and b). Px² also deletes the β tubulin gene (Kimble et al., 1990) and we found a decrease in the β 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 Px² 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 Px² deletes both the β 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 Px² and Px homozygous embryos and the transheterozygous Px/Px² 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 (Kimble 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 description 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. Px² 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.](image-url)  
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.
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/Px² transheterozygous embryos (data not shown). Again, and as already observed by the analysis of the mutants with the crumbs antisera, 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 strong signal obtained after hybridization with the DSRF cDNA and subsequently with the pdm-1 cDNA.

**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 EcoRI and probed either with a pdm-1, a β3-tubulin or a DSRF cDNA (see Materials and Methods). DNA was isolated from Px² 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 Px² (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 Px² 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/Px² transheterozygous embryos (data not shown). Again, and as already observed by the analysis of the mutants with the crumbs antisera, 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 Pₓ² 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 that were centered in the distal regions of the terminal branches and had migrated towards the terminal anastomosis at the time when DSRF expression is first detectable.
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 P12 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. P12 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) P12 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 P12 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 P12 homozygous embryos (thin arrow) and 3-5 cells cluster near the CNS (F,G). A dorsal view of a P12 homozygous embryo reveals the absence of cells lining up along the dorsal-ventral axis to form a dorsal branch (thin arrow in H). Instead, 4-6 tracheal nuclei cluster in close proximity to the dorsal midline.
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/Px mutant flies 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 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 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 Px2 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.

We are indebted to Mark Krasnow for providing a preprint of a review on tracheal development in Drosophila and for numerous discussions and suggestions. We thank Mark Krasnow and Karen Guillemin for invaluable comments on the manuscript. We would like to thank B. Shilo for giving us fly stocks and antiserum essential to this study. We are also grateful to M. Krasnow, C. Klämbt, R. Renkawitz-
REFERENCES


(Accepted 20 December 1993)