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Dorsoventral development of the *Drosophila* embryo is controlled by a cascade of transcriptional regulators

CHRISTINE THISSE and BERNARD THISSE

LGME du CNRS, U184 de l'INSERM, Institut de Chimie Biologique, Faculté de Médecine, 11 rue Humann 67085 Strasbourg Cedex, France

Present address: Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, USA

Summary

Maternal genes involved in dorsoventral (D/V) patterning of the *Drosophila* embryo interact to establish a stable nuclear concentration gradient of the Dorsal protein which acts as the morphogen along this axis. This protein belongs to the *rel* proto-oncogene and NF-KB transcriptional factor family and acts by controlling zygotic gene expression. In the ventral part of the embryo, *dorsal* specifically activates transcription of the gene *twist* and ventrally and laterally *dorsal* represses the expression of *zerknüllt*, a gene involved in the formation of dorsal derivatives. The extent of *dorsal* action is closely related to the affinity and the number of *dorsal* response elements present in these zygotic gene promoters.

***twist* is one of the first zygotic genes necessary for mesoderm formation. It codes for a 'b-HLH' DNA-binding protein which can dimerize and bind to DNA in vitro and to polytene chromosomes in vivo. In addition, in cultured cells *twist* has been shown to be a transcriptional activator. Thus, the first events of embryonic development along the D/V axis are controlled at the transcriptional level.**

Key words: transcriptional regulation, dorsoventral differentiation, *dorsal*, *twist*.

Introduction

Genetic analysis of early development in the *Drosophila* embryo has revealed that embryonic pattern formation depends on both maternal and zygotic genes. The establishment of the anterioposterior (A/P) pattern, along which the body plan and metameric segmentation are defined, requires three sets of maternal genes: the anterior genes for the head and thorax (Frohnhöfer and Nüsslein-Volhard, 1986, 1987), the posterior genes for the abdomen (Lehmann and Nüsslein-Volhard, 1986, 1987), and the terminal gene system for both acron and telson (Schüpbach and Wieschaus, 1986; Klingler et al., 1988).

By contrast the dorsoventral (D/V) axis, along which the embryonic germ layers are defined, requires only one set of genes: the dorsal-ventral system, which include eleven genes of the dorsal group and the gene *cactus* (Anderson, 1987; Roth et al., 1989; for review see Nüsslein-Volhard, 1991; St Johnston and Nüsslein-Volhard, 1992). Each gene in the dorsal group displays a complete dorsalization as the lack-of-function phenotype: only elements that normally derive from the dorsalmost region of the egg are formed, while ventral and lateral elements are lacking (Nüsslein-Volhard, 1979). The twelfth gene, *cactus*, shows partial ventralization as a lack-of-function phenotype: pattern elements normally derived from the dorsal and dorsolateral regions are absent in mutant embryos, while ventral and ventrolateral elements are formed along the entire D/V axis (Schüpbach and Wieschaus, 1989; Roth et al., 1989).

These twelve genes act in a complex way to establish the spatial coordinates of the D/V axis (for review see St Johnston and Nüsslein-Volhard, 1992). Analysis of the phenotype observed in mutants belonging to the D/V system initially suggested that the position along this axis is defined by the local concentration of a morphogen (Nüsslein-Volhard, 1979). Several lines of evidence suggest the *dorsal* (*dl*) gene to be at the end of this cascade of regulation, making it the best candidate for the gene encoding this morphogen: first, *dorsal* is the only mutation that produces a dorsalized phenotype in double mutants with loss-of-function ventralizing *cactus* alleles (Roth et al., 1989, 1991). Thus, *dorsal*, but none of the other dorsal-group genes functions downstream of the *cactus* gene. Second, in transplantation experiments, a localized rescuing activity can be found only in the case of *dorsal*, and this localization only appears at the syncytial blastoderm stage (Santamaria and Nüsslein-Volhard, 1983). Finally, only in the case of *dorsal*, loss-of-function mutations show a dominant (dl^D) effect (Nüsslein-Volhard et al., 1980); that is at 29°C, *dl*/+ females lay eggs that do not develop mesoderm. Thus, this germ layer which corresponds to the most ventral part of the embryo requires a higher level of *dorsal* activity than the lateral and dorsal regions.

Molecular analysis has shown how the product of the

dorsal gene acts as a morphogen for the D/V axis, and how the Dorsal product is able to define spatial coordinates along this axis. The *dorsal* gene was cloned (Steward et al.,1984), its RNA and protein were shown to be synthesized during oogenesis and to be uniformly distributed in the cytoplasm of the egg (Steward et al., 1985; Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). After the ninth cleavage division, the Dorsal protein becomes highly concentrated in the nuclei on the ventral side of the embryo (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). Laterally, the nuclear and cytoplasmic concentration of Dorsal are approximately equal, and dorsally, the Dorsal protein is excluded from the nuclei and remains in the cytoplasm. Thus, positional information along the D/V axis is defined by a gradient of concentration of Dorsal protein in cell nuclei.

The nucleotide sequence of *dorsal* (Steward, 1987) showed that it encodes a protein with strong sequence similarities to the Rel family proteins, which includes the proto-oncogene c-*rel* and NF-KB, a nearly ubiquitous eukaryotic transcription factor (Ghosh et al., 1990; Kieran et al., 1990; Bours et al., 1990; Nolan et al., 1991). Proteins of this family have several common characteristics (Fig. 1; Gilmore, 1991). They share a highly conserved 300 amino-acid domain located in their amino-terminal part and each Rel protein has its own unique carboxy-terminal half. Rel family proteins can form protein complexes with other family members and other unrelated cellular proteins (such as inhibitor IKB). Proteins in the Rel family appear to be regulated by subcellular localization: they are sequestered in an inactive form in the cytoplasm of cells and become active by translocation into the nucleus. Most of the Rel proteins that have been described are able both to activate and to repress transcription. These proteins have gene activation and cytoplasmic anchoring function within their carboxy-terminal domain. The highly related amino-terminal domain of Rel proteins contains several different functional motifs: a region involved in the formation of homodimers and heterodimers (Ghosh et al., 1990; Kieran et al., 1990; Nolan et al., 1991), a DNA-binding region (Ghosh et al., 1990; Kieran et al., 1990; Nolan et al., 1991). An IKB binding region (Ghosh et al., 1990), a region that inhibits the carboxy-terminal gene activation domain (Bull et al., 1990; Richardson and Gilmore, 1991), and a stretch of basic amino-acids that functions as nuclear translocation signal were also identified (Gilmore and Temin, 1988; Capobianco et al., 1990). A serine residue is located within a consensus recognition sequence for phosphorylation by protein kinase A and is positioned approximately 20 amino-acids amino-terminal to the nuclear targeting signal.

As *dorsal* belongs to the Rel family, it should be a DNA-binding protein and thus would be predicted to act as a transcriptional activator and/or repressor.

Zygotic genes affecting the D/V pattern

Mutations in several zygotic loci affecting the D/V pattern have been identified (Nüsslein-Volhard et al., 1984; Wieschaus et al., 1984; Jürgens et al., 1984). Generally these loci affect only one of the three D/V pattern elements (Fig. 2): dorsal ectoderm (Irish and Gelbart 1987; Rushlow et al., 1987a), ventral ectoderm (Mayer and Nüsslein-Volhard, 1988) and mesoderm (Simpson, 1983).

With such genetic analysis, seven loci required for the specification of dorsal structures have been identified: *decapentaplegic* (*dpp*), *zerknüllt* (*zen*), *screw* (*scw*), *tolloid* (*tld*), *shrew* (*srw*), *twisted gastrulation* (*tsg*) and *short gastrulation* (*sog*). Mutations in these loci cause a general loss of amnioserosa, dorsal ectoderm and dorsolaterally derived structures of the acron and telson (Anderson, 1987; Rushlow and Arora, 1990). Accompanying this loss of dorsal structures is an expansion of ventrolateral pattern elements. Two other zygotic loci, *twist* (*twi*) and *snail* (*sna*), are required for the formation of the ventralmost part of the embryo, the mesoderm. In such mutant embryos, the cells on the ventral side do not invaginate and no ventral furrow is formed (Simpson, 1983; Leptin and Grünewald, 1990) and the resulting embryos lack all derivatives of the mesoderm. This phenotype is very similar to the dl^D phenotype, the weakest phenotype of *dorsal*.

Some of these genes have been cloned and studies of their pattern of expression in wild-type and mutant embryos strongly support the hypothesis that the expression of these zygotic genes depends directly on the *dorsal* protein. In early wild-type embryos, *dpp* and *zen* are both expressed in the dorsalmost 40% of the blastoderm embryo. Their transcripts extend also around both anterior and posterior poles and label some ventral cells (St Johnston and Gelbart, 1987; Rushlow et al., 1987b). Ventrally, transcripts of *twi* and *sna* are first detected in a single continuous stripe, comprising the ventralmost 20% of the embryo, and extending up to and around both poles (Thisse et al., 1987, 1988; Alberga et al., 1991). For all of these zygotic genes, after this initial phase each of these pattern sharpens into a

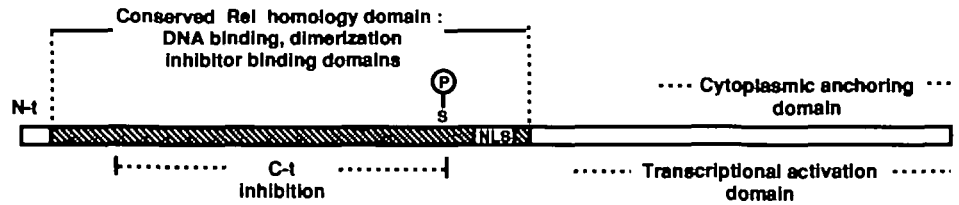

Fig. 1. Summary of structural information concerning the Rel family proteins. The domain of similarities (hatched box) includes a region important for DNA binding, dimerization, inhibitor binding (e. g. IKB), nuclear localization signal (NLS), inhibition of the unique carboxy terminal gene activation domain (Ct inhibition) and a consensus site for phosphorylation by protein kinase A (S-P). The amino-terminal domains vary in length. The carboxy-terminal domain contains transcriptional activation and cytoplasmic anchoring domains (modified from Gilmore, 1991).

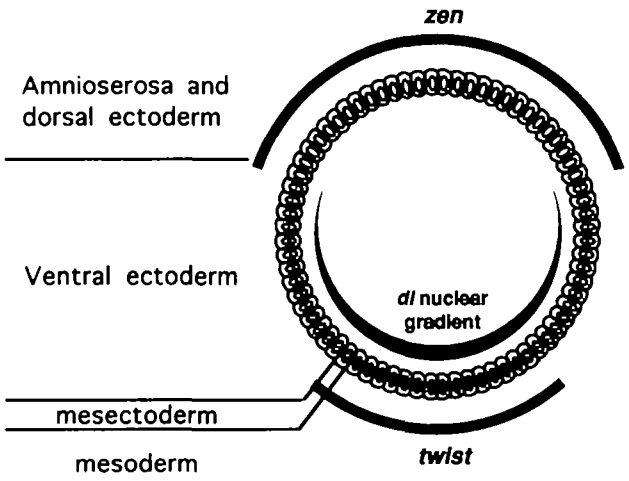

Fig. 2. Dorsoventral fate map of the blastoderm embryo in cross section and expression territories of Dorsal, Twi and Zen proteins. From dorsal to ventral: the amnioserosa and dorsal ectoderm, ventral ectoderm and mesoderm. Cells that connect mesoderm to ectoderm, and express Twi, form the mesectoderm. These cells are involved later in nervous system development. Dorsal protein is observed as a gradient of nuclear concentration with a maximum in the ventral region. In this region, its concentration is sufficient to allow the transcription of the *twi* gene. Due to a high affinity for binding sites on the *zen* promoter, Dorsal is also able to repress *zen* expression in ventral and lateral regions, thus restricting *zen* expression to the dorsal ectoderm and the amnioserosa (see details in the text).

refined pattern which is a derivative of the initial pattern (Ray et al., 1991).

In loss-of-function dorsal group mutations, in which Dorsal protein is excluded from the nuclei at all positions around the D/V axis, the dorsal zygotic genes *zen* and *dpp* are expressed everywhere, while the ventral genes *twi* and *sna* are not expressed anywhere (Roth et al., 1989; St Johnston and Nüsslein-Volhard, 1992). In such mutant embryos, all cells adopt a dorsal fate. Conversely, in the strongest ventralizing mutants, Dorsal protein localizes to all of the nuclei. In these mutants, *twi* and *sna* are expressed all around the circumference of the embryo while *zen* and *dpp* expression is repressed. Finally, in mutant combinations that produce a lateralized phenotype, Dorsal protein is evenly distributed between the nuclei and the cytoplasm and neither the dorsal nor ventral zygotic genes are expressed in the embryo (excepted for the polar regions).

These observations suggest strongly that the nuclear concentration of Dorsal determines the dorsoventral pattern by controlling the expression of the zygotic genes: high nuclear concentration of Dorsal seems to initiate the expression of the *twi* and *sna* genes, which are responsible for the differentiation of the mesoderm in ventral regions, and repress expression of dorsal zygotic genes *dpp* and *zen*, restricting the expression to dorsal regions where they are responsible for the differentiation of dorsal ectodermal derivatives.

Molecular analysis of the Dorsal protein

Regulatory elements of two putative target genes for the Dorsal protein, *zen* and *twi* have been extensively analyzed at the molecular level. For the *zen* gene, a region responsible for its ventral repression was localized between −1.4 and −1.1 kb (Doyle et al., 1989; Ip et al., 1991). This distal regulatory element has the property of a silencer element and can act over a distance to repress ventral expression of a heterologous promoter (Doyle et al., 1989; Ip et al., 1991). As a high nuclear concentration of the Dorsal protein seems to be responsible for the ventral repression of *zen*, this silencer element in the *zen* promoter is a good candidate for carrying Dorsal response elements (DREs). Using gel shift assay experiments, with a bacterially expressed Dorsal protein, in vitro experiments revealed that it is able to bind to the DNA of this particular region (Ip et al., 1991). Three of the four Dorsal binding sites characterized in that study are located within the limits of the ventral repressor element. These observations correlate well with the expected repressor effect of the Dorsal protein on *zen* gene expression. The conserved sequence motif recognized by the Dorsal protein on the *zen* promoter is: G G G several As C C (Ip et al., 1991). These binding sites are closely related to the consensus recognition site of the NF-KB transcriptional factor.

While *dorsal* represses *zen* gene activity, it activates the *twi* gene. The first evidence of a regulation of *twi* transcription by *dorsal* came from northern blot experiments: *twi* RNA is not detected in embryos derived from eggs laid by mutant *dorsal* females (Thisse et al., 1987). Subsequently, an extensive analysis of the regulatory sequences of the *twi* gene was performed by using in vivo P-mediated rescue experiments. These studies revealed different regulatory regions: one negative region between −7 kb and −3 kb; two positive quantitative regions, one located between −3 kb and −0.8 kb, the second one located between +3.2 kb and +6.2 kb. As no *twi* RNA is found in embryos lacking the Dorsal protein, and as 0.8 kb is suffficient to allow transcription of the *twi* gene, these observations suggest that within the 0.8 kb minimal promoter, there are Dorsal response elements (Thisse et al., 1991).

Using different combinations of *twi* 5′ flanking sequences fused to a *lac*Z reporter gene and expressed in P-transformed embryos, two regions containing DREs were defined: the first one, or proximal region, was localized in the minimal promoter between −0.18 kb and −0.4 kb; the second one, or distal region, was localized between −0.8 kb and −1.4 kb (Thisse et al., 1991; Jiang et al., 1991; Pan et al., 1991). Deletion of this distal element causes a reduction of the *twi* pattern of expression. Expression is lost at both poles and is restricted to the ventralmost part of the embryo (Thisse et al., 1991., and Fig. 3). In addition, as judged from in situ hybridization, a substantial narrowing in the lateral limits of *twi* expression was observed for constructs lacking the distal element (Jiang et al., 1991; Pan et al., 1991). These two observations demonstrate that both proximal and distal regions are required for a wild-type expression pattern of the *twi* gene. Nevertheless, a β-galactosidase construct carrying the 3′ *twi* quantitative enhancer does not reveal the ventral restriction observed by in situ hybridization experiments, as mesectodermal cells (the more dorsally *twi*-expressing cells in wild-type embryos) are clearly labelled during gastrulation (Thisse et al., 1991

and Fig. 3). The ventral restriction observed by in situ hybridization corresponds to a reduction of the level, but not to a complete loss of *twi* expression. On the contrary, the expression in the poles of the embryos is never seen with such a truncated promoter even if the 3′ regulatory sequences are present. Evidently, the control of *twi* expression in the poles is different from its control in the ventral part of the embryo. As was recently proposed (Ray et al., 1991), the terminal gene system could act through or with *dorsal* to affect expression of the *twi* gene at the poles.

Cotransfection assays in cultured Schneider 2 cells demonstrated that the Dorsal protein specifically activates expression from the *twi* promoter (Thisse et al., 1991). These experiments defined two activation regions which correspond to activation regions previously determined in phenotypic rescue and β-galactosidase expression experiments. Some interactions between regions of activation on the *twi* promoter and sequences localized on the vector used were also observed. This result suggests that cooperative interactions between different DREs may play an important role in the regulation of *twi* by *dorsal*.

In vitro studies were performed in order to define sequences of the *twi* promoter bound by the Dorsal protein. Gel shift and DNAase I foot printing assays, using a bacterially expressed Dorsal protein identified binding sites in the proximal and distal activation regions (Thisse et al., 1991; Jiang et al., 1991; Pan et al., 1991; see also Fig. 4). Two major binding sites were identified in the proximal region. The sequence motifs, GAGAAAACCC and GGGAAAATGC are closely related to the consensus sequence of Dorsal binding sites described for the *zen* promoter. Surprisingly, other Dorsal binding sites, present on the *twi* promoter, and described by three independent laboratories are different. Such differences may be due to different bacterially expressed Dorsal protein extracts. We used a full-length Dorsal protein which was solubilized in urea and then progressively renaturated (Thisse et al., 1991). Two other laboratories used a truncated Dorsal pro-

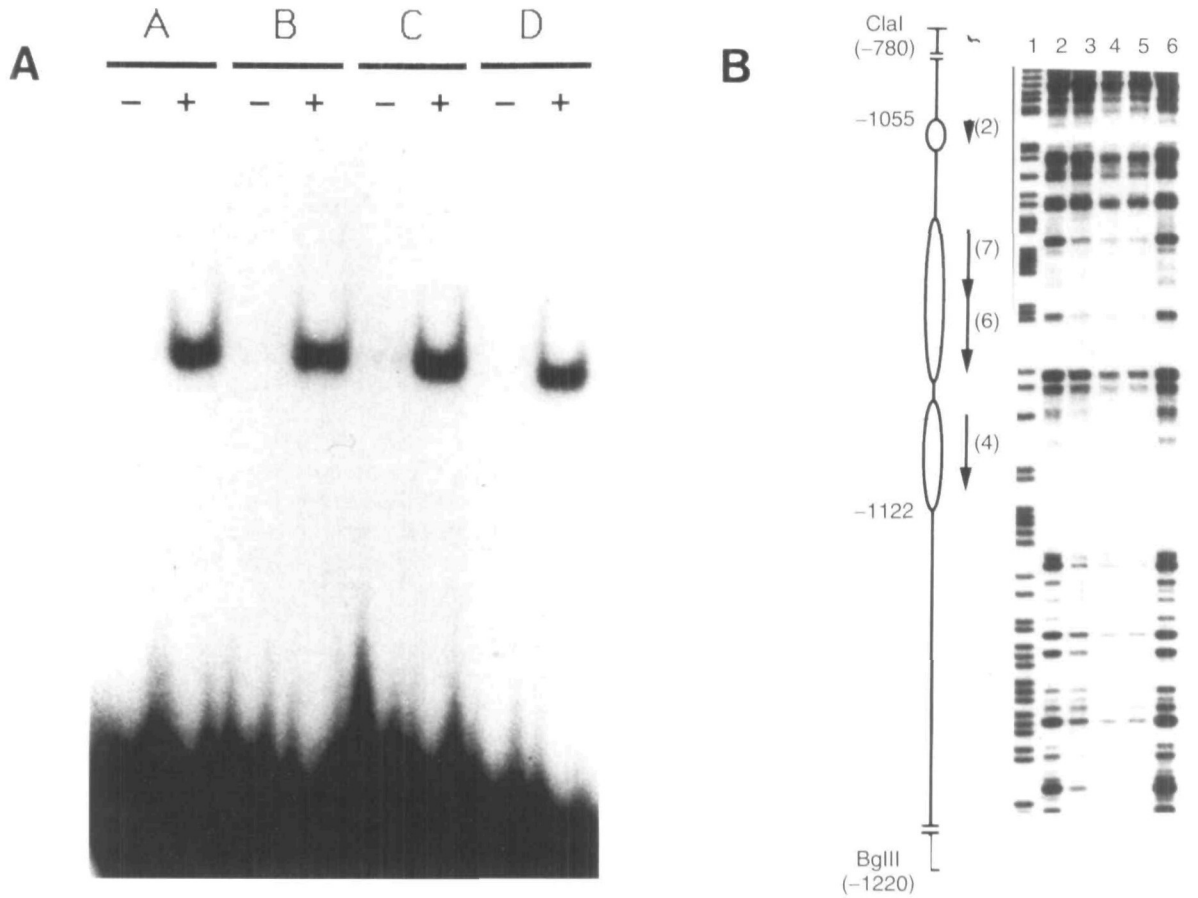

Fig. 4. (A) Gel retardation assays showing the binding of the bacterially synthesized Dorsal protein on *twist* promoter and pUC motifs. 2 μg of protein of an extract from IPTG-induced *E. coli*, were used with different ^{32}P-labeled probes in gel retardation assays. (A) Oligonucleotide corresponding to one site of the proximal region of activation by *dorsal*. (B and C) Oligonucleotides corresponding to motifs of the distal region. (D) Oligonucleotide corresponding to the most conserved motif in the pUC vector. +: extract from *E. coli* expressing *dorsal*. −: extract from IPTG induced *E. coli* containing the vector without insert (Thisse et al., 1991). (B) Footprint reaction using the bacterially induced full-length Dorsal protein. Example showing the DNAase I footprinting analysis of coding strand of region A. Lane 1: G+A product of chemical sequencing reactions. Lanes 2 and 6: DNAase I digestion of naked DNA. Lanes 3-5: pattern of protection with respectively 4, 8 and 16 μg of bacterial Dorsal protein. The diagram to the left of the autoradiogram represents the binding of the protein. The vertical arrows indicate the location and orientation of the conserved motifs (Thisse et al., 1991).

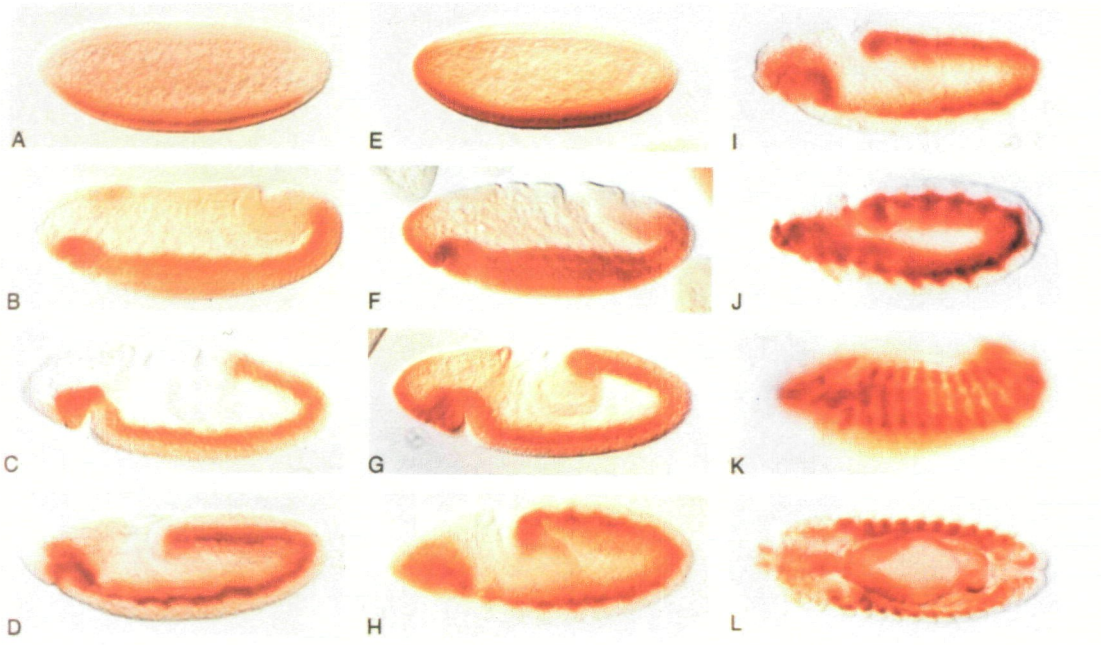

Fig. 3. Distribution of β-galactosidase expression in whole-mount embryos of different *twi*-β-galactosidase lines. Embryos of *twi*-β-galactosidase lines (with the 3′ region of *twi*) were stained using anti-β-galactosidase antibodies. Whole-mount preparations were photographed using Nomarski optics. (A-D) Embryos of the 0.8 kb *twi*-β-galactosidase lines lacking *twi* expression at their poles. (E-L) Embryos of 1.4 kb *twi*-β-galactosidase lines lacking *twi* expression at posterior pole during blastoderm stage due to the low rate of β-galacosidase synthesis (Thisse et al. 1991). (A and E) Cellular blastoderm embryos (stage 5), (B and F) gastrulating embryos (late stage 6), (C and G) embryos at germband extension (stage 7), (D and H) embryos at extended germband stage (stage 9), (I) embryo at stage 10, (J) embryo at stage 11, (K-L) embryos at stage 14. L is a dorsal view. Note for I to L that all the embryonic tissues involved in the larval muscle formation are labelled. Orientation of the embryos in A to K are lateral views, L is a top view. Staging is according to Campos-Ortega and Hartenstein (1985).

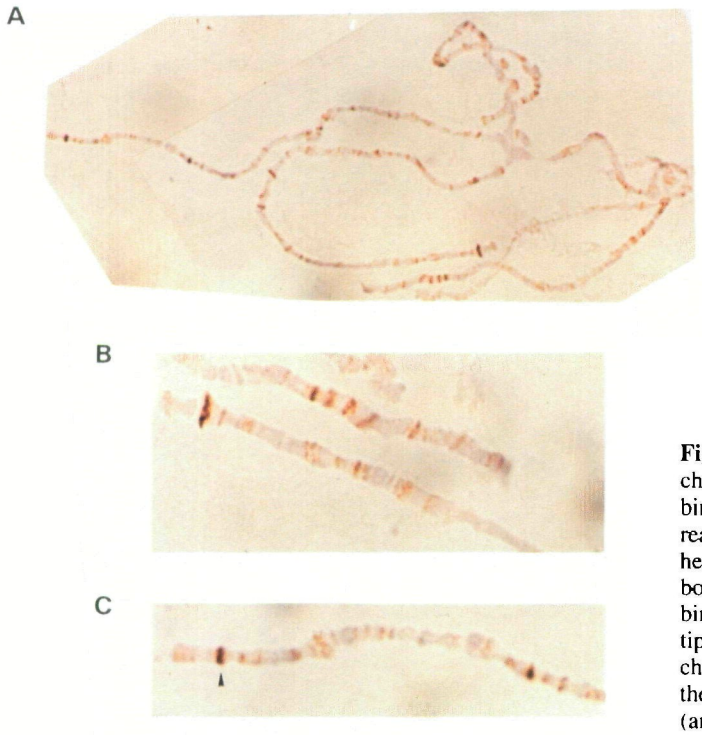

Fig. 6. Localization of Twi binding sites on polytene chromosomes of salivary glands of third instar larvae. Twi binding sites have been identified by immunocytochemical reaction using anti-Twi antisera. Chromosomes are stained with hematoxylin. (A) Entire nucleus showing chromosomes stained both with hematoxylin and anti-Twi antibodies. About 60 major binding sites have been identified. (B) Enlargment showing the tips of the right arm (top) and the left arm (bottom) of the third chromosome. (C) Enlargment showing the tip of the right arm of the second chromosome. One major band is located in 60B (arrow), which corresponds to the β*3 tubulin* locus.

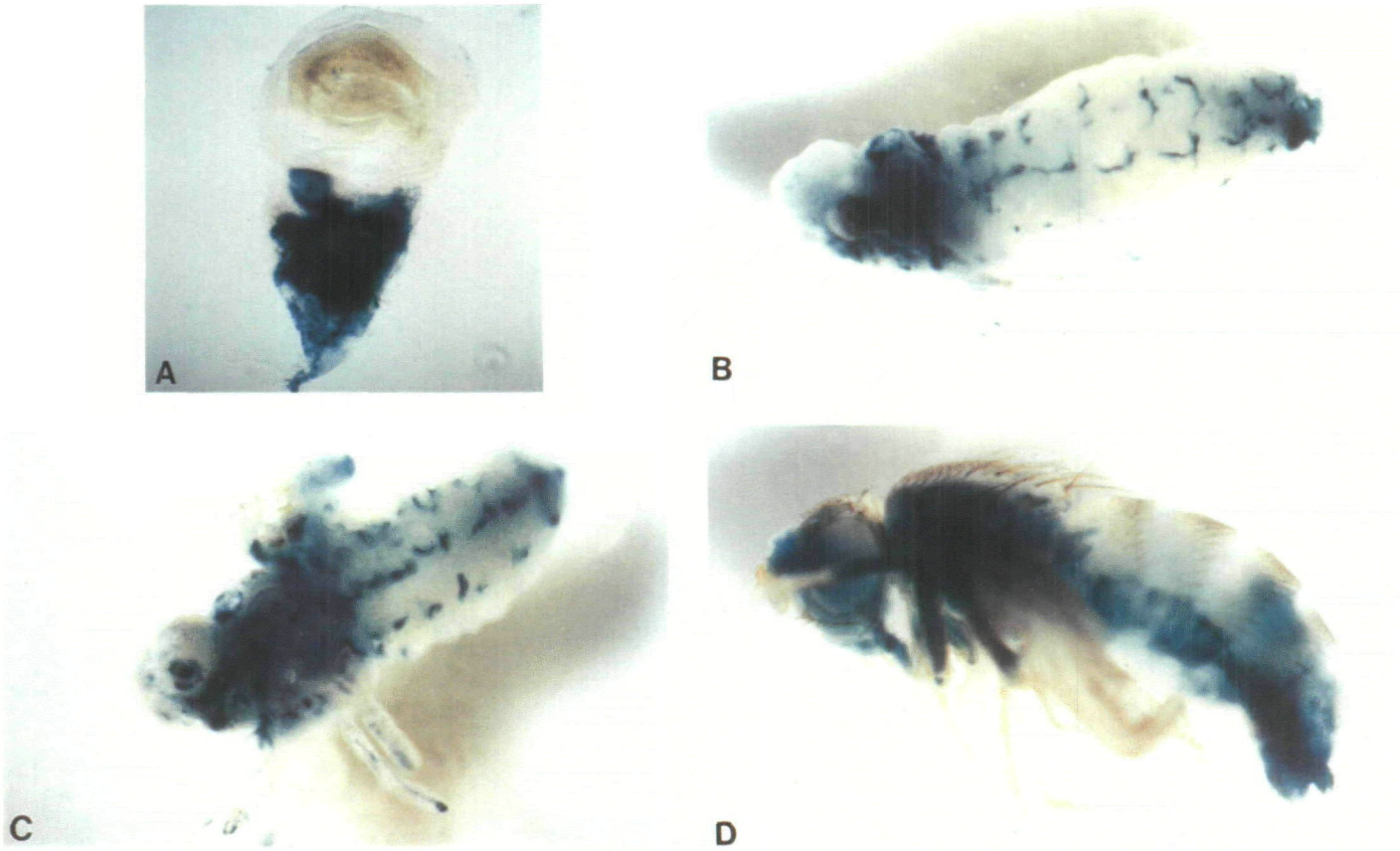

Fig. 8. Late distribution of β-galactosidase in *twi-lacZ* transgenic lines. β-galactosidase expression was revealed by X-Gal staining on whole-mount pupae and adult or dissected imaginal discs. (A) Wing imaginal disc: X-Gal staining is observed in adepithelial cells located in the thoracic part of the disc. These cells are involved in the formation of muscles necessary for flight. (B, C) β-galactosidase expression in pupae: staining is observed in all cells involved in adult muscle development. (D) β-galactosidase expression in an adult just after emergence: expression of β-galactosidase is revealed in all the adult muscles (low staining observed for abdominal dorsal muscles probably result from less diffusion of the X-Gal in this experiment).

tein (Jiang et al., 1991; Pan et al., 1991). Denaturation or partial deletion of this protein may modify some characteristics in the binding properties. Possibly, all these different sites would correspond in vivo to binding sites of native Dorsal protein. They could be low affinity sites and then cooperative mechanisms would increase the specificity of Dorsal action.

In addition, physiologically, the affinity of Dorsal for the *twi* promoter must be low, as *twi* transcription is initiated by Dorsal only in the ventral region of the embryo, where its nuclear concentration is maximal. In fact, binding affinity of Dorsal protein for *twi* and *zen* motifs have been compared in vitro. The affinity of Dorsal for *twi* motifs was found to be five times lower than the affinity of Dorsal for *zen* motifs (Thisse et al., 1991; Jiang et al., 1991; see also Fig. 5). This result correlates well with data concerning the spatial expression territories of these two genes, that is the minimum nuclear concentration of Dorsal able to activate *twi* is clearly higher than the maximum nuclear concentration of Dorsal able to repress *zen* (Fig. 2). Recently it was observed that the same Dorsal binding site can mediate either activation or repression depending upon its context within the promoter (Courey et al., 1992).

Thus, as for the anterior system for which the transmission of the maternal information to the zygote is mediated by the transcriptional activation of zygotic genes such as *hunchback* by the Bicoid protein (Driever and Nüsslein-Volhard, 1989), the transmission of the maternal information to the zygote along the D/V axis is mediated by transcriptional control of zygotic gene expression by the maternal gene product Dorsal.

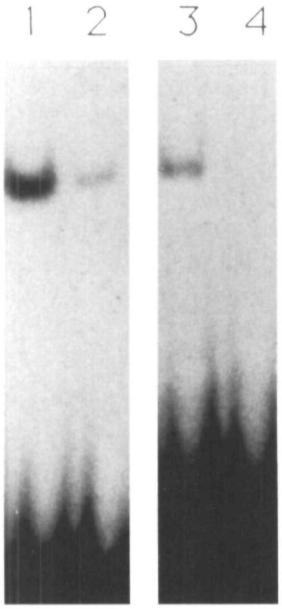

Fig. 5. Gel retardation assays showing the difference of binding affinity of Dorsal for *twist* and *zen* motifs. 0.5 μg (1 and 3) or 0.2 μg (2 and 4) of protein from IPTG induced *E. coli* were used with different ^{32}P-labeled probes in gel retardation assays. 1 and 2: gel retardation assay with a synthetic oligonucleotide corresponding to sequences −1534 to −1558 of the *zen* promoter ('strong' Dorsal binding sites described by Ip et al., (1991)), 3 and 4: gel retardation assay with the oligonucleotide B (see Fig. 4) from the distal region of activation by Dorsal on the *twi* promoter (Thisse et al., 1991).

Function of the zygotic genes

Four zygotic genes involved in D/V patterning have been extensively analyzed. The gene *zen* is necessary for formation of dorsal structures and it encodes a homeodomain-containing protein that binds to DNA (Rushlow et al., 1987a) and thus probably acts by regulating expression of downstream zygotic genes. The *dpp* gene is required for the formation of dorsal ectoderm and encodes a protein sharing strong sequence similarities with TGFβ (Gelbart, 1989), but its function in establishing the dorsal epidermis is not clearly understood. Two zygotic genes, involved in the formation of ventral structures, *sna* and *twi* encode putative DNA-binding proteins. The protein product of *sna* is a member of the zinc finger family (Boulay et al., 1987), and therefore is probably involved in transcriptional regulation. There is no direct molecular evidence at this time that the Sna protein regulates transcription, but the expression pattern of early embryonic markers in *sna* mutant embryos suggests that *sna* functions as a ventral repressor of laterally expressed genes, such as *single minded* (Leptin, 1991). Finally, *twi* encodes a protein containing a b-HLH domain, a domain described in a growing number of proteins involved in cellular differentiation and regulation of transcription (Murre et al., 1989). The b-HLH domain consists of a basic DNA-binding motif and helix-loop-helix dimerization motif (Murre et al., 1989). Presence of this domain in the Twi protein suggests that *twi* encodes a DNA-binding protein that would be able to dimerize and may act by regulating the expression of downstream mesodermal genes. Molecular arguments favor these predictions.

First, the Twi protein is able to homodimerize in vitro. Our finding based on glutaraldehyde cross-linking data, using a bacterially expressed Twi protein, is that one Twi monomer can complex with another and form a dimer. Under these experimental conditions, on SDS-PAGE, we observed a band that did not exist in a control extract with un-crosslinked Twi protein (data not shown). The apparent relative molecular mass corresponding to this band is twice that of monomeric Twi . This band is recognized by anti-Twi antibodies, strongly suggesting that it corresponds to the covalently linked Twi homodimer.

Secondly, we looked at the ability of the Twi protein to bind to DNA. We used one of the avantages of *Drosophila melanogaster*, that is the existence of polytene chromosomes in salivary glands of third instar larvae. The *twi* gene was cloned in an expression vector under the control of the promoter of the *sgs3* gene (salivary gland secretion protein 3) specific to salivary glands of third instar larvae (a gift of M. Martin). Transgenic flies were obtained that express *twi* in their salivary glands. By using anti-Twi antibodies, we detected Twi protein in the nuclei of cells of these salivary glands and localized Twi binding sites on polytene chromosomes (Fig. 6). Sixty major binding sites were observed. One of these sites was in 60B near the distal extremity of the right arm of chromosome 2 (Fig. 6B). This

site is at or near the β3 *tubulin* locus. This gene encodes a mesodermal variant of the β tubulin (Gash et al., 1989) that is not expressed in *twi* mutant embryos (Leiss et al., 1988), suggesting that *twi* is necessary for expression of this mesodermal gene, thus making it a potential target for the Twi protein. Unfortunately, complementary experiments using both cotransfection in cultured Schneider 2 cells and binding studies of the Twi protein on polytene chromosomes of transformed β3 *tubulin* lines carrying all the regulatory sequences of the β3 *tubulin* gene (a gift from R. Renkawitz-Pohl) reveal that Twi is not able to bind specifically to β3 *tubulin* regulatory sequences.

Such methods allowing the characterization of targeted genes have been previously described for some DNA-binding proteins, for example: *zeste* (Pirrotta et al., 1988), *polycomb* (Zink and Paro, 1989), *serendipity* β (Payre et al., 1990) and *polyhomeotic* (DeCamillis et al., 1992). All these genes are expressed in salivary glands of third instar larvae in normal development, but that is not the case for *twi*. In addition, all Twi binding sites were detected in the interband regions of chromosomes and thus this binding seems to be highly dependent of the chromatin structure. An absence of correlation between cytologically mapped binding sites on third instar larvae polytene chromosomes and in vitro DNA-binding specificities has also been described (Payre and Vincent, 1991). These authors proposed that only a subset of recognition sites for a given DNA-binding protein is occupied in a specific tissue at a given development stage. If this is true, this in vivo approach may not allow characterization of target genes, in the case of a gene like *twi* that is normally expressed during early embryonic development. Nevertheless, by using this method we have demonstrated that Twi is able to bind to specific sites on chromosomes.

In order to study the function of *twi*, we tested its ability to regulate its own transcription. In embryos mutant for strong *twi* alleles, *twi* RNA is initially transcribed normally, but during cellularization, the RNA begins to fade and finally disappears at the beginning of gastrulation. Thus, the early *twi* transcription pattern is established but its expression cannot be maintained in absence of the *twi* function (Leptin, 1991), consistent with the notion that *twi* expression is initiated by maternal genes, but maintained by an auto-regulatory function. Using cotransfection assays in cultured Schneider 2 cells we studied whether Twi protein could transactivate a CAT reporter gene under the control of the *twi* regulatory sequences. No transactivation of the reporter gene was detected, showing that Twi homodimer is not able to regulate its own expression. Surprisingly, in control experiments we observed a strong transactivation of the CAT gene from the pBLCAT2 vector we used (Fig. 7). Then looking in the pBLCAT2 vector sequence we found some CANNTG motifs. This motif, also called the E box, was previously described as a consensus binding site for other b-HLH proteins (Kingston, 1989). Three of these E boxes are localized upstream of the tk promoter of the pBLCAT2 vector in positions −72, −341 and −364. Deletion of a short restriction fragment upstream of the tk promoter eliminates the two distal E boxes and abolishes completely the transactivation by *twi* of the CAT gene (Fig. 7). This result strongly suggests that E boxes present in the

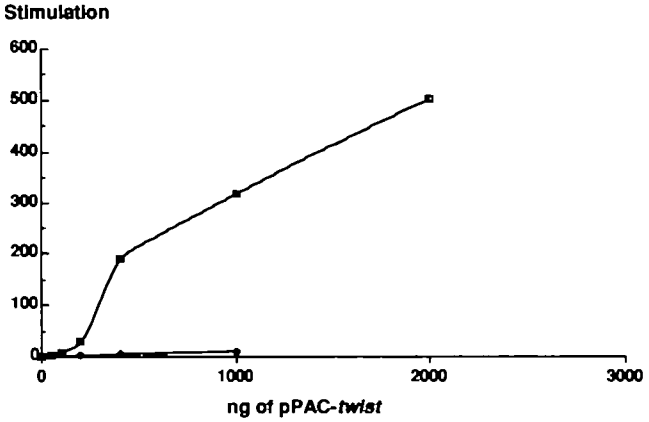

Fig. 7. Activation of CAT gene transcription by the Twi protein on the pBLCAT2 and pBLCAT2Δ vectors. 5 μg of pBLCAT2 (upper curve) and pBLCAT2Δ (lower curve) were cotransfected with the indicated amounts of pPAC-*twi*, a vector expressing *twi* under the control of the *actin 5C* promoter. The expression of β-galactosidase from the pACH vector used as control (Thisse et al., 1991) is not affected by the Twi protein.

region we deleted are involved in the transcriptional activation by Twi and that Twi is able to bind such sequences. This last hypothesis was tested in vitro using gel retardation assays, with a bacterially expressed Twi protein and an oligonucleotide corresponding to the pBLCAT2 sequence carrying the two distal E boxes. A specific retarded band was observed demonstrating that Twi is able to bind E boxes in vitro (data not shown).

In conclusion, the *twi* gene encodes a b-HLH protein, that homodimerizes in vitro and binds to chromosomes in vivo. It is also able to bind to DNA and it recognizes E box motifs and acts as a transcriptional activator.

Physiological targets of the Twi protein have yet to be well characterized but several genes are good candidates. For example, *tinman* (previously *msh*-2), is expressed in the early mesoderm between the end of the blastoderm stage and germ band elongation but is not expressed in *twi* mutant embryos (Bodmer et al., 1990). The second candidate is PS2α. This gene is expressed in cells of the presumptive mesoderm from the blastoderm stage until germ band retraction (Bogaert et al., 1987). PS2α is detected at the surface of basal cells of visceral myoblasts and is involved in the attachment of mesodermal cells to the ectoderm. No expression of this gene is detected in mutant *twi* embryos (Ingham, 1988; Leptin, 1991). Present and future work will determine whether *twi* is able to activate the transcription of these genes.

Phenotypic analysis of *twi* mutants showed that its activity is required for the invagination of the mesodermal layer at gastrulation (see the article by Leptin et al., in this issue). Molecular studies revealed that *twi* expression and probably function extends after these early steps of embryonic development. In fact, the *twi* product is detected in mesodermal territories until its differentiation in splanchnopleuric and somatopleuric mesoderm derivatives. After completion of germ band shortening, *twi* expression remains in a small residual population of cells of each segment (Thisse et al., 1988; Bate et al., 1991). This popula-

tion of cells proliferates during larval life. During the third instar larval stage, *twi* expression is detected in all imaginal discs (Bate et al., 1991; Broadie and Bate, 1991; see also Fig. 8A) and in some cells of each abdominal segment (Bate et al., 1991; Currie and Bate 1991; Broadie and Bate, 1991; and personal observations). For example, in a wing disc, *twi* expression is observed in adepithelial cells which are presumptive myoblasts of the adult musculature. Observation of *twi* expression during larval and pupal life and cell ablation experiments showed that cells with persistent *twi* expression are the embryonic precursors of adult muscles (Bate et al., 1991; Broadie and Bate, 1991). Using the advantage of the great stability of ß-galactosidase, it is possible to follow muscle formation in transgenic *twi-lacZ* lines, from precursor cells, during larval life, to the completely differentiated muscles of the adult (Fig. 8).

The Twi protein is observed in muscle precursors during early pupal stage, then disappears when muscle differentiation markers appear (Bate et al., 1991). Thus *twi* expression is only observed in undifferentiated cells, and *twi* may act by retaining these cells in an embryonic state prior to the onset of differentiation. This late function of *twi* in the muscle pathway is necessarily different from its function during mesodermal invagination at the gastrulation stage and, of course, the kind of genes regulated by *twi* during these two different processes must be different. We postulate that these two functions are mediated by two different sets of protein complexes. As Twi is a b-HLH protein and is able to form homodimers, it may also form heterodimer(s) with other uncharacterized b-HLH proteins. Such heterodimers may have new binding specificities and thus regulate new sets of genes. The early function of *twi* would be mediated by one oligomeric form (homodimeric or heterodimeric in a complex with b-HLH protein expressed at blastoderm stage) and for its later function, under an another oligomeric form, as a complex with other uncharacterized b-HLH protein expressed during late development and eventually with a more restricted tissue specificity.

Concluding remarks

During these last few years, one of the most interesting questions concerning the early development of the *Drosophila* embryo, namely, how the information coming from the mother is transmitted to the zygote, has been resolved by a combination of genetic and molecular analyses. First, genetic studies allowed the characterization of genes involved in this process; then the study of gene hierarchies allowed the classification of genes into a regulatory cascade resulting in the storage in the egg of molecular positional informations. Four localized maternal signals define the basic organization and polarity of the two major embryonic axes. These signals specify cell states and provide a prepattern of development. The anterior-posterior prepattern is formed by the spatially regulated transcription of the gap genes (St Johnston and Nüsslein-Volhard, 1992). For the D/V axis, the coordinate system results from a gradient of nuclear concentration of the Dorsal protein. This protein acts as a transcriptional activator or repressor depending of the target promoters. These two properties of activation and repression carried out by the same protein allows the division of the D/V prepattern into three regions: dorsally, the dorsal ectoderm expressing only dorsal zygotic genes; ventrally, the mesoderm, expressing only genes involved in ventral structures; and laterally, the ventral ectoderm that expresses neither dorsal nor ventral zygotic genes.

Interestingly, many of the zygotic genes regulated by the morphogen of the D/V axis encode putative transcriptional factors that are able to amplify and refine the early maternal signal. This succession of transcriptional regulation would probably continue during the next downstream steps as several DNA-binding proteins expected to act as transcriptional factors are putative targets for genes such as *twi* and *sna* (for example, *tinman* coding for a homeodomain-containing protein may be a target for *twi*).

In fact, function of early zygotic genes (immediately downstream of the maternal signal) like *twi* or *sna*, is probably complex. These genes act during the first step of embryonic development, that is just when the first movements occur in the embryo. As the time between the initiation of their expression (during the blastoderm stage) and gastrulation is too short to allow a complex cascade of transcriptional regulation, these genes must act directly on this process. This observation suggests that genes coding for proteins involved in cell shape and/or cell movements during ventral furrow formation would be early targets for *twi* and *sna*. Later, *twi* and *sna* would act via the activation and/or repression of other transcriptional factors.

An additional level of regulation would be a consequence of the properties of homo and heterodimerization of b-HLH proteins. These proteins could form heterodimers with other b-HLH proteins expressed at different stages of embryogenesis and with a possible tissue specificity. *twi*, in such heterodimers might have some new binding properties, allowing the transcriptional control of a new set of genes. Such mechanisms could be involved in the later function of *twi* in the myoblastic pathway.

Thus, with only a very small number of initial genes that are directly regulated by the maternal information, it would be possible to control in time and in space a large number of genes whose expression will be responsible for progressive cell differentiation and development of the embryo.

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