Two FGF-receptor homologues of *Drosophila*: one is expressed in mesodermal primordium in early embryos

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SUMMARY

The fibroblast growth factor (FGF)/receptor system is thought to mediate various developmental events in vertebrates. We examined molecular structures and expression of *DFR1* and *DFR2*, two *Drosophila* genes closely related to vertebrate FGF-receptor genes. *DFR1* and *DFR2* proteins contain two and five immunoglobulin-like domains, respectively, in the extracellular region, and a split tyrosine kinase domain in the intracellular region. In early embryos, *DFR1* RNA expression, requiring both *twist* and *snail* proteins, is specific to mesodermal primordium and invaginated mesodermal cells. At later stages, putative muscle pre-

INTRODUCTION

Fibroblast growth factors (FGFs) constitute a growth factor family of at least seven members (Burgess and Maciag, 1989). In mammals, FGFs are not only capable of inducing cell proliferation and chemotaxis (Burgess and Maciag, 1989), but also are potent inducers of angiogenesis (formation of new blood vessel; Cross and Dexter, 1991). Response to FGFs is mediated by high-affinity cell-surface receptors (FGF-Rs) through activation of intracellular tyrosine kinase (Lee et al., 1989). FGF-Rs are widely distributed in various embryonic cells in mammals so that mammalian embryogenesis may require pleiotropic functions of FGFs (Wanaka et al., 1991).

The FGF/FGF-R signaling system has also been shown quite likely essential for mesoderm formation in *Xenopus* (Jessell and Melton, 1992; Whitman and Melton, 1992). Most, if not all, FGFs can mimic the mesoderm-inducing activity of vegetal pole cells in the early blastula and, hence, may possibly be natural inducers of mesoderm formation (Slack et al., 1987; Kimelman et al., 1988). An injection of mRNA encoding a dominant-negative form of FGF-R into embryos has been demonstrated to cause significant deformation in mesodermal structures of tadpoles (Amaya et al., 1991).

Molecular analysis of FGF-Rs in chicken, humans and *Xenopus* has indicated that FGF-Rs constitute a family of receptor-tyrosine kinases (Lee et al., 1989; Ruta et al., 1989; Seno et al., 1991; Friesel et al., 1991). Each FGF-R mole-

cursor cells and cells in the central nervous system (CNS) express *DFR1*. *DFR2* expression occurs in endodermal precursor cells, CNS midline cells and certain ectodermal cells such as those of trachea and salivary duct. FGF-receptor homologues in *Drosophila* would thus appear essential for generation of mesodermal and endodermal layers, invaginations of various types of cells, and CNS formation.

Key words: fibroblast growth factor, *Drosophila melanogaster*, tyrosine kinase, immunogloblin superfamily, mesoderm, central nervous system

cule has more than two extracellular immunoglobulin-like domains (Ig-domains) as is also the case for receptors of the platelet-derived growth factor (PDGF) and colony stimulating factor-1 (CSF-1; Williams, 1988). However, unlike receptors for PDGF and CSF-1, receptors for FGFs possess in common additional characteristics such as a relatively long juxtamembrane region, kinase catalytic sequences split by a short stretch of amino acids and a short carboxyl terminal tail.

Understanding the physiological roles of the FGF/FGF-R signaling system may be facilitated by clarification of the roles of homologous genes in Drosophila, in which both genetic and developmental analyses are applicable. In a previous experiment (Shishido et al., 1991), we searched for genomic DNA fragments of Drosophila, encoding novel tyrosine kinases, by polymerase chain reaction (PCR). Two of the seven DNA fragments isolated (dtk1 and dtk2) were found partly to encode polypeptides highly homologous in amino acid sequence to vertebrate FGF-Rs. Using a different approach, Glazer and Shilo (1991) independently identified a Drosophila FGF-R homologue (DFGF-R). Unlike our dtk1 and dtk2 products, the D-FGFR protein appeared to have a long substitution at or near subdomain VIII, a tyrosine kinase subdomain conserved in all tyrosine kinases so far examined (Hanks et al., 1988).

For clarification of the above and further extension of our analysis, complete nucleotide sequences of the coding regions of dtk1 and dtk2 genes were determined and their expression patterns in embryos were examined. dtk1 and

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dtk2, respectively, were found to be *Drosophila* FGF-R homologues having two and five Ig-domains. *dtk1* expression was specific to mesoderm in early embryos, while *dtk2*, seemingly corresponding to *DFGF-R*, was expressed in endodermal precursors.

MATERIALS AND METHODS

Fly strains

Canton-S (wild type) was obtained from Y. Hotta (University of Tokyo). Fly strains with twi^{ID96} , sna^{IIG} were obtained from C. Nüsslein-Volhard (Max Plank Institute, Tübingen). Strain Df(1)260-1/FM4 was obtained from UMEÅ Stock Center. Df(1)260-1 is a mutation, in which the whole AS-C region is deficient (Gonzalez et al., 1989). Strain sr16 /TM1 (De La Pompa et al., 1989) and a Dmyd-lacZ transformant line, 14.1(II) (Paterson et al., 1991) were obtained from A. Ferrus (Instituto Cajal, Madrid) and W. J. Gehring (University of Basel), respectively.

Molecular analyses

Genomic DNA and cDNA clones were isolated from our genomic DNA and pupal cDNA libraries, respectively (Kojima et al., 1991). Nucleotide sequences were determined as described previously (Emori et al., 1985). All other procedures for molecular analyses were essentially as described by Sambrook et al. (1989).

In situ hybridization to whole embryos

Fixation and pretreatment of embryos were performed as described by Tautz and Pfeifle (1989). RNA probes were prepared as follows. cDNA inserts in cFR4-6 (*DFR1*), cFR5-1 (*DFR2*) and PCR-amplified *twist* genomic DNA (Thisse et al., 1988) were cloned into Bluescript plasmid vectors (Stratagene). Purified plasmid DNA was linealized by a suitable restriction emzyme and used as a template. Antisense RNA was prepared by in vitro transcription using digoxigenin-11-UTP (Boehringer Mannheim) and T7 or T3 RNA polymerase. The reaction was carried out according to the manufacturer's standard protocol. After fragmentation into an average size of 150 nucleotides by incubation at 60°C in a buffer containing 40 mM NaHCO₃, 60 mM Na₂CO₃, and 50 mM DTT, RNA was used as a probe for hybridization.

Hybridization was carried out essentially as described by Yokouchi et al. (1991). Embryos were incubated overnight at 50°C in a solution containing 20 mM Tris-HCl (pH 8.0), 2.5 mM EDTA (pH 8.0), 1× Denhardt's solution (0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 300 mM NaCl, 1 mg/ml *Escherichia coli* tRNA, 50% formamide, 10% dextran sulfate and 0.5 µg/ml of digoxigenin-labeled RNA probe. After hybridization, embryos were washed with 2× SSC containing 50% formamide for 1 hour at 50°C, followed by RNase treatment in 10 mM Tris-HCl (pH 8.0) with 500 mM NaCl and 20 µg/ml RNaseA for 30 minutes at room temperature. Then, embryos were washed again with 2× SSC containing 50% formamide for 1 hour at 50°C and then with 1× SSC in 50% formamide for 1 hour at 50°C. Immunological detection was carried out essentially as described by Tautz and Pfeifle (1989).

The crosses

To examine *DFR1* expression in *AS-C*⁻ embryos, crosses were made with Df(1)260-1/+ females derived from Df(1)260-1/FM4 and wild-type flies, and wild-type males. Consistent aberrations observed in one quarter of the embryos were noted as mutant phenotypes. P[Dmyd-lacZ]/+; sr16/+ flies were obtained by crossing flies homozygous for the P insertion (on the second chromosome) and sr16/TM1 flies. P[Dmyd-lacZ]/+; sr16/+ flies were crossed to

each other and the resultant embryos (progeny) were stained with anti- -galactosidase antibody as described (Higashijima et al., 1992). Consistent aberrations observed in one quarter of the *lacZ*expressing embryos were noted as mutant phenotypes.

Sectioning of embryos

After dehydration through an ethanol series, stained embryos were embedded in polyester wax (BHD, England), incubated at 37°C and sectioned at 10°C. Samples thus obtained were treated with 100% ethanol and mounted in Euparal (Chroma-gesellschaft, Germany).

RESULTS

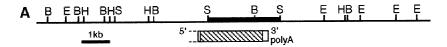
Molecular cloning of two *Drosophila* FGF-R gene homologues

Using *dtk1* and *dtk2* fragements as probes, genomic DNA clones were isolated. cDNA clones were isolated using suitable genomic DNA probes. Since, as described below, polypeptides encoded by cDNA inserts are very similar in sequence and organization to vertebrate FGF-Rs, *dtk1* and *dtk2*, respectively, were renamed *DFR1* and *DFR2* (*Drosophila* <u>FGF-receptor</u> homologues 1 and 2). In situ hybridization to polytene chromosomes showed *DFR1* and *DFR2* to be mapped at 90D-E and 70C, respectively (data not shown).

DFR1 encodes a *Drosophila* FGF-receptor having two Ig-domains

The complete nucleotide sequence of the cDNA insert in cFR4-6 and its relevant genomic regions were determined (Fig. 1B). As schematically shown in Fig. 1A, the DFR1 gene included no intron. The cFR4-6 insert contains a single long open reading frame (ORF) of 2251 bp, followed by 523 bp of the 3 non-coding sequence with a truncated poly(A) tail. The analysis of genomic sequences indicated the in-frame termination codon to be located at nucleotide positions -56 to -54 and no additional in-frame initiation codon to be present within the region from -1 to -53. Regions with the first and second ATG codons (nucleotide positions, 61-67 and 106-112, respectively) matched the Drosophila consensus sequence for the translational initiation (Cavener, 1987). However, only the second ATG codon may possibly be functional, since, as in the case of vertebrate FGF-R genes, it is directly followed by a nucleotide sequence coding for a hydrophobic stretch of 22 amino acids capable of serving as a signal peptide. The expected cleavage site of the putative signal peptide may be between Ala-19 and Val-20 (von Heijne, 1983). The second hydrophobic stretch was detected in the middle of the ORF (amino acid positions, 295-318), presumably representing the transmembrane domain. Thus, the extracellular and intracellular domains of the DFR1 protein were assigned to amino acid residues 20-294 and 319-714, respectively.

As in the case of vertebrate FGF-Rs (Lee et al., 1989), the intracellular region contains a relatively long juxtamembrane domain (77 amino acids), split tyrosine kinase catalytic sequence (278 amino acids) and a short carboxyl terminal tail (41 amino acids). All 11 tyrosine kinase subdomains (I to XI; Hanks et al., 1988) were found to be conserved. The *DFR1* kinase insert (19 amino acids) is slightly



Β, CTGTTGCACATTCGAGGGTGCTGCCGCCGGCCAGGGGTAGAGTTACAAAAATAAAATATACCAAAAATGGCTGCCGCCTGGAGTTGGCGAGCCAGTCACTCAACG m a a a w s w r a s h s t 104 ATCACAATGACAAGCGGATCGGTGGTGGTGGTCTTCCTGTTGCTTACGATTTGGCAGCCGGCGGGTGGAAGGACGACGACGACGACGAATGGCCAATAGCCAGGAG F 1 i t <u>m t s g s v v v l f l l l t i w o p a v o</u> v e g r r o m a n s o e 209 atgatc<mark>aaggatcatttgggaggccaggagtcaaaagacaccaggcatcacgaccatggagtggatttggatgatggggg</mark> P V O V E G R R Q M A N S OE 34 M I K D H L G A R S Q N K T P A I T N N A N Q S S T S S V D L DDG Α 314 GCAGATGACGATGATAACAAGGCCGATTTGCCGGTGAATGTCAGTTCGAAACCCTACTGGCGGAATCCCAAGAAGATGAGTTTCTGCAGACGCGACCCTCGGGC D D D D N K A D L P V N V S S K P Y W R N P K K M S F L Q T R P S G 69 419 TCACTGCTCACCCTCAATTGCCATGCCCTGGGCAATCCTGAGCCCAATATCACATGGTATCGCAATGGCACCGTCGACTGGACGCGCGGCTATGGCAGCCTGAAG 104 S L L T L N (C) H A L G N P E P N I T W Y R N G T V D W T R G Y G S L K 524 CGGAATCGATGGACCCTGACGATGGAGGATCTGGTGCCGGCGGATTGTGGCAACTACACCTGCAAGGTGTGCAATTCATTGGGTTGCATTCGTCACGACACCCCAG 139 R N R W T L T M E D L V P A D C G N Y T (C) K V C N S L G C I R H D T Q 629 GTGATTGTAAGCGATCGAGTTAATCACAAACCGATCCTAATGACCGGCCCATTGAATCTCACACTGGTGGTCAATTCCACAGGCAGCATGCACTGCAAATATCTA IVSDRVNHKPILMTGPLNLTLVVNSTGSMH(C)KYL 174 V 734 TCGGACTTGACCAGCAAGAAGGCGTGGATCTTTGTGCCCTGCCATGGCATGACCAACTGCTCCAACAATCGATCCATTGCCGAGGATAAGGATCAGTTGGAC 209 S D L T S K K A W I F V P C H G M T N C S N N R S I I A E D K D Q L D TTCGTGAACGTTAGGATGGAGGAGGAGGGGCTGGTACACGTGCGTCGAAAGCAACAGCCTGGGTCAATCCAATAGCACTGCCATATCTGCGAGTGGTGCGAAGTCTT 839 244 F V N V R M E Q E G W Y T (C) V E S N S L G Q S N S T A Y L R V V R SL 944 CACGTCTTGGAAGCCGGAGTGGCCAGTGGTTCACTTCATCGTCGACCAGCTTCGTGTGTACATCTTTGGCGGACTCATTTTCATCTTTATGACCACCCTGTTT 279 H V L E A G V A S G S L H S T S F V Y I F V F G G L I F I F M T LF 1049 GTCTTCTATGCCATCCGAAAGATGAAACATGAAAAGGTGCTGAAACAACGCATCGAAACCGTTCACCAGTGGACCAAGAAAGTCATCATCTTCAAGCCCGAAGGT 314 **FYAI**RKMKHEKVLKQRIETVHQWTKKVIIFKPEG 1154 GGCGGAGACTCCAGCGGTTCCATGGACACCATGATTATGCCGGTGGTTAGGATACAGAAACAGCGCACCACTGTCCTTCAGAATGGCAACGAGCCGGCTCCATTC 349 G G D S S G S M D T M I <u>M P V V R I Q K Q R T T V L Q N G N E</u> P 1259 AATGAATATGAATTTCCACTGGAACTGGGAACTGGGCAGAAGTCATTTGGTACTGGGTGCCACTTTGGGAGAAGGTGCTTTCGGACGAATGGTCATGGCG <u>EYEFPLDSNWE</u>LPRSHLVLGATLGEGAFGRMVMA 384 1364 E V N N A I V A V K M V K E G H T D D I A S L V R E M E V M K I I G 419 CGACATATCAATATTATTAACCTACTTGGTTGCTGCAGTCAAAATGGTCCGGCTCTATGTGATTGTCGAGTATGCGCCACACGGAAATCTCAAGGACTTCCTCTAT 1469 454 R HINIINLLGCCSONGPLYVIVEYAPHGNLKDFL Y 1574 489 RNRRSGR DODRD S SQPPPSPPA H <u>VITEKDLIKFA</u> Ħ 1679 524 QIARGMDYLASRRCI<u>H_R_D_L_A_A_R_N_L_L_Y_S_D_V_Y_L_R_I</u>A 1784 <u>p_F_G_L_A_R_D_I_Q_S_T_D_Y_Y_R_K_N_T_N_G_R_L_P_I_K_W_M_A_P_E_S_L_Q_E_K</u> 559 1889 TTCTATGATTCCAAGAGCGATGTCTGGTCATATGGCATCCTGCTGTGGGAGATCATGACCTATGGGCAGCAACCATATCCAACTATCATGTCCGCGGGAGGAGCTG 594 F<u>Y_D_S_K_S_D_Y_W_S_Y_G_I_L</u>LWEIMTYGQQPYPTIMSAEE L 1994 TACACCTATCTCATGTCCGGTCAGCGGATGGAGAAGCCAGCGAAATGCTCCATGAACATTTACATCCTGATGCGACAATGTTGGCATTTCAACGCCGACGATCGG 629 Y T Y L M S G Q R M <u>E</u> <u>K P A</u> N I 0 н K C SM Y M R С W F N D D CCACCTTTTACGGAAATCGTTGAGTATATGGATAAGCTGCTCCAGACGAAGGAGGACTACCTCGATGTGGATATCGCCAATCTGGATACGCCGCCCCCGACGAGT 2099 664 P <u>FTEIVEYM</u>DKLLQTKED<u>A</u>LDVDIANLDTPPSTS P 699 D E E E D E T D N L Q K W C N Y 2414 ACACTCGAATTTATGTATATAAACTCTCGTTTAAGTCTGTAAATCTCTTTGAAAACATTTTACCAAAATTGCACCAAAAGGCGTAAAACCCAAAACAAGCAGCAGCTGC 2519 ARTITACTICIAGITIAAITGITGATTTAAGATATTACCATAAAAAATCTACTAGTATATGCGIAGTTCTAAGITGAACITAAGACICGGCIGIAITTGCICIGI 2624 ATTTCATATTGTACTGATCTTTGTAGTATTCGCAGTTTTATACTCGCTANATTATACTCGTAGCCCATAAATATAAAAACAATGTTCTATCTTAGCGTTAGTTGCA

Fig. 1. (A) Restriction map of the *DFR1* genomic region and organization of the *DFR1* transcrption unit. The *DFR1* exon is boxed. The hatched region represents a possible coding region. The solid bar shows the region used as a probe for cDNA screening. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I. (B) Nucleotide sequence of the *DFR1* gene and amino acid sequence of its putative translation product. Lower-case letters shows the amino acid sequence between the first and second methionines. The thick underline shows the putative signal peptide. Eleven potential N-glycosylation sites (N-X-S/T) are shaded. Conserved Cys residues of Ig-domain are circled. The acidic region is enclosed by an open box. The shaded box indicates the predicted transmembrane region. The tyrosine kinase catalytic sequence is enclosed by a polygon. The kinase insertion sequence is shown by a thin underline. Conserved Tyr residue in the C-terminal tail is indicated by a triangle. The putative polyadenylation signal is shown by a wavy line and truncated poly(A), by a dotted line. The region corresponding to the *dtk1* sequence is labeled by a broken underline.

longer than counterparts in vertebrate FGF-Rs (14 amino acids). Except for the kinase insert, the overall sequence homology of the kinase domain between *DFR1* and vertebrate FGF-Rs was estimated as about 60%. Homologies with other types of vertebrate typosine kinases were approx-

imately 40%. The carboxyl terminal tail of *DFR1* includes a tripeptide sequence, Tyr-Leu-Asp, which may provide the tyrosine phosphorylation site supposedly required for binding of SH2 of phosphoinositide-specific phospholipaseC-(Mohammadi et al., 1991).

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Ir	nosívny tívéli kéyye érévv X (53) - stálážesztegyesztéssátássátáss	(Flg; Ruta et al., 1989) proteins.
		I-V, Ig-domains I-V (see Fig. 3).
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The extracellular domains of FGF-Rs are, in general, characterized by a series of Ig-domains, each containing two Cys residues, possibly connected by a disulfide bond, in an interval of several scores of residues capable of constituting -sheet structures (Williams and Barclay, 1988). The consensus sequence for the Ig-domains is Vx(I/L)xC(8x-12x)W(20x-50x)DxGxYxC (x=arbitrary amino acid). As shown in Figs 2 and 3, the DFR1 protein contains two Ig-domains. (Most vertebrate FGF-Rs have two or three Ig-domains (Lee et al., 1989; Ruta et al., 1989; Seno et al., 1991).) As in the case of vertebrate FGF-Rs, DFR1 extracellular domains contain a short stretch of acidic amino acids (Fig. 3). Locations of two potential N-glycosylation sites were found conserved between DFR1 and the flg, one of human FGF-Rs (Ruta et al., 1989).

Using a human FGF-R cDNA (Seno et al., 1991) as a probe, Southern blot analysis of the total genomic DNA of *Drosophila* was carried out. *DFR1*-derived fragments were found to be hybridized in all lanes under a less stringent condition (data not shown). From the above, the *DFR1* gene would appear quite likely to be a counterpart of vertebrate FGF-Rs in *Drosophila*.

DFR2 is another type of FGF-R gene homologue encoding a polypeptide with five Ig-domains

We next determined the nucleotide sequences of the cDNA insert in cFR5-1 and its relevant genomic regions. As schematically shown in Fig. 4A, the *DFR2* gene had an intron 85 bp long. The *DFR2* cDNA contained a single long ORF followed by 33 bp long, 3 non-coding sequence. Since neither possible signal peptide sequence nor probable translational initiation codon was found to be coded for by the cDNA, we sought to determine further 5 sequences using a *DFR2* genomic clone. Fig. 4B shows the composite nucleotide sequence thus obtained, which contains a 3159 bp long ORF following an in-frame termination codon of nucleotide positions, 121-123.

19 amino acids following the first methionine (amino acid positions, 2-20) may serve as a signal peptide, being highly hydrophobic. The second hydrophobic stretch, including 25 amino acids (amino acid positions, 601-625) may represent the transmembrane region, since it is followed by a putative tyrosine kinase sequence as described below.

As noted for DFR1, the intracellular region of DFR2 con-

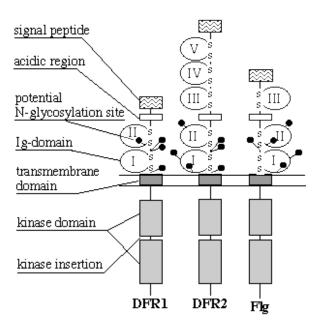
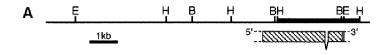


Fig. 3. Diagram showing structures of two *Drosophila* FGF-Rs and a human FGF-R, Flg. Only conserved N-glycosylation sites are indicated.

tained a split tyrosine kinase catalytic domain (Figs 3, 4B), highly homologous to those of vertebrate FGF-Rs. Homology between *DFR1* and *DFR2* kinase domains was 79%. The Tyr-Leu-Asp sequence was found near the carboxyl terminus of *DFR2*. The *DFR2* extracellular region included five Ig-domains, of which two were found in the region flanked by the acidic domain and transmembrane domain (Figs 2, 3). Three and four of nine N-glycosylation sites of *DFR2* were conserved in both *DFR1* and *flg*, respectively (Fig. 3).

DFR2 is virtually identical in nucleotide sequence to *DFGF-R*, whose sequence has been reported by Glazer and Shilo (1991). However, their original *DFGF-R* sequence appears to include some defects in two regions. One base deletion in an C stretch of *DFGF-R* (nucleotide positions, 865-868 in Fig. 4B) disrupted the coding region for the extracellular domain. Nucleotide changes in the region from 2850 to 2892 (see Fig. 4B) results in loss of the invariant glutamic acid of subdomain VIII of *DFGF-R*.



B₁ tcgattgaagatacccacaggaattgatattatttcgaccgcaactgtaacccatatgtacacgatgtgcccatccagatacgagatcttggtggcctgttgaac 106 TCAAGAGAATCCACTTGAATCATGGCAAAAGTGCCGATCACGCTGGTGATGATCATTGCGATCGTCGTCGTGCGGCGGCCGGGGATGCGATTATGGCCATCAT v v ν DLGCDYG н н 211 COTTGCTACATCGATGTGACTGTGGAAAACAGTCCCCCGCCAGCGCCATCTGCTCTCCCACATGGACATCACGCTGCAGTGCGACCCATGGCCAAATGGTCC 29 R (C) Y I D V T V E N S P R Q R H L L S D M D I T L Q C V R P M A K W F 64 Y E D K F Q L R A T L L R L E R A Q S G N S G N Y G (C) L D S Q N R W Y 421 ANTATTTCCCTGGTTATTGGCCATAAGGAACCGGTTGGCAATGATATCGCCAGCTTTGTCAAGTTGGAAGATGCACCGGCCATCCCGGAGTCAGATCTCTTTTTC 99 N I S L V I G H K E P V G N D I A S F V K L E D A P DLFF A IPES 526 CAGCCCCTGAATGAGAGTCGATCCTTGAAGTTGCTGCAACCCCTACCCAAAACCGTACAACGGACCGCAGGTGGCCTTTTTCAGTTGAACTGCAGTCCCATGGAT 134 Q P L N K S R S L K L L Q P L P K T V Q R T A G G L F Q L N (C) S P M D 631 CCGGATGCAAAGGGTGTTAATATATCCTGGCTGCACATTGATACTCAAATCCTTGGTGGACGTGGACGCATTAAGCTTAAAAGGTGGTCATTAACAGTGGGGCAA 169 P D A K G V N I S W L H I D T Q I L G G R G R I K L K R W S L T V G Q 736 CTGCAGCCGGAGGATGCTGGAAGCTATCACTGTGAGCTGTGGGTCGAACAGGACTGCCAAAGGAGTAATCCGACCCAATTGGAGGTGATAAGCAGAAAGCACACG 204 L Q P E D A G S Y H (C) E L C V E Q D C Q R S N P T Q L E V I S R K H T 841 GTGCCCATGCTAAAACCCGGATATCCCCGCAATACCAGCATTGCCCTCGGGGACAATGTGAGCATTGAATGCCTACTTGAGGACTCTGCCCTGGAGCCGAAGATC 239 V P M L K P G Y P R N T S I A L G D N V S I E (C) L L E D S A L E P K I 274 T W L H K G N A D N I D D L L Q R L R E Q S Q L P V D V T R L I T R M 1051 GATGAACCGCAGGTACTTCGTCTTGGAAATGTCCTGATGGAGGATGGCGGATGGTACATATGCATAGCCGAGAACCAGGTTGGACGCACTGTGGCCGCCCCCCTAT 309 DE PQVL R L G N V L M E D G G W Y I (C) I A E N OVGR T VAA 1156 GTGGATCTTTACAGTCCTTCGGACACTACCACAGTTCGCACGACCACTACCACCGTGGCCAGCCCAATACCCACTGCATCCACCGGAGAGGACAACGATGAC 344 V D L Y S P S D T T T V R T T T T T T V A S P I P T A S T G <mark>E D N D D</mark> 1261 GACGTGGAAAATCCAGCAGCGGACGCATCAGGCGGCGTTGGTCCACCTGTGTTCAGAAAAGAGCTGAAACGATTGCAGCACTCATTATCGGGAAATACTGTCAAT 379 DVENPAADASGGVGPPVFRKELKRLOHSLSGNTVN 1366 CTAGCCTGTCCAGTGTACGGCAAAGCGAACATCACCTGGACGAAGGACAAGAAGCCGCTAAACCGTGAACTCGGCGTCTATGTGCAAAAGAACTGGACACTCGC 414 L A (C) P V Y G K A N I T W T K D K K P L N R E L G V Y V QK NWTLR 1471 TTCGTGGAGGCCACCAGCGAGGATTCTGGACTATACAACTGTAAGGTGTGCAATGCGTGGGGCTGCACCAGTTTGACTTCAGTGTCCAAATAAACGATCGCACG 449 F V E A T S E D S G L Y N (C) K V C N A W G C I Q F D F S V Q I N D R T 1576 AGATCCGCACCAATAATCGTGGTGCCGCCAAAATCAGACGGTGAAAGTCAATGGCAGCTTGGTGATGAAGTGCACCGTCTACTCTGCATCTGCATCCTACAGTCAGC 484 R S A P I I V V P Q N Q T V K V N G S L V M K (C) T V Y S D L H P T V S 1681 TGGAAAAGGGTTGTCCTCAAGAATGCCTCATTGGACGGCCTTCAGTCTGTGGAAATCCCAGAATCTCAACCTTCACCGTGACGAATGACTCCGTAGTTTTAACGCTT N D S V V L 519 W RVVLK NASLDGLQSV EIQNLNFTVT 1786 CGAAATGTGACCTTTGACCAGGAGGGCTGGTACAGCTGTTTGGCCTCCAGGGCTGGGAAGGAGTAACTCCAGTGTGTACCTTAGGGTAGTGAGTCCTCTGCCA 554 R N V T F D Q E G W Y S (C) L A S S G L G R S N S S V Y L R V V S P L P 1891 CCTTTGGAGATCTACGCCCTGCTGCACGCCCATCCTCTCGGATTCACCTTGGCCGCTATTACGATAGTGGCTTTGTTCCTTTTGGGCACGCGCTTTCATAACGTTC 589 PLEIYALLHAHP<mark>LGPTLAAITIVALPLLGSAFITP</mark> 624 M L R R L R R EKLLKLRIETVHQWTKKV II YRP GGE 2101 AGTGGCTGCAGCTCCCGGCGATCTTCAGATGCCAGTGATAAGGATCGAGAAGCAGGAACCAGCGTTTCCACAACGGGAACTGGCGGTACTGATCCCCGCCCAGGGC 659 S G C S S G D L O M P V I R I E X QR т T v S T G т G G T D ₽ 0 G 2206 TTTAATGAGTATGAGTTCCCGCTGGACTCAAACTGGGAGAATTCCCAGGCAGCAGCATGCTTGGGCTCCATTTTGGGTGAGGGCGCCCTTTGGACGCGTAGTGATG <u>NEYEFPLDSNWE</u>IPRQQLSLGSILGEG**AF**GRVVM 694 2311 GCGGAAGCGGAGGGTCTCCCTAGGAGTCCACAACTGGCCGAAACCATCGTGGCCGTCAAGATGGTCAAGGAGGAGCACACGGATACGGACATGGCCAGCTTGGTG 729 AEAEGLPRSP QLAETIV A V K M V K E E н т DTD M A s LV 2416 CGTGAAATGGAGGTGATGAAAATGATTGGCAAGCAACATAAACATCAATCTGCTAGGCTGCTGCAGCCAGGGCGGTCCTCTGTGGGTGATCGTGGAGTATGCT 764 REMEVMENIGEHINIINLLGCCSOGGPLWVIVEYA CCACACGGAAACCTCAAGGACTTCCTCAAGCAGAATCGACCTGGTGCTCCACAGCGACGGAGTGACAGTGATGGGTATCTAGATGACAAGCCACTGATTTCCACT 2521 H G N L K D F L K Q N R P G A P Q R R S D S D G Y L D 799 P D X _L G E K E L T K F P F Q I A R G M E Y L A S R R C I <u>H _ R D _L A _A R</u> 834 2731 AATGTCCTTGTAAGCGATGGATACGTTATGAAGATCGCCGACTTTGGTCTGGCGAGGGATATCCAAGATACGGAGTACTACCGAAAAAAATACCAATGGACGGTTG N _V_ L _V_ S _D_ G _Y_ Y _M_ K _I_ A _D_ F _G_ L _A_ R _D_ I _Q_ D _T_ E _Y_ Y _R_ K _N_ T _N_ G _R_ L 869 cccattaagtggatggcgccccgagtcgctgcaggagaagaagtacgactcacagagcgacgtctggagctatggtgttctgctgtgggagatcatgacctacggg 2836 904 P_I_K_W_M_A_P_E_S_L_Q_E_K_K_Y_D_S_Q_S_D_Y_W_S_Y_G_V_L L W E I M T Y G 2941 GATCAACCATACCCGCACATACTGTCTGCCGAGGAGCTCTACAGCTACCTGATTACGGGTCAGCGCATGGAGAAACCGGCCAAGTGCTCCCTGAACATTTACGTG 939 D Q P Y P H I L S A E E L Y S Y L I T G Q R M <u>E</u> ĸ P K С s N A Τ. 77 3046 STAATGCGACAGTGCTGGCACTTTGAGTCCTGTGCACGACCCACGTTCGCGGAGCTGGTAGAGAGTTTGGATGGGATCCTGCAGCAGGCTAGTAGCAATCCAAAC 974 <mark>V M R Q C W H F E S C A R P T F A E L V E S F</mark> D G I L Q Q A S S N P N 3151 GATGCCTATCTGGACCTTTCGATGCCGATGCTGGAGACGCCGCCCCCATCGGGGGATGAGGATGATGCTCCGACACCCGAAACATTCCGGGAAACGTCCCCGCCT 1009 D A 🖄 L D L S M P M L E T P P S S G D E D D G S D T E T F R ETSPL 3256 AGATATCAGTACACCTATAAGTTTAATTAGTTCGTAGTATAAGGAGACCAAAAAGAATTC 1044 R Y Q Y T Y K F N

Fig. 4. (A) Restriction map of the *DFR2* genomic region and organization of the *DFR2* transcription unit. (B) Nucleotide sequence of the *DFR2* gene and amino acid sequence of its putative translation product. The arrowhead indicates the location of an 85 bp intron. All symbols and abbreviations are the same as in Fig. 1.

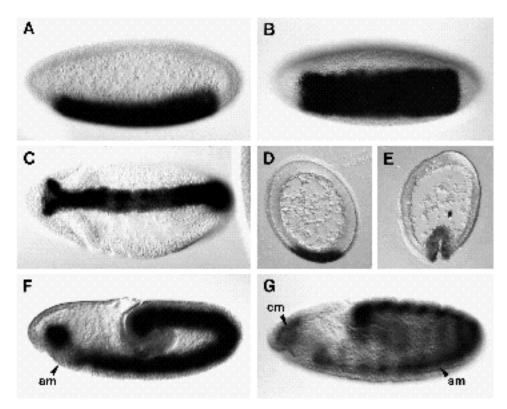


Fig. 5. DFR1 RNA expression in early embryos. Spatial distribution of DFR1 RNA was examined by in situ hybridization using digoxigenin-labeled RNA as a probe. (A,F,G, lateral views; B,C, ventral views. Anterior is the left and dorsal is up. D,E, transverse sections; dorsal side up). (A,B,D) Stage 5 embryos (cellular blastoderm). Note that DFR1 is exclusively expressed in mesodermal primordium situated at the ventral side. (C,E) Stage 6-7 embryos. (F) Stage 8 embryo (germ-band extension). Only invaginated mesoderm appears to express DFR1. am, anterior midgut. (G) Stage 9 embryo (fully extended germ band). DFR1 expression appears to segment. cm, cephalic mesoderm; sm, somatic mesoderm.

Specific expression of *DFR1* in prospective mesoderm during early embryogenesis

The spatial and temporal expression of DFR1 RNA was examined by in situ hybridization of whole embryos. The expression of DFR1 RNA was first detected at the cellular blastoderm stage (stage 5; Campos-Ortega and Hartenstein, 1985), about 150 minutes following egg fertilization, just before the onset of gastrulation. The ventral surface of the embryo, 16-17 cells wide and from 10 to 85% egg length, was hybridized with the DFR1 probe (Fig. 5A,B,D). This region of DFR1-expressing cells corresponds to mesodermal primordium, which eventually participates in the ventral furrow formation. Note that at stages 6-7 (about 3 hours after egg fertilization), all DFR1-expressing cells invaginated to form mesoderm (Fig. 5C,E). Fig. 5F shows the staining pattern of an embryo at stage 8 (3 hours 30 minutes; stage for germ-band extension). The anterior part of the DFR1-positive region appears to be separate from the main body in forming the cephalic mesoderm. This is most probably due to invagination of midgut primordium (see an arrowhead labeled am in Fig. 5F). At stage 9 (about 4 hours), the DFR1-positive region began to segment (Fig. 5G). These findings indicate the expression of DFR1 to be mesoderm-specific at least in early embryos.

DFR1 expression in somatic mesoderm and other tissues

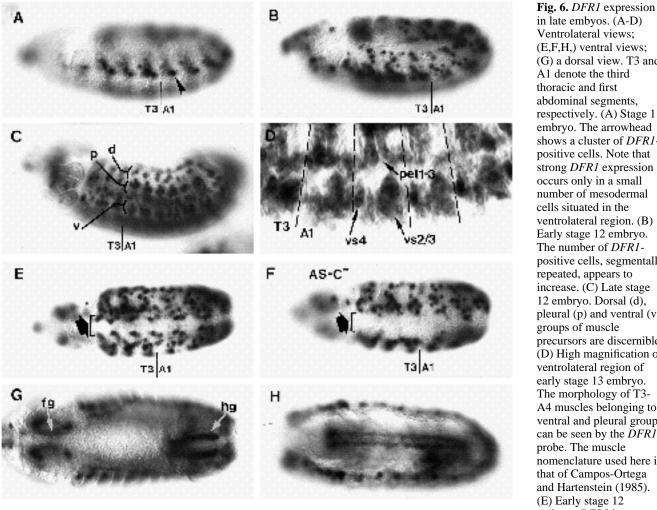
The invaginated mesodermal cells mainly differentiate into somatic and visceral muscles (Campos-Ortega and Hartenstein, 1985). *DFR1* expression in somatic mesoderm is described in the following.

DFR1 expression dramatically changes at stage 11 (6-7

hours), when germ-band fully extends and intersegmental furrows become much more discernible. As shown in Fig. 6A, the overall expression of DFR1 in the mesoderm is virtually extinct and, instead, a small number of cells strongly expressing DFR1 emerge in the ventrolateral region of each segment. Since such DFR1-positive cells were found in all of 50 embryos derived from $AS-C^-/+$ parents, the *achaete-scute* gene products may not be required for their presence. Thus, these DFR1-positive cells are suggested not to be neural cells. During germband retraction (stage 12), isolated DFR1-positive cells increase in number and scatter in the entire region of the somatic mesoderm (Fig. 6B).

At late stage12 (about 8 hours), three lows of *DFR1*-positive cells are distinguishable in bodywall (Fig. 6C). These cells appear to be precursor cells for ventral (v), pleural (p) or dorsal (d) groups of muscles. When the germ-band has completely retracted, cell fusions to produce the muscle pattern finish (Bate, 1991). As expected, at early stage 13 (9.5 hours), vs 2-4 (muscles 26,27,25; ventral group; Crossley, 1978) and pet 1-3 (muscles 21-23; pleural group) were clearly *DFR1*-positive (Fig. 6D). Thus, the majority of *DFR1*-positive cells appear to eventually differentiate into muscle.

DFR1 expression also occurred in tissues other than somatic mesoderm. In the middle of stage 12, mid-ventral, *DFR1*-positive cells were detected (Fig. 6E). They may be ventral nerve cord cells, since their presence requires the *achaete-scute* gene products (Fig. 6F). *DFR1* expression in CNS persists at least until stage 16 (13 hours; Fig. 6H). Cells surrounding the hindgut and foregut were noted to be *DFR1*-positive at stage 12-13 (Fig. 6G).



in late embyos. (A-D) Ventrolateral views; (E,F,H,) ventral views; (G) a dorsal view. T3 and A1 denote the third thoracic and first abdominal segments, respectively. (A) Stage 11 embryo. The arrowhead shows a cluster of DFR1positive cells. Note that strong DFR1 expression occurs only in a small number of mesodermal cells situated in the ventrolateral region. (B) Early stage 12 embryo. The number of DFR1positive cells, segmentally repeated, appears to increase. (C) Late stage 12 embryo. Dorsal (d), pleural (p) and ventral (v) groups of muscle precursors are discernible. (D) High magnification of ventrolateral region of early stage 13 embryo. The morphology of T3-A4 muscles belonging to ventral and pleural groups can be seen by the DFR1probe. The muscle nomenclature used here is that of Campos-Ortega and Hartenstein (1985). (E) Early stage 12 embryo. DFR1 is

expressed in cells of ventral nerve cord, which is indicated by the arrow. (F) Early stage 12 Df(1)260-1 embryo lacking the expression of the achaete-scute genes. As indicated by the arrow, the DFR1 expression in the region corresponding to the ventral nerve cord is eliminated in the AS-C- embryos. (G) Late stage 12 wild-type embryo. DFR1 is expressed in cells surrounding foregut (fg) and hindut (hg). (H) Stage 16. DFRI RNA distribution can be detected along longitudinal connectives of CNS.

DFR2 expression in invaginating endodermal and ectodermal cells

The expression of DFR2 RNA is first observed in primordia of anterior midgut and posterior midgut in the begining of germ-band extension (stages 6-7, about 3 hours; Fig. 7A). This expression becomes much more prominent at stage 8 (about 3.5 hours), when anterior and posterior midguts invaginate as shown in Fig. 7B. Other expression patterns of DFR2 RNA are essentially identical to those described by Glazer and Shilo in the case of DFGF-R (1991). At stages 8, DFR2-positive regions 1-2 cells wide become discernible (Fig. 7C). They appear to correspond to CNS midline cells and/or their precursors. At stage 10 (about 5 hours), cells contributing to tracheal pit formation begin to express DFR2 RNA (Fig. 7D). At stage 13, DFR2positive signal can also be observed in cells correponding to the presumptive salivary duct and salivary tubes (data not shown). These observations suggest that DFR2 is involved in the formation of the endodermal layer (midgut),

and development of mesectoderm and tubular organs derived from ectoderm.

DFR1 expression in gastrulation-defective mutants, twist and snail

Embryos of null mutations in twist or snail fail to form a normal ventral furrow; and cannot form mesodermal cell layer (Nüsslein-Volhard et al., 1984; St. Johnston and Nüsslein-Volhard, 1992; Leptin, 1991). Examination was made of the distribution of DFR1 RNA in twist- and snailembryos. A quarter of progeny derived from either twist/+ or snail/+ parents was found negative to the DFR1 probe (255 and 179 embryos were examined, respectively; Fig. 8A,B,D). The morphology of DFR1-negative embryos was abnormal, showing twist or snail phenotypes (Fig. 8B,D). Thus, concomitant expression of twist and snail gene products may be required for mesoderm-specific DFR1 expression in early embryos. At later stages, a small number of DFR1-positive cells were detected in the ventral region

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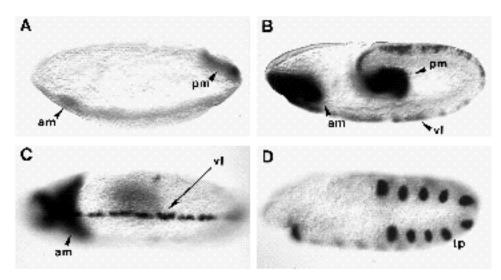


Fig. 7. *DFR2* expression in wildtype embryos. Whole embryos were hybridized with *DFR2* probe. (A,B,D) Lateral views; (C) a ventral view. (A) Stages 6-7 embryo. am, anterior midgut; pm, posterior midgut. (B,C) Stage 8 embryos. Note that *DFR2* is expressed in ventral midline (vl). (D) Stage 11 embryo. Tracheal pits (tp) are labeled with *DFR2* probe.

of *twist*⁻ and *snail*⁻ embryos (Fig. 8C,E). From their positions and size, these cells appeared to be neuronal cells.

DFR1 may be required not for gastrulation but for the formation of somatic mesoderm

During in situ hybridization experiments, we noticed that the *DFR1* gene is located within a short deletion of *sr16* at the 90D-E region (De La Pompa et al., 1989; Bellen et al., 1992). This deletion was estimated to be 70-300 kb in length; it is known to include at least two genes *couch potato* (*cpo*) and *stripe* (*sr*). *cpo* is apparently unrelated to *DFR1* (Bellen et al., 1992). We first examined the gastrulation of 3-5 hour embryos derived from *sr16*/+ parents, by following *twist* RNA expression in presumptive mesodermal cells (Thisse et al., 1988). No abnormal invagination was observed in more than 100 embryos at least until stage 8. Germ-band-extending embryos from the same parents were also hybridized with a mixture of *DFR1* and *DFR2* probes. *DFR1*⁻*DFR2*⁺ embryos should correspond to those homozygous for *sr16*. As shown in Fig. 9A, *sr16/sr16* embryos were essentially normal in *DFR2* expression in midline cells. In contrast, aberrant *DFR2*-expression was observed in *twist*⁻ embryos defective in gastrulation (Fig. 9B). Although we do not know possible morphological changes in 5- to 7-hour-old *DFR1*-defective embryos, it should thus be possible for normal gastrulation to occur without the *DFR1* gene product.

To examine the muscle formation in sr16 embryos, we utilized P[Dmyd-lacZ], by which Dmyd expression can be

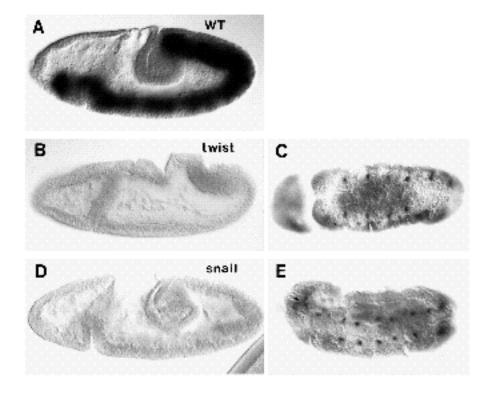


Fig. 8. *DFR1* expression pattern in gastrulation-defective mutant embryos. Embryos were hybridized with the *DFR1* probe. (A,B,D) Lateral views; (C,E) ventral views. (A) Wild-type embryo at stage 8 (3.5 hour). (B) Germ-bandextending *twi*^{*ID96*} embryo. (C) Germband-retracting *twi*^{*ID96*} embryo. (D) Germ-band-extending *sna*^{*IIG*} embryo. (E) Germ-band-retracting *sna*^{*IIG*} embryo. (E) Germ-band-retracting *sna*^{*IIG*} embryo. Note that, in *twist*⁻ or *snai1*⁻ mutant embyos, *DFR1* is not expressed at early stages (B,D), but a small number of *DFR1*-expressing, presumably neural cells can be seen in ventral region at later stages (C, E).

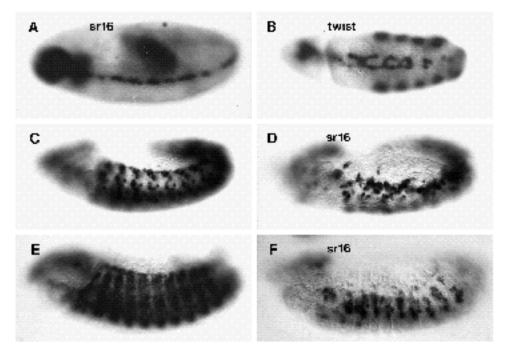


Fig. 9. Gastrulation and Dmvd expression in sr16 mutant embryos lacking DFR1 expression. (A,B) Ventral views; (C-F) lateral views. (A) Germband-extending sr16 embryo hybridized with a mixture of DFR1 and DFR2 probes. No DFR1 expression was observed. Normal DFR2 expression in midline cells suggests that DFR1 is not required for normal midline formation. (B) Germband-extending twi^{ID96} embryo hybridized with the DFR2 probe. Note the aberrant DFR2 expression in midline cells. (C-F) Expression of Dmyd-lacZ fusion gene in wild-type embryos (C and E) and sr16 embryos (D and F), determined by anti- -galactosidase antibody.

Wild-type patterns of muscle precursors at stage 12 (C) are destroyed in sr16 embryo (D). At stage 13, the number of *Dmyd*-expressing cells is extensively reduced in sr16 embryo, particularly in the dorsal region (compare E and F).

detected as *lacZ* gene expression (Paterson et al., 1991). *Dmyd*, also called *nautilus*, is a *Drosophila* homologue of the vertebrate *MyoD*-family genes, specifically expressed in a subset of muscle precursors (Michelson et al., 1990; Paterson et al., 1991). The effect of *sr16* on *Dmyd* expression was examined. At stages 12-13, the number of *Dmyd*-expressing cells was found extensively reduced, particularly, in the dorsal region (Fig. 9C-F). At later stages, reduction of muscle fiber content was evident under a microscope with Nomarski optics (data not shown). These results show *DFR1* possibly to be required for the formation or extension of muscle precursor cells.

DISCUSSION

The *Drosophila* genome includes two homologues of mammalian FGF-R genes, *DFR1* and *DFR2*. Their expression dynamically changes during embryogenesis. At earlier stages, *DFR1* is expressed in mesodermal primordium, and *DFR2* in the primordia of the anterior and posterior midguts. At later stages, *DFR1* RNA is expressed in developing tissues derived from mesoderm and neuroectoderm, including muscle precursors and CNS. *DFR2* RNA appears to be expressed in those tissues derived from ectoderm and mesectoderm, including the trachea, salivary tubes and CNS.

Structural and evolutional relationship between Drosophila and vertebrate FGF-Rs

A comparison of the amino acid sequences of kinase domains indicated *DFR1* to have about 80% and 60% homology with *DFR2* and any member of vertebrate FGF-Rs, respectively. *DFR1* and *DFR2* genes may thus quite

possibly be derivatives of a common ancestral arthropod gene, diverging from an ancient vertebrate FGF-R gene at the time of the major phylogenetic branch leading to arthropods and chordates.

Extracellular regions of *Drosophila* and vertebrate FGF-Rs are much more diversified than kinase regions. *DFR1* and *DFR2* have two and five Ig-domains, respectively. (Most members of the vertebrate FGF-R family contain three Ig-domains, while the remaining have two.) *DFR1* and *DFR2* may recognize different ligands. Our sequence analysis showed the extracellular region flanked by the acidic region and transmembrane domain to be considerably conserved in both sequence and organization. In particular, some N-glycosylation sites were found invariant in position among *DFR1*, *DFR2* and *flg* protein. (see Fig. 3). This region may possibly be essential for common functions of the FGF-R family members.

Possible roles of *DFR1* in mesoderm formation and subsequent differentiation of mesodermal cells

FGFs secreted by non-mesodermal cells may induce mesoderm formation in vertebrates (Jessell and Melton, 1992; Whitman and Melton, 1992) but, so far, nothing has been reported as to the possible involvement of FGF or FGFlike factors in mesoderm formation in *Drosophila*. Only the nuclear concentration of the *dorsal* protein is considered important in determining mesodermal fate in *Drosophila* (Roth et al., 1989; Thisse et al., 1991). Thus, our finding of specific expression of *DFR1* in mesodermal cells is of particular importance. Recent molecular-cloning experiments show that other mesodermal genes, which include *twist, snail* and *MyoD*, are also shared in common by *Drosophila* and vertebrates (St. Johnston and Nüsslein-Vol-

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hard, 1992; Michelson et al., 1990; Paterson et al., 1991) and, thus, it is reasonable to consider that *Drosophila* and vertebrates share in common some essential mechanisms underlying mesoderm formation and/or subsequent differentiation of mesodermal cells.

Our results suggest that DFR1 expression is not necessarily mandatory for ventral furrow formation. In its place, DFR1 would rather appear required for patterning of muscle precursor cells (somatic mesoderm), since DFR1 RNA is predominantly expressed in embryonic muscle precursor cells and its absence results in extensive reduction in the number of Dmyd/nau-expressing muscle precursor cells, particularly, in the dorsal region (see Fig. 9D,F). During the early stages of muscle formation, some muscle precursor cells migrate from the ventral to lateral or dorsal region so as to produce the muscle pattern (Dohrman et al., 1991). Although there is no direct evidence to date, muscle patterning is supposed to be specified by the underlying ectoderm (Bate, 1991). The above findings suggest the possible involvement of DFR1 (and maybe its ligand) in cell migration during muscle formation.

The *stripe* gene included in the *sr16* deletion is required for the normal development of adult flight muscles and CNS (De La Pompa et al., 1989). Although, at present, the relation between *stripe* and *DFR1* at the molecular level is unknown, similarity in expression/(mutant phenotype) and chromosomal locus suggest that they are genes intimately related, if not identical, to each other.

Possible involvement of FGF-receptors in CNS formation

In Drosophila, various members of an immunoglobulin superfamily are expressed in developing nervous system; they include fasciclins, neuroglian, receptor-type phosphatases and a Drosphila homologue of mammalian trk (Grenningloh et al., 1990; Yang et al., 1991; Tian et al., 1991; Pulido et al., 1992). Some are homologous in amino acid sequence to vertebrate neural cell adhesion molecules and proteins with Ig-domains may possibly essential for neural development. As seen in Fig. 7C, DFR2 RNA is expressed in most midline cells, which probably play a key role in the formation of axon commissures. DFR1 RNA expression may occur along a pair of logitudinal connectives (see Fig. 6H). Since neurotrophins and FGFs are possibly required for neural cell differentiation in vertebrates (Jessell and Melton, 1992), the above findings imply important functions of DFR1 and DFR2 proteins in CNS formation in Drosophila.

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