The *vrille* Gene of Drosophila Is a Maternal Enhancer of *decapentaplegic* and Encodes a New Member of the bZIP Family of Transcription Factors

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ABSTRACT

We report here the genetical and molecular characterization of a new Drosophila zygotic lethal locus, *vrille* (*vri*). *vri* alleles act not only as dominant maternal enhancers of embryonic dorsoventral patterning defects caused by *easter* and *decapentaplegic* (*dpp*) mutations, but also as dominant zygotic enhancers of *dpp* alleles for phenotypes in wing. The *vri* gene encodes a new member of the bZIP family of transcription factors closely related to gene *9* of *Xenopus laevis*, induced by thyroid hormone during the tadpole tail resorption program, and NF-IL3A, a human T cell transcription factor that transactivates the interleukin-3 promoter. NF-IL3A shares 93% similarity and 60% identity with Vri for a stretch of 68 amino acids that includes the bZIP domain. Although all the alleles tested behave like antimorphs, the dominant enhancement is also seen with a nonsense mutation allele that prevents translation of the bZIP domain. Because of the strong dominant enhancement of *dpp* phenotypes by *vri* alleles in both embryo and wing, and also the similarity between the wing vein phenotypes caused by the *vri* and *shortvein dpp* alleles, we postulate that *vri* interacts either directly or indirectly with certain components of the *dpp* (a TGFβ homologue) signal transduction pathway.

IN higher organisms, members of the transforming growth factor β (TGFβ) superfamily of signaling molecules control many aspects of cellular communication, such as cell division and determination of cell fate (reviewed by MASSAGUÉ *et al.* 1994; MIYAZONO *et al.* 1994). TGFβ ligands transduce their signal across cell membranes through two types of serine-threonine kinase receptors. TGFβ binds directly to the constitutively active type II receptor kinase leading to its association with the type I receptor. This results in the transphosphorylation of the type I receptor-specific GS domain. TGFβ also binds to nonsignaling proteoglycan cell surface receptors. These receptors, also called type III receptors, probably modulate the activity of growth factors. TGFβ activities include growth inhibition, expression of genes encoding nuclear transcription factors, production of extracellular matrix proteins and induction of apoptosis. TGFβ induces cell cycle arrest in mid to late G_1 phase of the cell cycle by inhibiting various cyclin dependant kinases (Cdk), which leads to the accumulation of the unphosphorylated growth suppressive form of the retinoblastoma anti-oncogene product (Rb). Members of the *Mothers against dpp* (*Mad*)-related genes (RAFTERY *et al.* 1995; SEKELSKY *et al.* 1995) transduce signals from TGFβ members in *Drosophila melanogaster* (NEWFELD *et al.* 1996; WIERSDORFF *et al.* 1996), *Caenorhabditis elegans* and in vertebrates (reviewed by WRANA and ATTISANO 1996). MAD proteins are associated with the ligand-bound receptor complex and following phosphorylation are translocated into the nucleus where they activate transcription. They could possibly mediate response to various TGFβ homologues by heterodimerization with other MAD proteins. The TGFβ gene, as well as genes for signaling pathway components, including the receptor complex and members of the MAD family, act as human tumor suppressor genes.

The *D. melanogaster dpp* gene encodes a TGFβ homologue closely related to the mammalian bone morphogenetic proteins BMP2 and BMP4 (PADGETT *et al.* 1987). *dpp* is required at numerous crucial stages of development. In the early embryo *dpp* acts as a morphogen for the specification of cell fate along the dorsoventral axis (FERGUSON and ANDERSON 1992b; WHARTON *et al.* 1993). A gradient of *dpp* activity specifies a precise pattern in the dorsal 40% of the dorsoventral axis. The dorsal-most cells of the blastoderm embryo are specified as amnioserosa by high levels of *dpp* activity, whereas lower levels specify the cells in the dorso-lateral region that will differentiate as dorsal ectoderm. In null *dpp* mutants all cells in the dorsal half of the embryo adopt a ventral fate and differentiate as ventral ectoderm (IRISH and GELBART 1987). Later in embryogenesis, *dpp* is required for setting aside the imaginal disk precursor nests from adjacent epidermal cells (COHEN *et al.* 1993), for dorsal mesoderm specification and endoderm morphogenesis (IMMERGLUCK *et al.* 1990; PANGANIBAN *et al.* 1990; HURSH *et al.* 1993; STAEHLING-HAMPTON *et al.* 1994). Finally *dpp* is required during the larval and pupal stages for the proximo-distal and antero-pos-

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terior patterning of adult appendages (BASLER and STRUHL 1994) and for the outgrowth of imaginal disks (SPENCER *et al.* 1982).

Genes, other than *dpp*, that are required for correct embryonic dorsal and dorso-lateral cell specification include *zerknüllt* (a homeobox transcription factor), *shrew*, *tolloid* (a BMP1 homologue), *screw* (a TGFβ homologue), *short gastrulation* and *twisted gastrulation* (SHIMELL *et al.* 1991; ARORA and NÜSSLEIN-VOLHARD 1992; FERGUSON and ANDERSON 1992a,b; WHARTON *et al.* 1993; ARORA *et al.* 1994). DPP serine-threonine kinase receptors have been identified. The *saxophone* and *thick veins* genes encode type I transmembrane receptors (BRUMMEL *et al.* 1994; NELLEN *et al.* 1994; PENTON *et al.* 1994; XIE *et al.* 1994), while the *punt* gene encodes a type II receptor (CHILDS *et al.* 1993; LETSOU *et al.* 1995; RUBERTE *et al.* 1995). Genes other than *Mad* acting downstream in the *dpp* pathway have been identified. *schnurri* (ARORA *et al.* 1995; GRIEDER *et al.* 1995; STAEHLING-HAMPTON *et al.* 1995) encodes a zinc finger transcription factor. *shortsighted*, required for eye development, encodes a homologue of the TSC-22 protein induced, in mammals, in response to TGFβ (TREISMAN *et al.* 1995). *spalt* and *spalt-related*, two zinc finger proteins, and *optomotor-blind*, a member of the T-box family of transcription factors, are target genes for *dpp* signaling in the developing wing (DE CELIS *et al.* 1996; LECUIT *et al.* 1996; NELLEN *et al.* 1996).

In this article we describe a new gene, *vri*, mutations in which act as dominant maternal enhancers of the ventralization caused by *dpp* mutations. The gene is expressed throughout development. Transcripts are provided maternally and zygotic transcripts are detected in the pharynx, foregut, and hindgut of the embryo, and in the larval gut and disks. Some of these localizations are compatible with a requirement for *vri* in larval gut development. Furthermore, *vri* alleles result in shortened wing vein phenotypes very similar to the defects caused by *shortvein dpp* alleles and enhance *dpp* phenotypes in the wing, suggesting a requirement for *vri* in wing vein differentiation. We have cloned the *vri* gene and show that it encodes a new member of the bZIP family of transcription factors homologous to Xenopus and human proteins required in cell death and growth events, respectively. We propose that *vri* could be a new component of, or could interfere with, the *dpp* signal transduction pathway.

MATERIALS AND METHODS

Strains and genetic analysis: Flies were reared on standard media and all crosses were performed at 25°. The wild-type strain used was Oregon R. 25D rearrangements are described in REUTER and SZIDONYA (1983) and SZIDONYA and REUTER (1988). vri^1 and vri^2 correspond, respectively, to the $l(2)jf23^{Sz7}$ and $l(2)jf23^{Sz36}$ lethal EMS-induced mutations isolated by SZIDONYA and REUTER (1988). vri^3 corresponds to the maternal effect EMS-induced mutation, *mat(2)earlyRS32* (SCHÜPBACH and WIESCHAUS 1989). The lethal *P* stocks are described in FLYBASE (1994), and their localization, by *in situ* hybridizations, were performed by the Berkeley Drosophila Genome Project (BDGP) (personal communication). The isolation of *l(2)k16713* (25D1-2) and the *vri* lethal enhancer trap lines are described in TÖRÖK *et al.* (1993). The vri^P alleles were renamed vri^4, *l(2)k03801* (25D4-6); vri^5, *l(2)k05901* (25D4-5); vri^6, *l(2)k09602* (25D4-6); vri^7, *l(2)k09713* (25D4-5); and vri^8, *l(2)k11805* (25D4-5). The isolation of the lethal enhancer trap *P* stocks, *l(2)04415* (25D1-2) and *l(2)03771* (25D4-6), is described in MLODZIK and HIROMI (1992). These stocks were provided by the Bloomington Stock Center and the BDGP. The *tkv* strains are described in TERRACOL and LENGYEL (1994). dpp^{hr27} *and* dpp^{hr4} are described in SPENCER *et al.* (1982), dpp^{d6} in LINDSLEY and ZIMM (1992), Mad^6 in SEKELSKY *et al.* (1995) and $ea^{161.13}$ in JIN and ANDERSON (1990). shn^{IB} and put^{135} (JÜRGENS *et al.* 1984; NUSSLEIN-VOLHARD *et al.* 1984) were provided by the Tübingen Stock Center. Embryonic cuticles were prepared as described in WIESCHAUS and NÜSSLEIN-VOLHARD (1986). Wings were dissected, collected in 70% ethanol and mounted in Euparal. Balancer chromosomes and w^{1118} strain are described in LINDSLEY and ZIMM (1992). The *vri P* elements were mobilized by providing the Δ2-3 external source of transposase (ROBERTSON *et al.* 1988).

Germline clones: To generate mutant germline clones we recombined *vri* alleles onto a *P[hs-neo; FRT]* 40A chromosome and used a $P[ovo^{D1}]$, *P[hs-neo; FRT]* 40A (CHOU *et al.* 1993) second chromosome (Bloomington Stock Center). Germline clones were heat shock induced by 1-hr exposure to 38° during the larval period. FLP-induced germline clone-bearing females were fertilized with *vri/CyO* or wild-type males.

Genomic and cDNA libraries: Nucleic acids preparation: The chromosomal walk was performed using the CoSpeR iso-1 cosmid and λEMBL3 libraries made from Canton S genomic DNA (TAMKUN *et al.* 1992). P1 clones were provided by M. ASHBURNER and M. MLODZIK. P1 phage DNA was prepared according to SMOLLER *et al.* (1991). Embryonic Oregon R cDNAs were isolated from 0–3-hr, 3–12-hr (POOLE *et al.* 1985) and 1.5–5-hr (gift from M. GOLDSCHMIDT-CLERMONT) λgt10 libraries and from 0–4-hr, 4–12-hr pNB40 libraries prepared from an isogenic *dp cn bw* strain (BROWN and KAFATOS 1988). RNAs were extracted with pH 5 hot phenol at 60° (SATO *et al.* 1982), enriched for poly A^+ RNAs by oligo dT cellulose chromatography and run in 1% formaldehyde agarose gels. Northern blots were hybridized in 50% formamide, 5× SSC, 5× Denhardt's, 0.1% SDS at 42° and then washed at high stringency. Plasmid rescue was performed according to PIRROTTA (1986).

DNA sequencing: Genomic DNA and cDNA were subcloned into the pBluescript KS+ vector (Stratagene). Nested exonuclease III deletions were selected (Pharmacia kit) and sequenced (U.S. Biochemicals sequencing kit) by the dideoxy-chain termination method (SANGER *et al.* 1977). The sense cDNA strands and the antisense genomic strand were sequenced. 5′ end clones were obtained by two different techniques. The first consisted in the synthesis of cDNA 5′ ends from 8–12-hr embryonic polyA+ RNA followed by the rapid amplification of cDNA ends (5′RACE) by PCR (FROHMAN 1990). The second method consisted in 5′ end amplification from a directionally cloned 8–12 hr cDNA embryonic library (BROWN and KAFATOS 1988). In this case the 5′ end primer used was the SP6 primer from the pNB40 plasmid vector of the library. The first 3′ primer for 5′ RACE was 5′ACTCAG CACCTGTTCGCT3′ between positions 1268 and 1251 of the *vri* cDNA sequence (Figure 4). Primer 1 used to screen cDNA library was 5′TCTTCTCTCGACTCCGCTT3′ from position 1095 to 1077, and the second round of amplification was performed using a second internal primer, identical for both types of experiment: 5′GTTCCTTCTCCGGCGATC3′ from

position 1064 to 1047. EMS mutant DNA was amplified by PCR between positions 895 and 1606. Primer 1 was 5′GAAGCGGCTATGGAGATG3′ from positions 895 and 912 and primer 2, 5′TGTTGCTGCGGTGGCTGGGA3′ between positions 1606 and 1587. PCR products were cloned using the TA cloning kit (Invitrogen) and sequenced (Fidelity DNA sequencing system, Appligene). DNA sequences were compiled using the Genetics Computer Group software: GCG (DEVEREUX *et al.* 1984). The deduced amino acid sequences were compared with the GenPept database using the FASTA program (PEARSON and LIPMAN 1984).

Whole mount *in situ* hybridizations: Whole mount *in situ* hybridizations were performed, in embryos and ovaries, according to TAUTZ and PFEIFLE (1989) and MÉVEL-NINIO *et al.* (1991) and in imaginal disks according to MASUCCI *et al.* (1990). Purified restriction fragments were used to synthesize digoxigenin labeled probes (Boehringer Mannheim labeling kit).

Transformation experiments: Genomic DNA was cloned into *Pw6* or *Pw8* mini *white P*-element vectors (KLEMENZ *et al.* 1987). The *vri* cDNA4-7 was obtained after ligation between cDNA4 and cDNA7 at their *Eco*RI sites. It was cloned into the CaSpeR-*Hsp70* transformation vector (PIRROTTA 1988). DNA was injected with Δ2-3 helper transposase in pole cell region of w^{1118} preblastoderm embryos (SPRADLING and RUBIN 1982). The GenBank accession numbers for the sequence reported in this article are U92867 and Y11837.

RESULTS

***vri* embryonic lethal phenotypes:** The *vri* gene is located in the 25D region around map position 2-16. It is included in $Df(2L)tkv^{Sz2}$ (25D2-4; 25D6-E1) and excluded from the overlapping $Df(2L)cl^{h2}$ (25D5-6; 25F4-5) and $Df(2L)cl^{7}$ (25D5-6; 26A7-8) (SZIDONYA and REUTER 1988). The two initial *vri* mutations, $l(2)jf23^{Sz7}$ and $l(2)jf23^{Sz36}$, were previously recovered in a screen as lethal EMS-induced mutations on the second chromosome (SZIDONYA and REUTER 1988). They were renamed here vri^{1} and vri^{2}, respectively. Five other *vri* alleles were recovered by testing the *P*-induced lethal stocks included in $Df(2L)tkv^{Sz2}$. $Df(2L)tkv^{Sz2}$ includes at least four complementation groups: distally *tkv, vri,* and *l(2)03771,* and proximally, *l(2)jf24,* also included within $Df(2L)cl^{h2}$ and $Df(2L)cl^{7}$ (SZIDONYA and REUTER 1988). Consequently, $Df(2L)tkv^{Sz2}$ totally deletes *vri* and represents a null allele for *tkv, vri, l(2)03771* and probably *l(2)jf24*. $Df(2L)tkv^{Sz2}$ homozygous embryos show the typical *tkv* null phenotype (NÜSSLEIN-VOLHARD *et al.* 1984; TERRACOL and LENGYEL 1994) with no dorsal closure and head involution resulting in the absence of dorsal epidermis. In developing embryos, no defects are observed before dorsal closure. Principally, at the blastoderm stage the expression of the *zen* protein is normal and no obvious gastrulation defects are observed (TERRACOL and LENGYEL 1994).

The *vri* lethal mutants die in the embryo and, except with vri^{8}, no dominant maternal lethality is detectable when *vri/Cy* females are crossed to wild-type males. vri^{1}, vri^{2} as well as vri^{1}/vri^{2} homozygous embryos have very similar phenotypes. The embryos are shortened and the dorsal epidermis often appears wrinkled and reduced, and tracheae are interrupted. This dorsal shortening leads to a slight "tail up" phenotype (Figure 1B). Filzkörper are often internal (Figure 1C). Less frequently the head skeleton is abnormal and ventral denticles are fused or missing. The vri^{P} alleles result in similar phenotypes but these latter are stronger than those described above. The head skeleton is almost always defective (Figure 1D). In some embryos the germ band remains extended, suggesting defects in germ band retraction. Some embryos are convoluted, with ventral denticles extended laterally and Filzkörper internal and presenting an abnormal morphology (Figure 1E). This phenotype is similar to those observed in weakly ventralized embryos. These latter phenotypes have a low penetrance, however, and for this reason we were unable to determine (using the expression of *zen*) whether they result from defects occurring in the early stages of establishment of dorsoventral polarity or later on. All five *vri P* elements were mobilized in the presence of transposase leading to complete reversion of lethality and viability, which confirms that the phenotypes observed are due to the *P* insertion only. The vri^{P} alleles are probably not null alleles since the mutants show apparent defects in dorsoventral polarity, not observed with the tkv^{Sz2} null allele.

The zygotic phenotype of the total absence of the *vri* gene is difficult to detect, however, due to the presence of at least three other embryonic lethal genes within the smallest available deficiency, $Df(2L)tkv^{Sz2}$, mainly the *tkv* gene itself presenting strong head and dorsal cuticular defects. *l(2)03771,* represented by a single *P*-induced mutation, shows weakly ventralizing embryonic phenotypes and is clearly not a null allele. The *l(2)jf24* group includes five embryonic lethal mutant alleles with no detectable cuticle abnormalities. To obtain a better view of the null *vri* phenotype we observed the $Df(2L)tkv^{Sz2}$ homozygous phenotype in the presence of a *tkv* cDNA transgene, *P[Ubi-tkv-2]* (see Figure 3), which is capable of total rescue of *tkv* null mutants (BRUMMEL *et al.* 1994; G. MARQUÉS, personal communication). $Df(2L)tkv^{Sz2}/Df(2L)tkv^{Sz2}$; $P[Ubi\text{-}tkv\text{-}2]^{17}/+$ or $Df(2L)tkv^{Sz2}/Df(2L)tkv^{Sz3}$; $P[Ubi\text{-}tkv\text{-}2]^{17}/+$ (tkv^{Sz2} and tkv^{Sz3} deficiencies overlap in the *tkv* and *vri* region only) embryos die and possess a phenotype very similar to the homozygous phenotypes of vri^{1} or vri^{2}, which can therefore be considered null alleles. $vri/Df(2L)tkv^{Sz2}$ hemizygous progeny hatch, however, and die as larvae. Thus, the phenotype appears weaker when hemizygous than when homozygous. Although this delayed lethality could be due to a background effect, it would suggest that the alleles are not null or even hypomorphic, but rather neomorphic or antimorphic. Alternatively, it could be that one gene within the deficiency acts as a dominant suppressor of the embryonic lethality. The same results are observed with the *P*-induced alleles. To determine whether the alleles are neomorphic or antimorphic, we used a duplication of the 25D region (SZIDONYA and

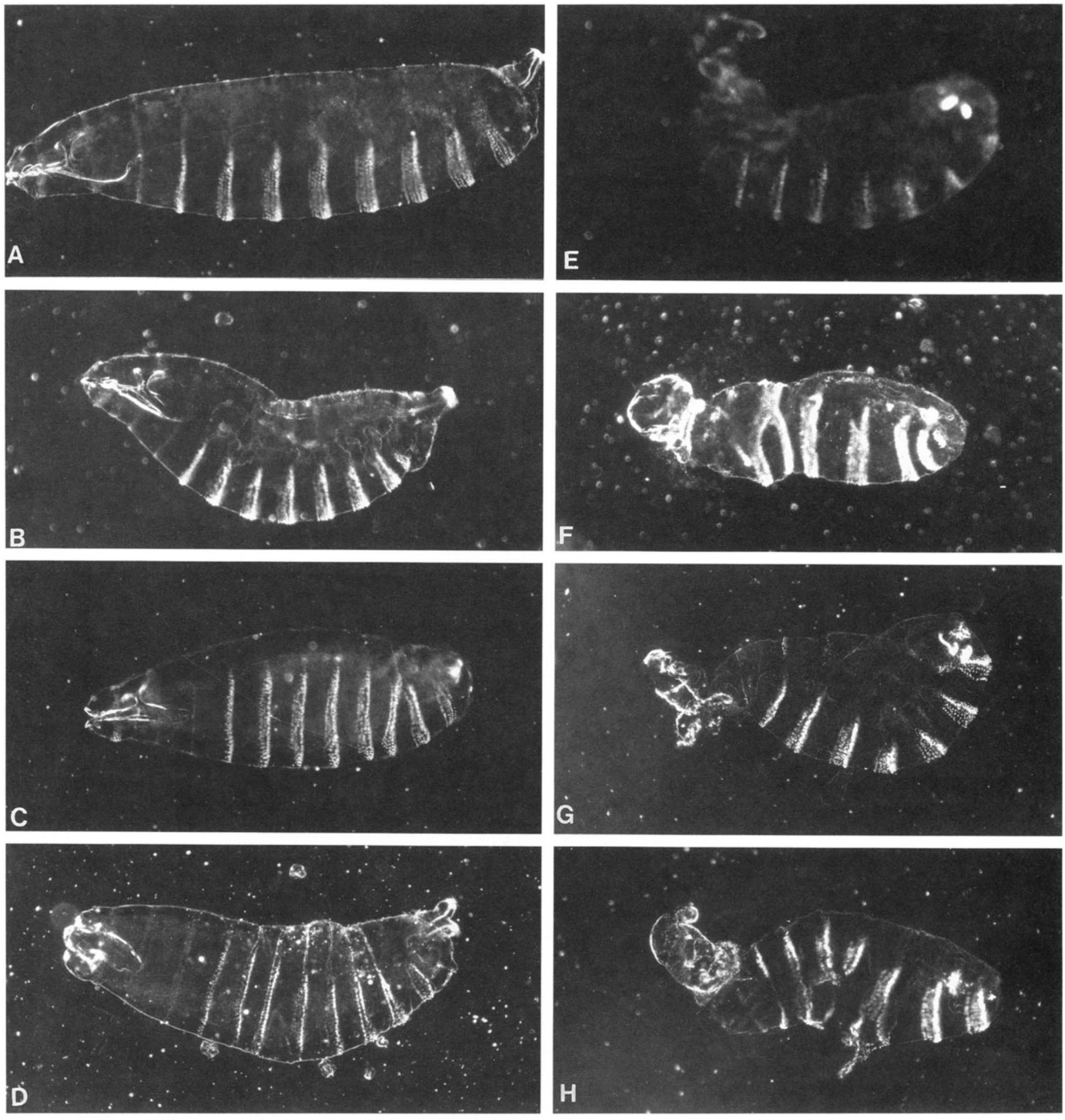

FIGURE 1.—Embryonic phenotypes. The embryos are oriented with anterior to the left and dorsal up. A wild-type larva is shown in A. (B–E) *vri* homozygous embryos. (B) vri^2/vri^2 embryo with wrinkled dorsal epidermis and interrupted tracheas. (C) vri^2/vri^2 embryo with internal Filzkörper. (D) vri^5/vri^5 embryo. The involution of the head is abnormal and the ventral denticles are compressed. (E) Weakly ventralized vri^6/vri^6 embryo with internal and abnormal Filzkörper (V4). (F) Moderately ventralized (V3) embryo from vri^5+; $ea^{161.13}$/+ females crossed to wild-type males. (G) V4 and (H) V3 ventralized embryos from vri^5/CyO females crossed to dpp^{hr27}/CyO males. The ventralized phenotypes are classified according to ROTH *et al.* (1991).

REUTER 1988). *Dp(2;1)B19* (9B/25F2;24D4/9C)/+; $vri/Df(2L)sc^{19\text{-}12}$ (25A4-5; 26A6-B1) as well as the *Dp(2;1)B19*/+; *vri/vri* flies are viable in every case, showing that none of the seven alleles is neomorphic. One hypothesis to explain why the hemizygous progeny die as larvae whereas homozygotes do not hatch is that the product of these alleles, including vri^1 and vri^2, is able to antagonize itself or the wild-type maternal product and therefore has a stronger effect than the total absence of Vri.

To recover new alleles and principally to confirm the null phenotype, a secondary *P*-induced mutagenesis was performed by excision of the vri^5P transposon. Among the 23 *w* lethal lines selected, of which nine are inde-

pendent (originating from different males), two categories of embryonic phenotype are observed. Seventeen lines show phenotypes similar to the vri^5 initial phenotype (with weakly ventralized phenotypes), and six show a phenotype similar to that of vri^1, vri^2 or $Df(2L)tkv^{Sz2}/Df(2L)tkv^{Sz2}$; $P[Ubi\text{-}tkv\text{-}2]^{17}/+$. These lines probably occurred by imperfect excision of the *P* element leading to deletions in the genomic flanking regions and could thus correspond to null alleles. Again, with all six lines, $vri/Df(2L)tkv^{Sz2}$ hemizygous progeny hatch and die as larvae.

***vri* mutations affect wing vein differentiation:** In a search for other genes reported in the 25D region, we focused our interest on the *mat(2)earlyRS32* locus, which is uncovered by $Df(2L)cl^1$ (25D2-4; 25F2-4) and is excluded from $Df(2L)cl^7$ (25D5-6; 26A7-8) like *tkv*, *vri* and *l(2)03771* and which was isolated as a recessive maternal effect mutation (SCHÜPBACH and WIESCHAUS 1989). Homozygous females lay eggs that do not develop and have the appearance of old unfertilized eggs. Homozygous *RS32* flies have wing defects consisting in a shortening of the L5 vein (Figure 2B), a phenotype also seen in $RS32/vri^1$ and $RS32/vri^2$ flies (Figure 2C). The effect is not totally penetrant with the other *vri* alleles. $RS32/Df(2L)tkv^{Sz2}$ flies show shortening of L2 and L5 wing veins (Figure 2D). Consequently, *mat(2)earlyRS32* is a new hypomorphic *vri* allele, which we renamed vri^3. This wing vein phenotype is reminiscent of that observed in *shortvein* alleles of *dpp* (SEGAL and GELBART 1985). In the *shortvein* phenotype all types of vein can be affected but a hierarchy in the frequency of occurrence was observed: L2 > L4 > L5 > L3.

***vri* enhances the maternal ventralizing effect of *easter*:** To test for a maternal requirement for *vri* in establishing the dorsoventral pattern of the embryo, we generated germline clones. As shown in Table 1 with vri^5, a large number of undeveloped eggs were recovered, which is probably due to defects in the maternal components necessary for fertilization or early cleavages. Nevertheless, since this phenotype appears to be rescued by a paternal contribution it is unlikely that it is due to a defect in fertilization. This phenotype is similar to the vri^3 maternal phenotype, although vri^3/vri^3 females lay 100% undeveloped eggs. It is possible that vri^3 alters a maternal-specific product while vri^5 alters mostly a zygotic component. In the second type of cross only vri^5/CyO progeny survive to adulthood. No strongly ventralized embryos are recovered and the dead embryos have phenotypes not significantly different from the zygotic phenotypes observed. Similar results are observed with vri^1 or vri^2 (not included in the Table 1). From this experiment it was not possible to detect any maternal component specifically required for establishment of dorsoventral polarity of the embryo.

To investigate further a maternal requirement in the specification of the dorsoventral axis, the different alleles were associated in double heterozygotes with a ventralizing *easter* allele (JIN and ANDERSON 1990). This test was previously used to detect a maternal requirement for *tkv* in the establishment of dorsoventral polarity of the embryo (TERRACOL and LENGYEL 1994). As shown in Table 2, 62% of the embryos from $ea^{161.13}/TM3$ females crossed to wild-type males die and are very weakly ventralized (V5). As previously described (TERRACOL and LENGYEL 1994) these embryos show a failure in head involution and 25% of them have a tail up phenotype due to an incomplete retraction of the germ band. When the *vri* alleles are associated maternally with the *ea* allele the lethality is greatly increased, up to 100% with the vri^5 and vri^7 alleles, and the phenotypes of the embryos are more strongly ventralized. Two new categories of ventralized embryos are found. Weakly ventralized embryos (V4) are observed, in which head involution has not occurred and the posterior segments are internalized with disorganized Filzkörper. Moderately ventralized embryos (V3) are also observed with a lateral extension of ventral denticle belts, disorganized and reduced Filzkörper, with posterior segments internalized, head structures missing and a convoluted thorax (Figure 1F). This enhancement is still observable with the six revertant lines obtained after vri^5 *P*-element excision and with phenotypes similar to vri^1 or vri^2 phenotype (vri^{5Rx}). It is noteworthy that the effect of $Df(2L)tkv^{Sz2}$, although it includes the two genes, *tkv* and *vri*, capable of enhancing *ea*, is no stronger than when *tkv* or *vri* single mutations are used. However, an increase in ventralization is still observable in eggs from $Df(2L)tkv^{Sz2}/+$; $P[Ubi\text{-}tkv\text{-}2]^{17}/ea$ females. One explanation for the stronger dominant maternal effect observed with point or regulation mutations would be that it is due to an antimorphic effect of both *tkv* and *vri* alleles. For comparison, two mutant alleles of genes of the *dpp* signal transduction pathway were tested, in the same context: *schnurri*, a transcription factor homologous to the human MBP family members (ARORA *et al.* 1995; GRIEDER *et al.* 1995), and *punt*, a type II TGFβ receptor (LETSOU *et al.* 1995; RUBERTE *et al.* 1995). shn^{IB} and put^{135} show a similar, although weaker, effect when compared with the *vri* alleles (Table 2). For instance, while the total number of dead embryos is >90%, shn^{IB} produces only 1% V3 embryos and put^{135} produces no V3 embryos at all. A maternal ventralizing effect was previously reported, by the germline clone procedure, for *punt*, but not for *schnurri*. In the case of *shn*, this discrepancy between the results of the two approaches may be due to the fact that *shn* acts in concert with another factor. The enhancement of *vri* is also stronger when compared with the effect of *tkv*, since among the five strong *tkv* alleles studied, including the deficiency, only 1% V3 embryos are obtained (TERRACOL and LENGYEL 1994). Therefore, *vri* is a stronger maternal enhancer of ventralization by *ea* than are the downstream components of the *dpp* pathway, *tkv*, *shn*, and *put*.

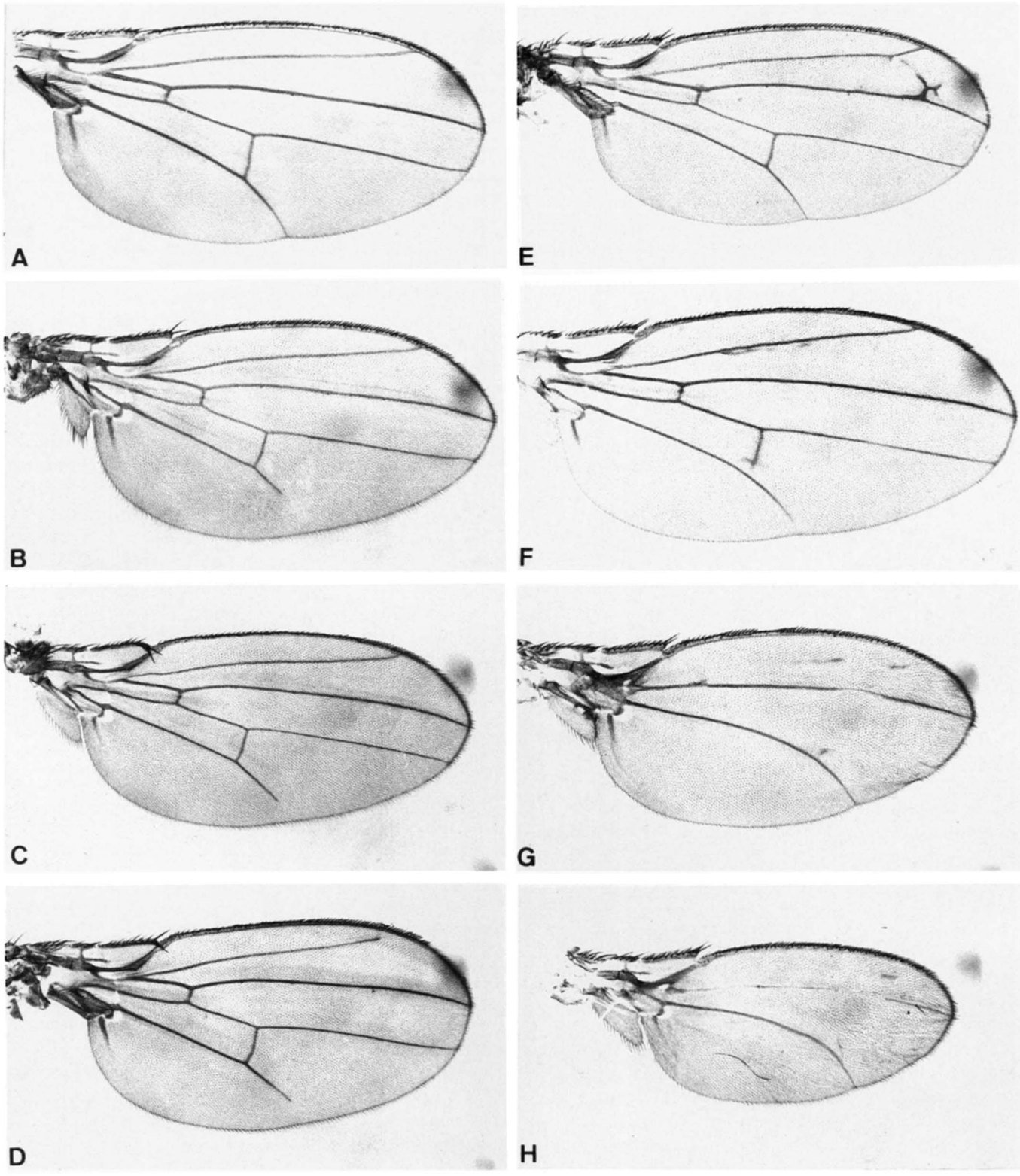

FIGURE 2.—*vri* wing vein phenotypes. (A) Wild-type wing. (B) vri^3/vri^3 wing with shorter L5 vein. (C) vri^3/vri^1 wing showing L5 vein shortening. (D) $vri^3/Df(2L)tkv^{Sz2}$ wing showing a shortening of both L2 and L5 veins. (E) $dpp^{hr27}+/+vri^7$ wing with extra vein material between L2 and L3. (F) $Mad^6+/+vri^2$ wing showing extra vein material along L2, shorter posterior crossvein and L5 vein. (G) dpp^{hr4}/dpp^{d6} wing, smaller than normal with reduced vein material. (H) $dpp^{hr4}vri^2/dpp^{d6}+$ wing, which is smaller and display more reduced vein material than the control (G).

TABLE 1

Germline clones

Genotype	Females screened	Total eggs	% hatched	% undeveloped	% abnormal
vri5/vri5 × +	20	569	88	8	3
vri5/vri5 × *vri5/CyO*	29	965	58	35	7

Experimental procedure is described in MATERIALS AND METHODS.

***vri* acts as a dominant maternal enhancer of *decapentaplegic*:** In a further search for a maternal and possibly a zygotic ventralizing product, we associated *vri* and *dpp* alleles in double heterozygotes. As shown in Table 3, when the vri^{1} allele was provided by the mother, 27% of the expected progeny was recovered when the allele provided by the father was dpp^{hr27}, and 45% with a weaker allele, dpp^{hr4}. With vri^{2} the values were 35% and 73%, respectively. When the vri^{4} and vri^{5} alleles were used in the same conditions with dpp^{hr27}, no dpp^{hr27} +/ + vri^{P} flies were observed. Similarly, when the dpp^{hr4} allele was used, 30% and 2% of the expected *dpp/vri* progeny were recovered, respectively. With the three other *vri* alleles, between 2% and 5% of the expected *dpp/vri* progeny were recovered with dpp^{hr27} and 23–39% with dpp^{hr4}. A similar decrease is generally observed in the *dpp/Cy* class, which indicates a strict maternal effect. This is confirmed by the reciprocal crosses in which, except with the vri^{4} allele, no significant effect is detected beyond a slight decrease attributable to the haploinsufficient effect of *dpp* (WHARTON *et al.* 1993). The six *w* lethal lines obtained after excision of the vri^{5} *P* element and with phenotypes similar to vri^{1} or vri^{2} phenotype (vri^{5Rx}) also strongly interact maternally with *dpp*. It is noteworthy that neither $Df(2L)tkv^{Sz2}$/+ nor $Df(2L)tkv^{Sz2}$/+; $P[Ubi\text{-}tkv\text{-}2]^{17}$/+ is able to enhance *dpp* maternally. Therefore, the strong maternal enhancement of *dpp*, by both *tkv* and *vri* point or *P* element mutations is probably due, as previously reported, to an antimorphic effect. The lethality is embryonic and these embryos are ventralized, with phenotypes varying from V4 to V3 (Figure 1, G and H); the haploinsuffi-

TABLE 2

Ventralizing maternal effect of *vri* alleles compared to alleles of genes of the *dpp* signal transduction pathway

Allele	% of dead embryos	Phenotypes (%) V5	V4	V3
$ea^{161.13}$	62	100	0	0
vri^{1}	99	34	61	5
vri^{2}	95	30	66	4
vri^{4}	95	7	83	10
vri^{5}	100	5	88	7
vri^{6}	99	8	80	12
vri^{7}	100	5	84	11
vri^{8}	90	7	84	9
$vri^{5R1.5}$	98	8	86	6
$vri^{5R4.11}$	97	10	85	5
$vri^{5R5.7}$	100	6	87	7
$vri^{5R5.24}$	100	5	87	8
$vri^{5R7.2}$	100	7	85	8
$vri^{5R8.4}$	96	11	84	5
tkv^{Sz2}	97	43	56	1
tkv^{Sz2}; $P[tkv^{+}]$	95	45	52	3
shn^{IB}	99	17	82	1
put^{135}	90	60	40	0

In the control, $ea^{161.13}$/*TM3* females were crossed to wild-type males. The other genes were combined maternally in double heterozygotes with $ea^{161.13}$ and crossed to wild-type males. About 300 embryos were counted in every experiment. The phenotypic categories are described in the text according to the nomenclature of ROTH *et al.* (1991).

TABLE 3

Maternal enhancement of *dpp* effects by *vri* alleles

Allele	From	% of expected *vri/dpp* adult progeny dpp^{hr27}	dpp^{hr4}
vri^{1}	F	27	45
	M	79	86
vri^{2}	F	35	73
	M	85	82
vri^{4}	F	0	30
	M	101	41
vri^{5}	F	0	2
	M	82	85
vri^{6}	F	5	39
	M	87	62
vri^{7}	F	3	23
	M	73	64
vri^{8}	F	2	34
	M	113	87
$vri^{5R1.5}$	F	4	—
$vri^{5R4.11}$	F	2	—
$vri^{5R5.7}$	F	0	—
$vri^{5R5.24}$	F	0	—
$vri^{5R7.2}$	F	0	—
$vri^{5R8.4}$	F	5	—
tkv^{Sz2}	F	92	—
tkv^{Sz2}; $P[tkv^{+}]$	F	99	—

The values are percentages of *vri/dpp* adult flies compared with the number of *vri/Cy* control progeny. To discriminate between *vri/Cy* and *dpp/Cy* progeny, two types of balancer chromosomes, *CyO* and *SM6b*, were used. At least 300 flies were counted in every experiment.

cient effect of dpp^{hr27} alone gives only V5 embryos. The more strongly ventralized embryos observed (Figure 1H) are similar to dpp^{hr27}/dpp^{hr27} homozygous embryos (V3). In comparison, in the same conditions, *tkv* point mutation alleles give only V4 embryos (TERRACOL and LENGYEL, 1994). *vri* mutations are thus stronger dominant maternal enhancers of *dpp* than *tkv* alleles.

***vri* interacts with *dpp* and *Mad* in adult flies:** The escaper + *vri*/*dpp* + adult flies from the previous experiment often show (~10%) split thorax, atrophic wings and wing vein defects (Figure 2E). We also observed that *Mad* acts as a dominant enhancer of *vri* phenotypes in wing. About 10% of Mad^{6} +/+ vri^{2} flies show a wing phenotype. The L5 vein is shortened and sometimes the posterior cross vein is also shortened and extra vein material is observed along the L2 vein (Figure 2F). The same phenotype is observed with vri^{1} whereas with the other alleles the effect is weaker. Since this phenotype in not observed in the *Mad*/+ and *vri*/+ controls, we conclude that it is due to the association of both genes. To investigate a possible interaction between *vri* and *dpp* in wing, we tested for a dominant effect of *vri* in a dpp^{-} context. The dpp^{hr4}/dpp^{d6} phenotype previously described by SEKELSKY *et al.* (1995) consists of a reduction of wing to about one half of the wild-type size (Figure 2G) and no defects in eyes or legs. When one dose of *vri* is associated with this genotype, in dpp^{hr4} vri^{2}/dpp^{d6} + flies a further reduction in wing size is observed with reduction of veins (Figure 2H). Furthermore, eyes are smaller with a rough aspect and legs are truncated (not shown). The enhancement of *dpp* phenotypes by vri^{2} is always observed, although the strength of the enhancement is variable. The same phenotypes are observed with vri^{1}.

Walk in the 25D chromosomal region: *Cloning of the vri gene:* The *vri* gene was isolated within a walk in the 25D region (Figure 3). The walk was initiated from the previously isolated IB150 phage DNA, which contains the *bsg25D* gene and the adjacent 4.4-kb transcript (here renamed e1). They both map to the 25D3 band (ROARK *et al.* 1985; BOYER *et al.* 1987). Genomic DNA was isolated from cosmid and phage libraries (TAMKUN *et al.* 1992). The two overlapping P1 phage clones (SMOLLER *et al.* 1991), DS06813 (25D4-7) and DS000714 (25D4-E1), were mapped at the proximal end of the walk. They appear to have the same distal end and we confirmed their initial localizations. The *thick veins* gene was relocated at the distal end of the walk in the 25D1–3 interval. The 5′ ends of two *tkv* cDNAs, tkv-1 and tkv-2 (BRUMMEL *et al.* 1994), were mapped within the phages λ123 and λ411, respectively. The 5′ end of tkv-2 maps very close to the 3′ end of the *bsg25D* gene (less than 400 bp), in a previously sequenced region (BOYER *et al.* 1987). The tkv-2 first intron was shown to extend over 30 kb, and the *tkv* gene itself over at least 50 kb. Three new adjacent embryonic cDNA groups, e2, e3 and e4, were isolated and correspond to two or three new genes. The 25D $Tp(2;3)tkv^{Sz3}$ (25A2-3;25D5-E1;69C) breakpoint (REUTER and SZIDONYA 1983) maps at the 3′ untranslated end of e2 cDNA (Figure 3). The transposition leads to a *tkv* mutation (REUTER and SZIDONYA 1983; TERRACOL and LENGYEL 1994) and is rescued by a *tkv*+ transgene. However, the *tkv* gene maps 40 kb upstream and both tkv-1 and tkv-2 cDNA transgenes (Figure 3) as well as a genomic transgene that includes sequences coding for tkv-1 cDNA are able to rescue the *tkv* phenotypes (BRUMMEL *et al.* 1994; G. MARQUÉS, personal communication; PENTON *et al.* 1994; Y. CHEN, personal communication). Therefore the *tkv* phenotype of the transposition is not due to the breakpoint but probably to another mutation within the *tkv* gene or to a partial inactivation of the gene consecutive to its new location.

Eight lethal *P*-insertion mutations fail to complement $Df(2L)tkv^{Sz2}$ (25D2-4; 25D6-E1) but complement $Df(2L)cl^{h2}$ (25D5-6;25F4-5) and $Df(2L)cl^{7}$ (25D5-6;26A7-8), and therefore map in the same interval as *tkv* and *vri*. They correspond to three complementation groups. These *P* insertions were mapped by plasmid rescue and their precise insertion site was sequenced with an internal 5′ end *P* primer. Two of them, *l(2)04415* and *l(2)k16713*, previously positioned within the 25D1-2 interval, fail to complement *tkv* null mutations and are new hypomorphic embryonic lethal *tkv* alleles. We mapped *l(2)04415* at the 5′ noncoding end of tkv-1 cDNA, at position 73, corresponding also to the second tkv-2 intron. *l(2)16713* maps 600 bp upstream of *l(2)04415* insertion site, within tkv-2 second intron.

Three vri^{P} alleles (25D4-6), *l(2)k03801:*vri^{4}, *l(2)k09602:*vri^{6} and *l(2)k11805:*vri^{8}, were mapped upstream of the longest e2 cDNA 5′ end, at positions −57 for vri^{8} and −66 for both vri^{4} and vri^{6}. In the vri^{4} allele a change from T to C at position −75 was also detected. The two other alleles, *l(2)k05901:*vri^{5} and *l(2)k09713:*vri^{7} (25D4-5), were mapped within the first e2 intron at positions 757 and 838, respectively, from the 5′ end of intron 1. Mobilization of all *vri P* elements led to reversion of lethality and viability, showing that the transposons were responsible for the mutant phenotypes. e2 cDNAs thus probably correspond to the *vri* gene. $Tp(2;3)tkv^{Sz3}$ complements all *vri* alleles and therefore does not alter *vri*, which shows that the gene does not extend beyond e2 cDNA 3′ end. Attempts to rescue the *vri* lethal phenotype, however, were unsuccessful. Three genomic transgenes, *Pw6*[X5], *Pw8*[SK10] and *Pw8*[K17], containing 5-, 10- and 17-kb DNA insertions (Figure 3) and the CaSper-[*Hsp70*-cDNA4-7] transgene including a coding sequence for the shorter Vri putative protein (Figure 4) were tested. No rescue was obtained with either one or two doses of the transgenes, or after heat shock treatment of the cDNA transgenic strains.

The *l(2)03771 P* insertion (25D4-6) maps at the other end of our walk, probably within the 25D6 band and corresponds to a new locus. Because vri^{P} and *l(2)03771*

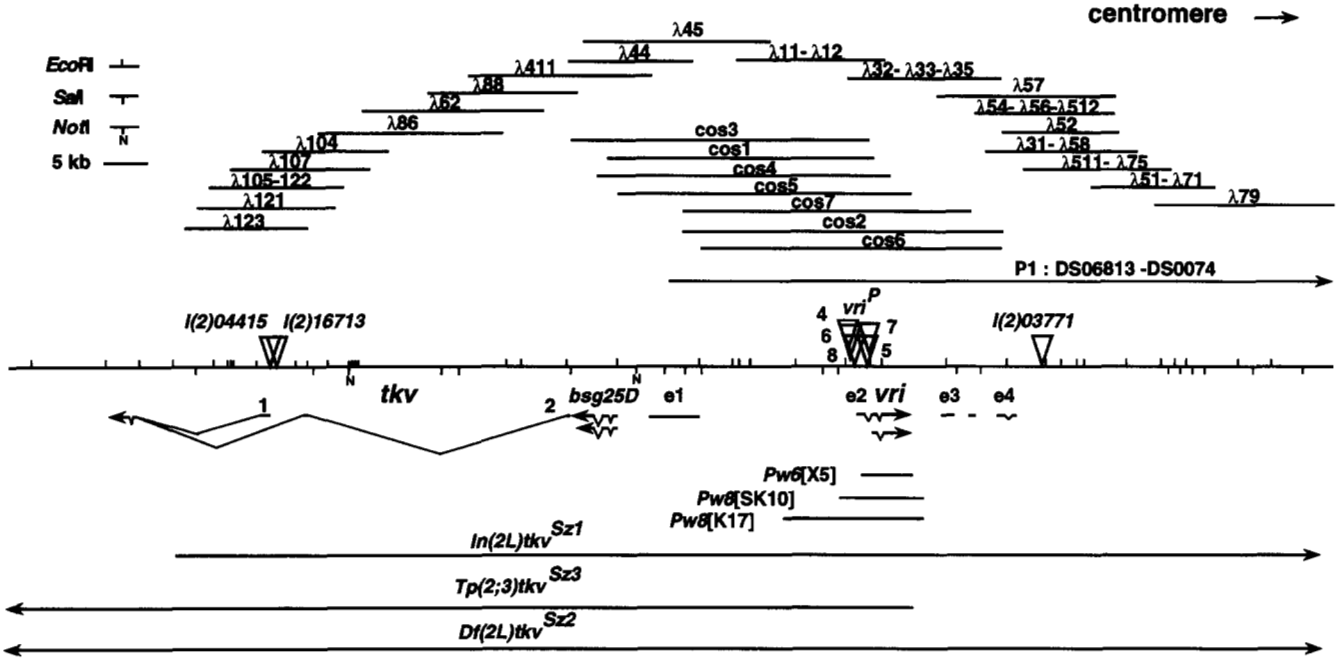

FIGURE 3.—Genomic map of the walk in 25D region. Localizations of cosmid, λ phage and P1 clones are shown. The *bsg25D* and *tkv* genes were mapped and cDNA positions are shown. The breakpoints were mapped by Southern blot analysis, and the lethal *P* mutations by plasmid rescue.

map 5′ to the *tkv* gene, we attempted to rule out the hypothesis whereby these elements map in regulatory regions of *tkv,* by testing rescue of all *vri* alleles (EMS- and *P*-element induced) and *l(2)03771* with a *tkv* transgene. When *vri/Cy* or *l(2)03771/CyO* females were crossed to $Df(2L)tkv^{Sz2}/CyO;\ P[Ubi\text{-}tkv\text{-}2]^{17}/+$ males no Cy^+ flies were observed in either case. Furthermore, *vri* alleles in $vri/+;\ P[Ubi\text{-}tkv\text{-}2]^{17}/+$ females still strongly enhance *dpp*. These results confirm that the *vri* and *l(2)03771* mutations do not alter *tkv* regulation and map in two other independent loci.

We showed that the whole walk is included within $Df(2L)tkv^{Sz2}$ by hybridizing genomic DNA from dead embryos homozygous for this deficiency to the phage DNA of the walk. The tkv^{Sz2}, cl^7 and cl^{h2} deficiency breakpoints were not recovered and probably map outside the walk. The initial extent of $Df(2L)tkv^{Sz2}$ (25D2-4; 25D6-E1) (SZIDONYA and REUTER 1988) was reassigned to the 25D1-25E1 interval.

cDNA and genomic sequencing: Three cDNAs, cDNA2, 7 and 8, were isolated from a 3–12-hr cDNA library (POOLE *et al.* 1985). 5′ ends were cloned by the 5′ RACE method (FROHMAN 1990) from poly A + RNA (8–12 hr) (cDNA4, 5, and 6), or by direct amplification from an 8–12-hr embryonic cDNA library (BROWN and KAFATOS 1988) (cDNA1 and 3). cDNAs and 6.55-kb genomic DNA were sequenced. Map positions are indicated in Figure 4. Sequence comparison predicts the presence of two introns. Intron 1 is located after position 459 and is 1396 bp long, intron 2 maps after position 807 and is 108 bp long. In both cases the predicted splice donor and acceptor sites (GT at 5′ and AG at 3′ splice sites) fit the invertebrate splice junction consensus (MOUNT *et al.* 1992). cDNA7 contains a polyA tail (24A) preceded by a consensus polyadenylation site (AATAAA) at position 3764. Two other potential polyadenylation sites were found at positions 3373 and 3385 but no corresponding polyadenylated cDNAs were isolated. The 5′ end of cDNA1 begins with a G, not present in the genomic sequence. This residue may have been introduced by the reverse transcriptase copying the cap site, indicating that cDNA1 is probably full-length. No TATA or CAT box is found, however, within the 300 bp mapping upstream from the 5′ cDNA1 end. At the 3′ intron 1 junction, −11 nucleotides upstream from the 5′ end of cDNA3, the CAGTTC sequence that fits the cap site consensus (HULTMARK *et al.* 1986) is found. Within intron 1 (not shown in Figure 4) at positions −86 and −104 upstream from the 5′ cDNA3 end are found two TATA box consensus and at position −121 a ACAAT sequence that fits the CAT box consensus is found. These potential transcriptional regulatory domains and the potential cap site sequence are consistent with the prediction that cDNA3 is an almost full-length cDNA. This hypothesis is fully supported by the fact that an identical 5′ cDNA end was found when using a 4–8-hr cDNA library (BROWN and KAFATOS 1988). The largest cDNA predicted from cDNA1 and cDNA7 is 3807 bp and encodes a putative 729-amino acid protein. The first ATG in frame at position 318, however, is preceded by the ACATT sequence, which does not perfectly fit the C/AAAA/C consensus of Drosophila translation initiation sites (CAVENER 1987). A second type of cDNA, 3340 bp long, is predicted from cDNA3 and

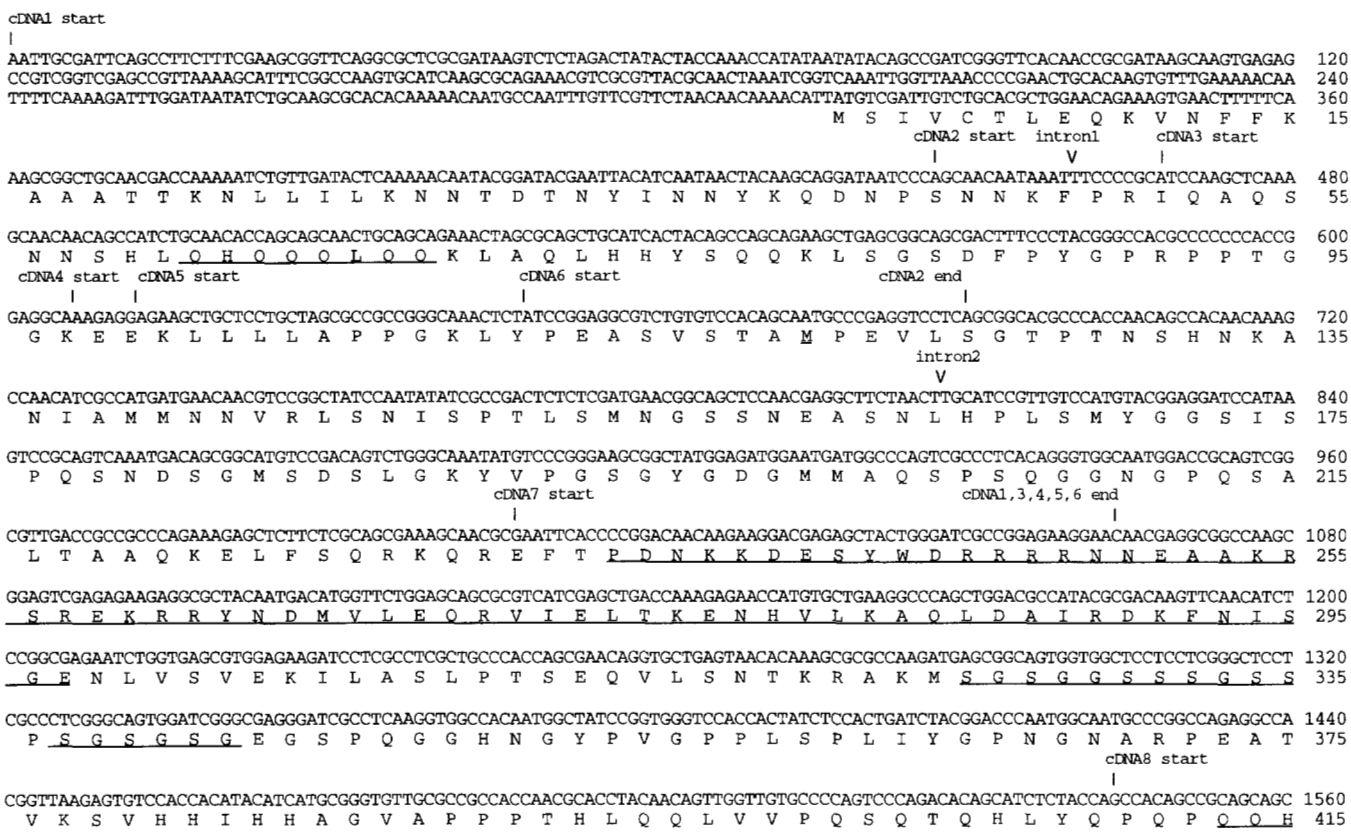

FIGURE 4.—*vri* cDNA sequence. Nucleotide sequence of the sense strand of the longest composite cDNA clone (3807 bp) and amino acid sequence of the two longest ORF encoded (729 and 610 amino acids). The ends of the other cDNAs and the positions of the two introns are indicated by an arrow. The OPA repeats, the region of the bZIP domain, and three polyadenylation sites are underlined.

cDNA7, and encodes a putative 610-amino acid protein starting at position 675 and lacking the first 119 amino acids. Between the first ATG of the ORF and the second intron (at position 807), three other ATG in frame are present, but the upstream nucleotide sequence does not fit the consensus translation initiation site described above for any of them.

The predicted 729-amino acid protein is rich in serine (14%), glutamine (8%) and proline (8%) residues. Two runs of histidines and glutamines (OPA repeats), frequently found in eukaryotic transcription factors, are detectable between positions 61 and 68 (seven over eight) and between positions 413 and 434 (17 over 22). A GS box consisting of a serine and glycine repeat is found between positions 325 and 342 (17 over 18). GS boxes have been described in serine-threonine kinase type I TGFβ receptors and are juxtamembrane intracellular domains with potential sites for phosphorylation on the serine residues (BRUMMEL *et al.* 1994; NELLEN *et al.* 1994; PENTON *et al.* 1994). Searches in the GenPept protein database revealed (between positions 234 and 297) a motif characteristic of the leucine zipper class of DNA binding proteins (VINSON *et al.* 1989) common to the two putative proteins. These proteins contain two contiguous domains, a basic DNA binding domain and the leucine zipper involved in homo- or hetero-dimerization of the proteins. Figure 5 shows a comparison between Vri and other bZIP proteins. The most closely related proteins are the bZIP encoded by the *gene 9* of *X. laevis* and the human NF-IL3A/E4BP4 protein. The Gene 9 protein (similar to Gene 8) is induced by thyroid hormone during metamorphosis in the tadpole resorption program and is 63% identical and 87% similar to Vri within 68 amino acids of the bZIP domain (BROWN *et al.* 1996). E4BP4, a placental human transcription factor, is identical to the human NF-IL3A protein that binds and transactivates the interleukin-3 (IL-3) promoter in T cells (COWELL *et al.* 1992; COWELL and HURST 1994; ZHANG *et al.* 1995). NF-IL3A/E4BP4 shares 60% identity and 93% similarity with Vri, across the 68 amino acids within the bZIP domain. NF-IL3A/E4BP4 binds specifically to regulatory sequences in the IL-3 promoter, the adenovirus E4 promoter and human gamma interferon promoter. NF-IL3A/E4BP4 is a multiple site phosphorylated bZIP factor and has been also isolated as a binding protein of the CREB/ATF-like sequence of the upstream regulatory region of human IL-1β gene (CHEN *et al.* 1995). As shown in Figure 5, Vri is also related to the PAR subfamily of bZIP proteins, which includes VBP/TEF, DBP and HLF (hepatic leukaemia factor). The PAR proteins (DROLET *et al.* 1991) display a high identity, not only within the bZIP domain

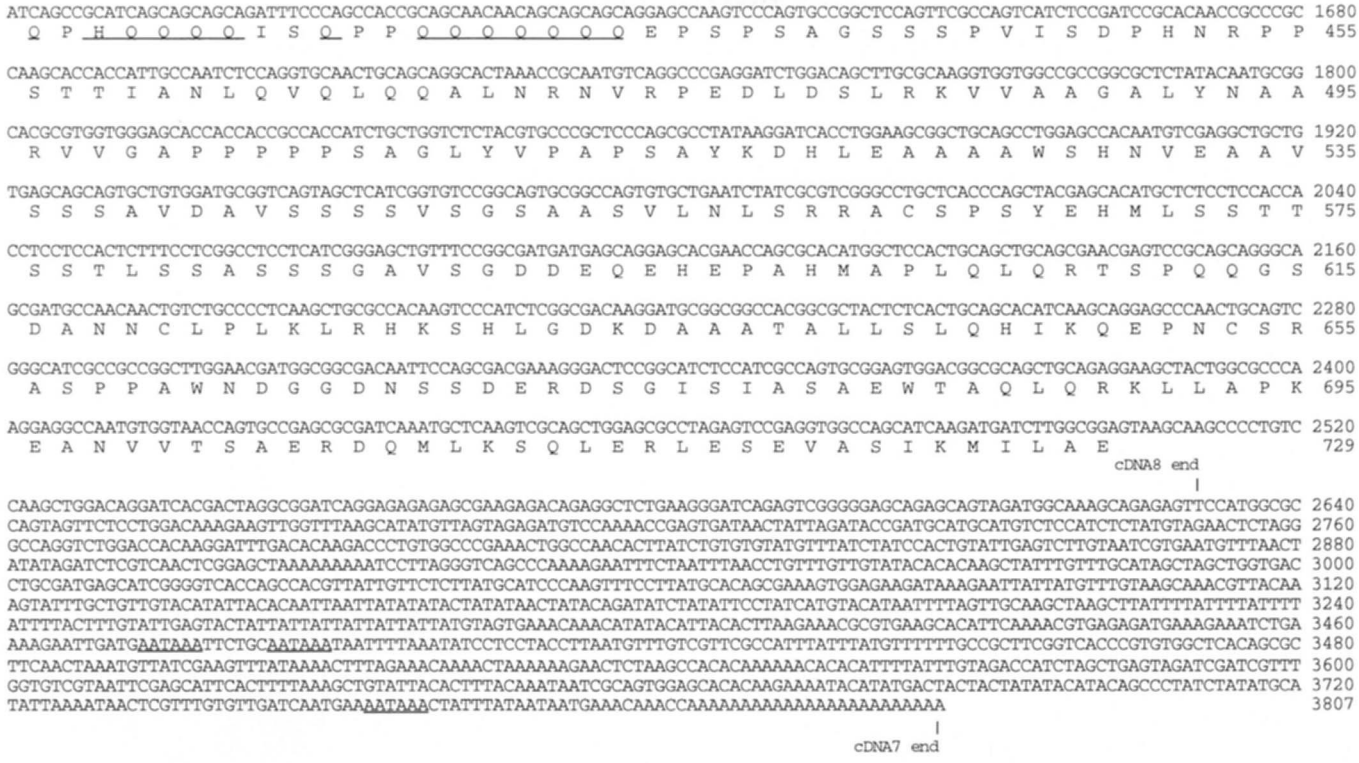

FIGURE 4.—*Continued*

but also over a conserved proline and acidic-amino-acid-rich (PAR) adjacent domain, at the amino terminal to the basic region (not shown in Figure 5). Vri, like NF-IL3A/E4BP4, Gene 9, the Giant factor of *D. melanogaster* and the product of the *cell-death specification* gene of *C. elegans*, CES-2 (METZSTEIN *et al.* 1996), although sharing a high identity within the bZIP domain with PAR proteins, does not contain a PAR domain. Nevertheless, NF-IL3A/E4BP4, Giant, CES-2 and the PAR proteins share similar consensus binding sites (HAAS *et al.* 1995; METZSTEIN *et al.* 1996). It is noteworthy that E4BP4, Giant and CES-2 have been shown to act as transcriptional repressors.

Localization of point mutations and new *P* excision-induced *vri* mutations: To confirm that *vri* mutants affect the bZIP protein, the essential domain of the protein, the bZIP region, was sequenced from the EMS-induced mutants. $vri^1/SM6b$ and $vri^2/SM6b$ genomic DNAs were PCR amplified between positions 895 and 1606 (Figure 4). Two or three independent PCR experiments were performed in both cases and at least three clones from each PCR amplification were sequenced. With vri^1 and vri^2, a transition from C to T was found at position 924 in about one half of the clones (the other half corresponding to the *SM6b* chromosome). We verified that the same PCR amplification experiment per-

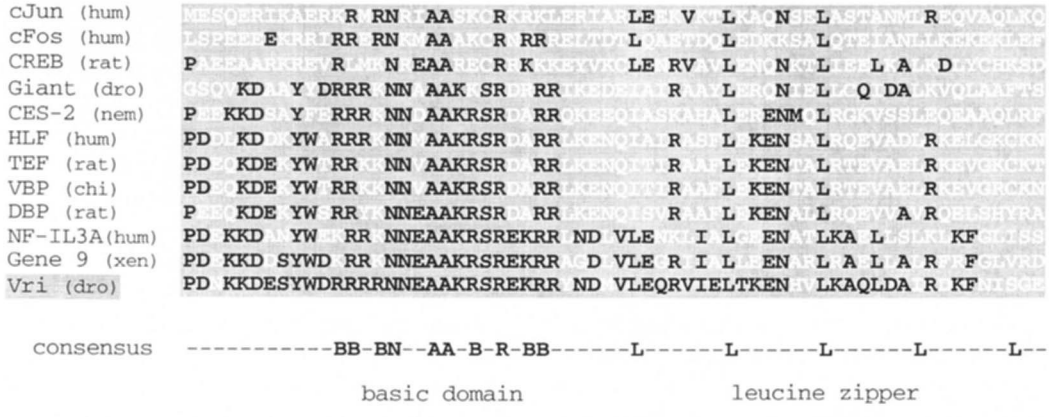

FIGURE 5.—Comparison of DNA binding domains of Vri with other typical bZIP proteins: cFos (VAN STRAATEN *et al.* 1983), cJun (BOHMANN *et al.* 1987), CREB (GONZALEZ *et al.* 1989), Giant (CAPOVILLA *et al.* 1992), CES-2 (METZSTEIN *et al.* 1996), HLF (HUNGER *et al.* 1992), TEF (DROLET *et al.* 1991), VBP (IYER *et al.* 1991), DBP (MUELLER *et al.* 1990), NF-IL3A/E4BP4 (COWELL *et al.* 1992; ZHANG *et al.* 1995), Gene 9 (BROWN *et al.* 1996).

formed with *Df(2L)tkv*^{Sz2}/*SM6b* flies, in which the bZIP region is present exclusively on the *SM6b* chromosome, produces only wild-type sequences. Consequently, in *vri*^{1} and *vri*^{2}, the glutamine (Q) at position 202 (CAG) is changed to a stop codon (TAG). The occurrence of a stop codon at position 924 predicts two types of putative truncated proteins, 202 and 83 amino acids long with no bZIP domain (located between the amino acids 234 and 297) and thus inactive as transcription factors. A transition from C to T corresponds well with what could be expected after EMS treatment. Surprisingly, both mutants show the same transition. These two mutants are the result, however, of the same mutagenesis and, moreover, the EMS-treated males were not crossed individually (SZIDONYA and REUTER 1988). It is therefore not possible to determine whether these mutations are independent. The same region was PCR amplified from *vri*^{3}/*Df(2L)tkv*^{Sz2} genomic DNA. The sequence shows that the *vri*^{3} mutation does not map in this domain.

Southern blot analyses from the 23 *w* lethal lines selected after secondary excisions of *vri*^{5} *P* element were performed. Seventeen lines with phenotypes similar to the original phenotype (presence of weakly ventralized embryos) originate from internal rearrangements inactivating the *white* gene. Deletions are observed on one side of the transposon in five other lines and on both sides in one line.

Transcription of *vri* RNAs is detectable throughout development: The maternal effect detected by the interaction between *ea* and *dpp* clearly predicts maternal and early embryonic accumulation of *vri* mRNA, but makes no prediction for other developmental functions. To address this question we carried out a developmental Northern analysis. Poly A^{+} RNA from Oregon R embryos, larvae, pupae and adults raised at 25° were hybridized to the 2.8-kb cDNA7. As shown in Figure 6, RNA expression is dynamic throughout development. In embryos aged from 0 to 4 hr (stages 1–8: germ band elongation completed) two major transcripts of 4.9 and 6.2 kb are present at a low level. In 4–8-hr embryos (stages 8–11: beginning of germ band retraction) we found two major types 3.3 and 3.8 kb long and also minor transcripts with higher molecular weights (up to more than 10 kb). The long transcripts do not hybridize with a 15-kb genomic probe mapping upstream cDNA1 and therefore the *vri* locus extends over at least 20 kb. The large number of bands observed in embryos aged between 0 to 8 hr is also observed when using a genomic probe mapping 5′ to cDNA7 with no bZIP domain and no OPA repeats. These bands are, therefore, not due to cross-hybridization. In older embryos aged from 8 to 24 hr (stages 11–17), the transcripts are more abundant and are represented exclusively by the two 3.3- and 3.8-kb major types. In third instar larvae, the 3.3- and 3.8-kb transcripts are still present, together with an abundant 1.5-kb transcript. In pupae (6–7 days of development), and in female adult flies, only the 3.3- and 3.8-kb transcripts are present at a high level. In males, an abundant 1.6-kb transcript is present. The 3.3- and 3.8-kb transcripts probably correspond respectively to the 3340- and 3807-bp putative cDNAs previously described. cDNAs corresponding to longer or shorter RNAs were not recovered and the origin of these RNAs is thus unknown. The expression of RNAs throughout development implicates many other functions besides those giving early embryonic phenotypes.

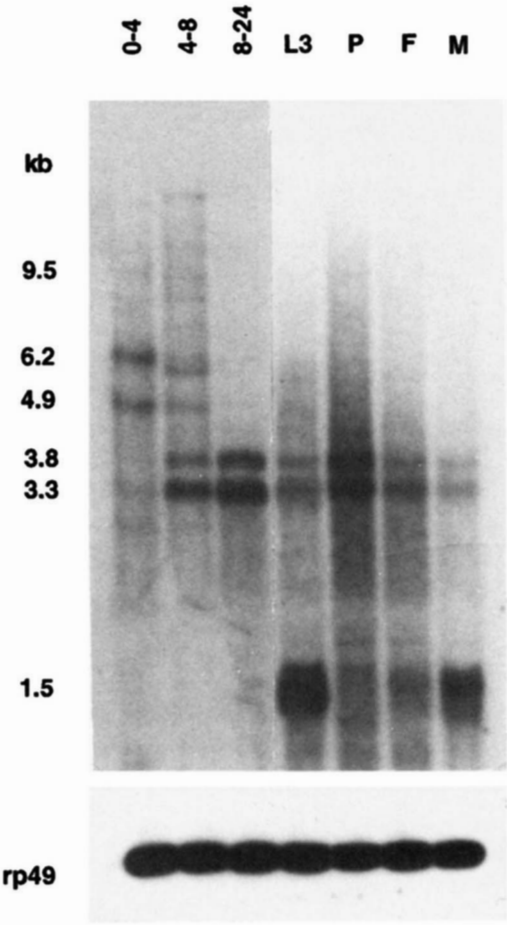

FIGURE 6.—Northern blot of staged Oregon R polyA + RNA. Ten micrograms of RNA were loaded on 1% formaldehyde agarose gel, run, transferred onto nitrocellulose filter and hybridized to the cDNA7 probe. Embryonic stages are as follows: 0–4, 0–8, and 8–24 hr after egg laying. L3, 3rd instar larval stage (3–5 days after egg laying); P, pupal stage (6–7 days after egg laying); F, adult females; M, adult males. Control for loading was performed with the ribosomal protein rp49.

Spatial expression of RNAs in embryos, ovaries and larvae: *In situ* hybridizations performed in embryos show a uniformly distributed maternal product at preblastoderm stage (Figure 7A). From stage 10 (germ band fully elongated), the transcripts begin to localize and can be seen at higher levels in the primordium of the foregut (Figure 7B). At stage 13, transcripts are present at high levels in the hypopharyngal lobe at the ventral opening of the stomodeum, the foregut, the proventriculus primordium, the hindgut, anal pads and posterior spiracles (Figure 7, C and D). At stage 14, during head involution

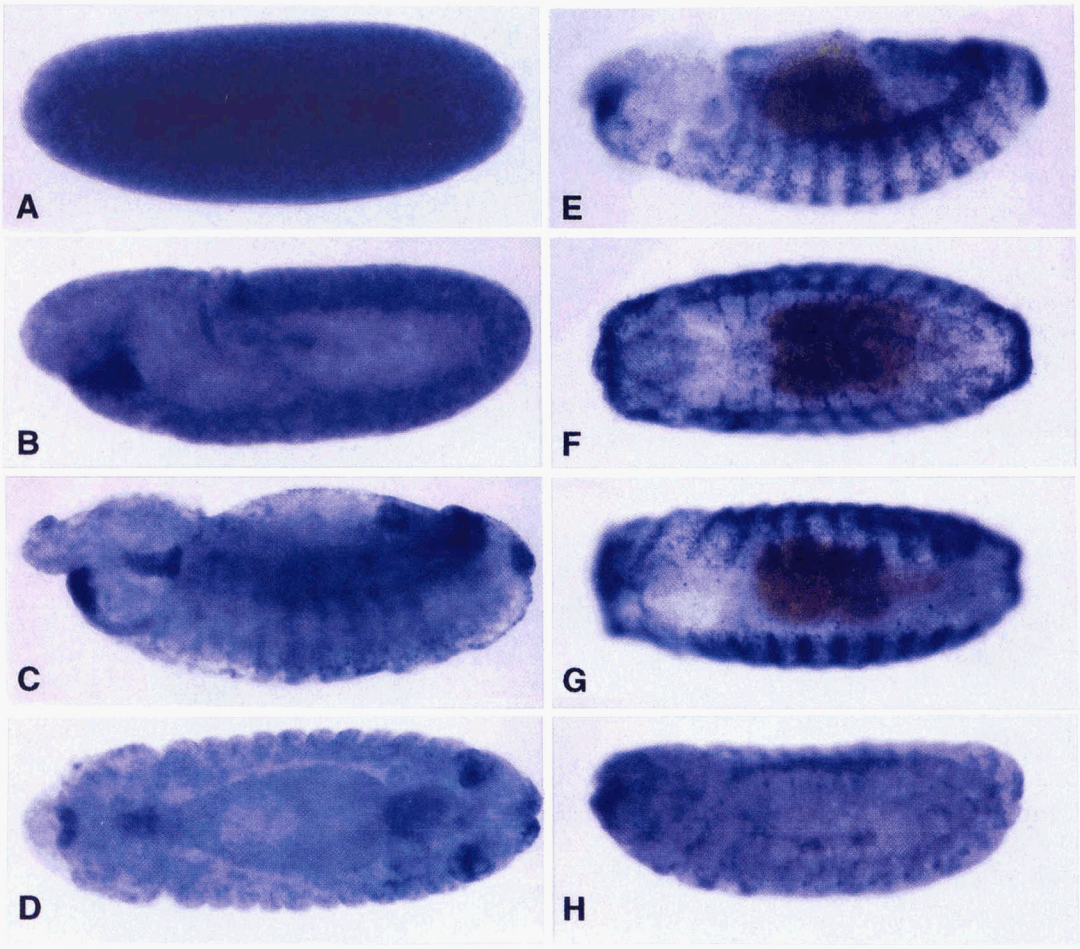

FIGURE 7.—Detection of the *vri* RNAs in embryos. We performed *in situ* hybridizations using a digoxigenin-labeled cDNA7 probe. Embryos are oriented with anterior to the left and dorsal up, unless otherwise specified. Stages are according to CAMPOS-ORTEGA and HARTENSTEIN (1985). (A) A stage 4 embryo (syncitial blastoderm) with maternal transcripts uniformly detected. (B) A stage 10 embryo (germ band fully elongated) with RNAs detectable in the primordium of the foregut. (C and D) Stage 13 embryos (end of germ-band retraction) with detectable transcripts in the hypopharyngal lobe, the proventriculus primordium, in the foregut, the hindgut, and in two longitudinal stripes between foregut and hindgut. The anal pads and the posterior spiracles (D, dorsal view) also express the transcripts. (E) A stage 14 embryo shows labeling in stripes along the anterior part of every segment in the lateral epidermis. (F) A stage 15 embryo (end of dorsal closure) with detectable transcripts in dorsal epidermis and amnioserosa. (G) The ventral-most epidermis and central nervous system are not labeled (stage 15). (H) A stage 16 embryo with labeling in the tracheal system. The brown color is due to fixative in the gut.

and dorsal closure (Figure 7E), the transcripts are located in stripes along the epidermis in the anterior part of each segment. The stomodeum, hindgut and anal pads are still strongly labeled and a longitudinal stripe is observed dorsally along the epidermis. At stage 15 (end of dorsal closure) thin stripes are seen dorsally across the closing epidermis and the amnioserosa is weakly labeled (Figure 7F). The ventral-most regions of the epidermis and the central nervous system are not labeled, although 50% of the ventral epidermis shows *vri* expression (Figure 7G). At stage 16, the transcripts are still present in the stomodeum, anal pads and in a network of lateral and dorsal cells probably corresponding to the tracheal track (Figure 7H).

The maternal effect of *vri* led us to examine its expression in ovaries. *vri* transcripts are not expressed in ovarian stem cells, oogonia or early cysts and are first detectable at stage 8 in the nucleus and cytoplasm of nurse cells, which is consistent with maternally provided RNAs, and in the columnar follicular epithelial cells (Figure 8, A and B). The transcripts are also expressed in gut, brain (Figure 8C) and imaginal discs (Figure 8D) of third instar larvae. Similar localizations or subpatterns were observed with the βgal staining of all the *PlacZ* mutant alleles, the differences between the five alleles being only quantitative. As shown in Figure 8, in embryos, the foregut, posterior spiracles and anal pads express *lacZ* (Figure 8E), and in ovaries the border cells and columnar epithelial cells are stained, but not the nurse cells (Figure 8F). This could reflect the fact that the *P* elements are localized in transcripts provided only zygotically or, on the contrary, that they completely abolish the production of the maternal RNAs. The staining in border cells was not observed with *in situ*

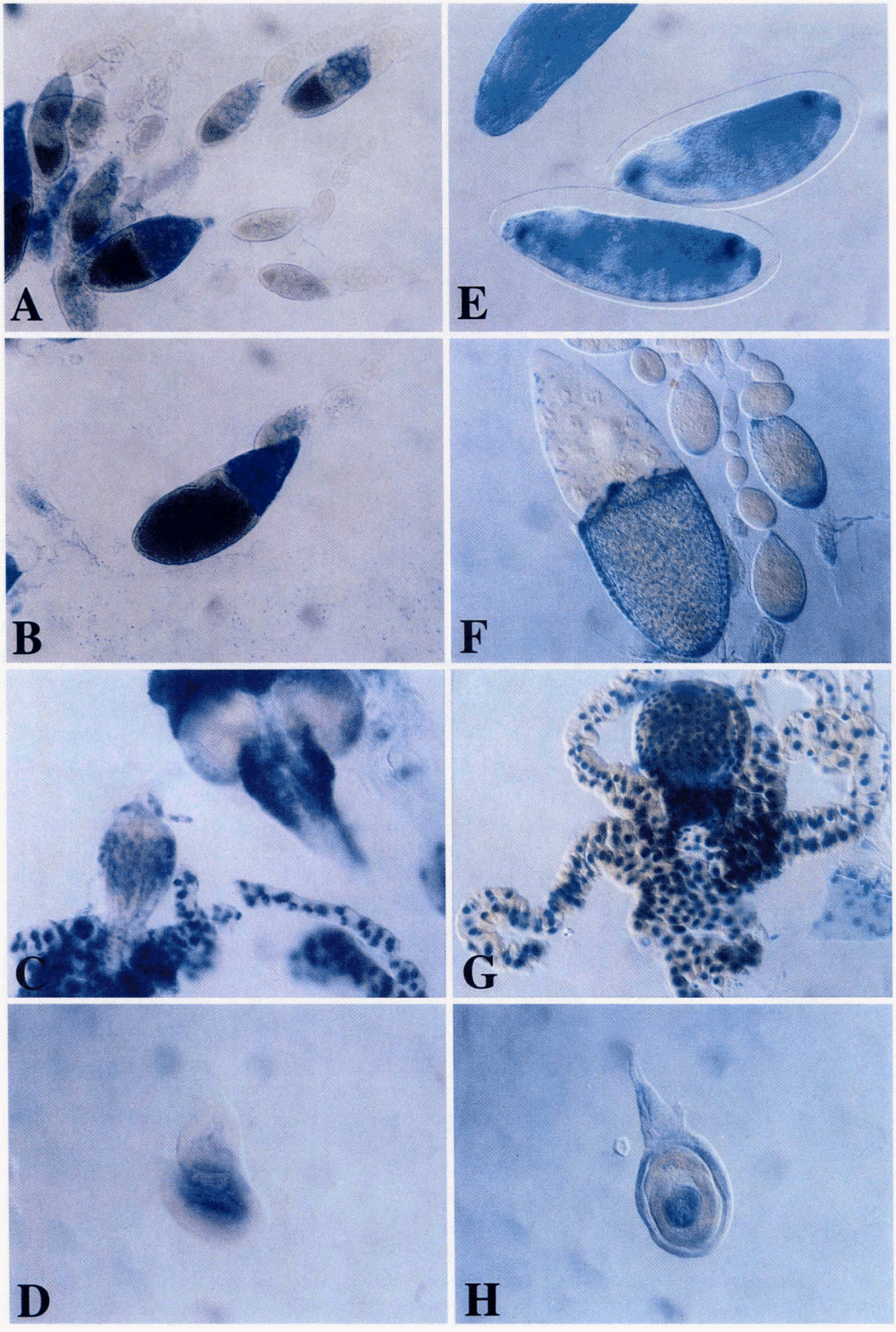

FIGURE 8.—Comparison of *in situ* hybridizations and *lacZ* stainings. *In situ* hybridizations were performed with a digoxigenin-labeled cDNA7 probe (A–D). (A and B) The transcripts are first detectable at stage 8 in the cytoplasm of nurse cells and in columnar follicular cells. (C) Larval gut and brain. (D) Imaginal disk. Expression in vri^{P} alleles (E–H). (E) vri^{4} embryo showing staining in foregut, posterior spiracles and anal pads. (F) vri^{8} ovaries showing staining in border cells and columnar follicular cells. (G) vri^{4} larval gut showing the gastric caecae. (H) vri^{8} imaginal disk stained in the central region.

hybridizations and we do not know at the present whether this is an ectopic localization unrelated to the real expression of the transcripts. Staining is observed in larval gut in a pattern very similar to that observed by *in situ* hybridization (Figure 8G) and in the central region of imaginal disks (Figure 8H). These results are consistent with the hypothesis whereby the *P*-element mutations alter the bZIP function.

DISCUSSION

***vri* enhances *dpp* phenotypes in both embryo and wing:** The *vri* gene represents a previously undescribed

locus. Three EMS, two lethal alleles and one viable allele, and five *P*-element insertion lethal induced alleles have been identified. New lethal alleles have also been selected after imperfect *P*-element excision. All types of lethal alleles act as dominant enhancers of ventralization in the embryo. An increased lethality and ventralization is observed in embryos from females carrying *vri* alleles associated in double heterozygotes with an *easter* weakly ventralizing allele. In the same context a maternal ventralizing effect is found for the *schnurri* gene, which encodes a zinc finger transcription factor and *punt*, a type II TGFβ receptor, both of which are active in the *dpp* signal transduction pathway (ARORA *et al.* 1995; GRIEDER *et al.* 1995). The level of ventralization is stronger with *vri* than with *punt, schnurri* or *tkv,* however (TERRACOL and LENGYEL 1994). Therefore, partial reduction of *vri* function in mothers enhances the slight ventralization induced by a weakly ventralizing *easter* allele. No additional increased ventralization is observed when a *vri* allele is provided also zygotically. Furthermore, the maternal effect of the *vri* gene enhances *dpp* ventralizing embryonic phenotypes. This is observed with all the available lethal *vri* alleles and two different *dpp* alleles. In the strongest interaction, no *vri/dpp* or *dpp/Cy* embryos survive and moderately ventralized embryos (V3) are observed, whereas in the same context with *tkv,* only V4 embryos were recovered (TERRACOL and LENGYEL 1994). With some *vri* alleles a zygotic effect is observed. In neither type of experiment is an increased ventralization obtained zygotically, however.

It is noteworthy that all the alleles tested behave like antimorphs since they show a dominant enhancement of *dpp* phenotypes stronger than a deficiency. The alleles behave also as loss of function mutations however, and we have shown, using a duplication, that they were not neomorphic. Therefore, a nonfunctional Vri protein, which is still able to bind to a substrate, might be expected to have a stronger effect than the simple absence of one copy of the gene. The same observation was made with *tkv*. $Df(2L)tkv^{Sx2}$, which deletes totally *tkv* and *vri,* does not enhance *dpp,* whereas *tkv* point mutations do. These alleles however display phenotypes similar to the deficiency phenotype and are therefore considered to be null alleles. Again this could be explained by an antimorphic effect of these alleles, which are able to produce truncated nonfunctional proteins. The occurence of a large number of antimorphic alleles is consistent with the nature of the two proteins, a bZIP transcription factor and a serine-threonine kinase receptor, which both act as dimers.

The strong maternal interaction of *vri* with dorsoventral patterning genes is consistent with a participation in the early stages of the establishment of the dorsoventral axis. Germline clone analysis, however, has not provided any direct evidence. One explanation for this paradox would be that *vri* acts maternally to establish dorsoventral polarity in concert with another gene, perhaps another bZIP protein. This function would, therefore, be detected only when *dpp* activity is weakened, either indirectly in an $ea^{161.13}/+$ maternal context, by extending the repression of *dpp* by Dorsal toward the dorsal regions of the embryo, or directly in a *dpp*/+ zygotic context.

One viable allele with shortened wing veins has been identified. This phenotype is similar to that observed in *shortvein* alleles of *dpp,* although it is not specific to *dpp* alleles and has also been described for genes apparently unrelated to *dpp.* Similarly, a dominant enhancement of *dpp* by *vri* is observed in wing. This enhancement is associated with roughened eyes and truncated legs. Furthermore, we have shown that a downstream component of the *dpp* pathway, *Mad,* also enhances *vri* phenotypes in wing.

***vri* encodes a new member of the bZIP family of transcription factors:** The gene was cloned within a walk in the 25D region, by localization of five vri^{P} mutations. Two were located within the first intron, and three upstream from the 5′ end of an embryonic cDNA encoding a bZIP transcription factor. The *P* elements from the five *vri* alleles were mobilized leading to a reversion to viability and fertility. Furthermore, partial sequencing from an EMS-induced allele has revealed the presence of a stop codon mutation upstream from the bZIP domain. The gene was shown to map in a highly transcribed region, close to the *thick veins* gene. Two new *P*-induced *tkv* hypomorphic alleles have been identified and mapped as well as another *P*-induced lethal mutation defining a new gene. Attempts to rescue the *vri* phenotype were, however, unsuccessful even with a 17-kb *P* element transgene or an *Hsp*-cDNA. We explain this result by the fact that the very long transcripts (more than 10 kb), not included in the longest transgene, are necessary for correct functioning of the gene. Furthermore, the cDNA transgene encodes only one of the putative bZIP proteins (the shorter) whereas both are probably required for correct function. Nevertheless, *lacZ* expression corresponds well with what one would expect if the bZIP cDNA indeed corresponds to the *vri* gene, since embryonic and larval stainings are very similar to the RNA *in situ* hybridizations.

bZIP transcription factors (VINSON *et al.* 1989) act as homo- or heterodimers. The most closely related proteins are Gene 9 of *X. laevis* (BROWN *et al.* 1996) and NF-IL3A, a human transcriptional activator of IL-3 (ZHANG *et al.* 1995) identical to the human placental transcription factor, E4BP4 (COWELL *et al.* 1992; COWELL and HURST 1994). IL-3 is a hematopoietic growth factor that stimulates the proliferation of progenitor cells and enhances the activity of mature effector cells. As with other lymphokines, IL-3 expression occurs following T-cell activation. *gene 9* is induced by thyroid hormone during metamorphosis in the tadpole tail resorption program. E4BP4 and CREB/ATF (GONZALEZ

et al. 1989) binding sites overlap, but unlike these factors E4BP4 acts like a transcriptional repressor whereas NF-IL3A binds and transactivates the human IL-3 promoter in T cells. Regulatory elements in the IL-3 promoter include a consensus AP-1 binding site, which can bind the c-Fos/c-Jun heterodimers. Therefore, the NF-IL3A/E4BP4 protein can act as an activator or a repressor. The strong sequence similarity between Gene 9, NF-IL3A/E4BP4 and Vri bZIP domains suggests that they bind to the same consensus sequences. It is noteworthy that NF-IL3A/E4BP4 shares consensus binding sites with the PAR bZIP factors, Giant, and CES-2, encoded by a cell death specification gene of *C. elegans* (HAAS *et al.* 1995; METZSTEIN *et al.* 1996). It has been proposed that the members of the CES-2/PAR family are evolutionarily conserved regulators of programmed cell death (METZSTEIN *et al.* 1996). Furthermore, both Gene 9 and NF-IL3A are implicated in cell death or growth events. While Gene 9 is induced by the thyroid hormone, a growth hormone, NF-IL3A activates the mitogen-responsiveness of the promoter of IL-3, a growth factor.

***vri* is expressed throughout development in localized regions:** *vri* transcripts are produced by ovarian nurse cells and found in preblastoderm embryos, which is consistent with the maternal requirement detected by genetic interactions. In ovaries, transcripts are also found in the follicular epithelial cells. These localizations do not correlate with *dpp* expression, which is restricted to the centripetally migrating and nurse cell-associated follicle cells, an anterior subset of somatic follicle cells (TWOMBLY *et al.* 1996). In the embryos the transcripts are first seen to localize during stage 10 in the primordium of the foregut, then at stage 13, in the stomodeum, the foregut, the proventriculus primordium and in the hindgut primordium. This restricted expression suggests that *vri* is required for gut development and is reminiscent of the *dpp* expression pattern, although the expression of *dpp* in the foregut is restricted to the anterior part (ST. JOHNSTON and GELBART 1987; MASUCCI *et al.* 1993). Unlike *dpp,* no localized expression was detected in the visceral mesoderm (IMMERGLÜCK *et al.* 1990; PANGANIBAN *et al.* 1990; REUTER *et al.* 1990). At stage 14 *vri* transcripts can be detected in the dorsal and dorso-lateral epidermis in a segmental pattern. The expression is then seen in the tracheal system, a localization that is consistent with the interrupted tracheae observed in *vri* mutants. It is noteworthy that the *dpp* receptors *thick veins* (AFFOLTER *et al.* 1994) and *punt* (RUBERTE *et al.* 1995) are required for tracheal development. In *tkv* mutants, the formation of the ventral trunk, and the ganglionic and dorsal branches are strongly affected while the dorsal trunk and visceral branches appear to be unaffected. *tkv* is expressed, at germ band extension, in the tracheal placodes before the tracheal cells start to migrate in the vicinity of the cells of the lateral ectoderm that express *dpp. punt* mutants fail to develop dorsal branches. The expression of *vri* transcripts in the tracheae occurs later. This could also reflect the fact that, at earlier stages, the *vri* transcripts are ubiquitous.

Potential role for *vri* in the *dpp* pathway: Genetic screens have been performed to identify other partners of *dpp.* In addition to yielding new alleles of the genes already described, new genes have been identified as dominant maternal enhancers of *dpp* phenotypes. This is the case for *Mothers against dpp* (*Mad*) and *Medea,* a MAD homolog (RAFTERY *et al.* 1995; SEKELSKY *et al.* 1995; NEWFELD *et al.* 1996; WIERSDORFF *et al.* 1996; L. RAFTERY, personal communication), which both appear to be essential components of the *dpp* pathway. *vri* was not found in this screen, neither was *tkv* nor *put.* This is understandable, however, since the screen was done with the dpp^{hr4} allele, whose phenotype is weakly enhanced by *vri* or *tkv.* Furthermore only mutations presenting a strong enhancement, with less than 10% survivors of the dpp^{hr4} progeny, were retained (RAFTERY *et al.* 1995). From our results, only vri^{5} and tkv^{IO} alleles (TERRACOL and LENGYEL 1994) would have been retained.

The fact that putative truncated *vri* proteins with no bZIP domain, as well as *P*-element-induced mutations and *P* excision secondary mutations, are able to aggravate maternally the embryonic ventralized *ea* and *dpp* phenotypes suggests a maternal participation in early establishment of dorsoventral polarity of the embryo. *vri* mutations alter wing vein differentiation, which leads to a phenotype very similar to that of *shortvein* alleles of *dpp.* Furthermore, *vri* enhances *dpp* phenotypes in wing and *Mad* acts as a dominant enhancer of the *vri* wing phenotype. *vri* is expressed both in embryonic-specific regions of the gut, and in the larval gut and imaginal disks. Although the function of the gene remains to be established, *vri* appears to be active during various stages of development and in different cell types.

Genetical analysis suggests that Vri acts in concert with another factor and, because bZIP transcription factors can act as heterodimers, it is possible that Vri binds to another bZIP protein. Three of the known *D. melanogaster* bZIP transcription factors are probably implicated in the *dpp* pathway. The homologue of the mammalian *fos* oncogene, dFRA, is expressed in embryos, in the head, dorsal ectoderm, amnioserosa, a subset of cells of the peripheral nervous system, a portion of the midgut, the hindgut and in the anal pads (PERKINS *et al.* 1990). The *fos* partner, the *jun* oncogene, *Djun,* is expressed throughout development (ZHANG *et al.* 1990). It is noteworthy that, in mammals, these genes are induced in response to TGFβ. The homologue of CREB, dCREB-A (SMOLICK *et al.* 1992), is expressed in ovarian columnar follicular cells and in male reproductive organs, in embryonic salivary glands, in the brain, the optic lobe, and the midgut of the adult. It is interesting

to note that it has been shown that dCREB-A is required for dorsoventral patterning of the larval cuticle, and it was proposed that it functions near the end of both DPP- and SPI-signaling cascades (ANDREW *et al.* 1997). Some localizations of these bZIP factors coincide with the *vri* expression pattern. Furthermore, putative Fos/Jun and CREB binding sites have been identified close to the Zerknüllt (a Hox transcription factor of the *dpp* pathway) binding sites in the RACE (an amnioserosa specific gene) promoter. It has been suggested that Hox-bZIP synergy is a common feature of *dpp* signaling (RUSCH *et al.* 1997). It will therefore be interesting to test for a genetical and molecular interaction between these factors and *vri*.

In conclusion, we propose that *vri* interferes with the *dpp* signal transduction pathway. The gene could mediate certain aspects of *dpp* function, upstream, downstream, or even in parallel. Alternatively, in view of the numerous possible functions attributable to the *vri* gene it is likely that *vri* also interferes with other pathways.

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