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Journal of Theoretical Biology 224 (2003) 517–537
Journal of Theoretical Biology
www.elsevier.com/locate/jtbi

Segmenting the fly embryo: a logical analysis of the *pair-rule* cross-regulatory module

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Received 13 November 2002; received in revised form 16 April 2003; accepted 9 May 2003

Abstract

This manuscript reports a dynamical analysis of the *pair-rule* cross-regulatory module controlling segmentation in *Drosophila melanogaster*. We propose a logical model accounting for the ability of the *pair-rule* module to determine the formation of alternate juxtaposed Engrailed- and Wingless-expressing cells that form the (para)segmental boundaries. This module has the intrinsic capacity to generate four distinct expression states, each characterized by the expression of a particular combination of *pair-rule* genes or *expression mode*. The selection of one of these expression modes depends on the maternal and gap inputs, but also crucially on cross-regulations among *pair-rule* genes. The latter are instrumental in the interpretation of the maternal-gap pre-pattern. Our logical model allows the qualitative reproduction of the patterns of *pair-rule* gene expressions corresponding to the wild type situation, to loss-of-function and *cis*-regulatory mutations, and to ectopic *pair-rule* expressions. Furthermore, this model provides a formal explanation for the morphogenetic role of the initial bell-shaped expression of the gene *even-skipped*, i.e. for the distinct effects of different levels of the Even-skipped protein on its target *pair-rule* genes. It also accounts for the requirement of Even-skipped for the formation of all Engrailed-stripes. Finally, it provides new insights into the roles and evolutionary origins of the apparent redundancies in the regulatory architecture of the *pair-rule* module.

Keywords: *Drosophila*; Segmentation; *Pair-rule* genes; Logical analysis; Regulatory circuit; Positive circuit

1. Introduction

The *Drosophila* embryo is composed of serially repeated units. This *segmental organization* also exists in the larval and adult stages, in which each unit has its own identity (morphology) depending on its position in the embryo. The first signs of segmentation, however, are transient grooves, which are visible on the surface of the embryo after gastrulation. These do not define adult segments but parasegments, the relevant developmental units in the segmentation of the *Drosophila* embryo (Martínez Arias and Lawrence, 1985). The posterior portion of one segment plus the anterior portion of the next most posterior segment make up an individual parasegment. Therefore, parasegments span the same length as segments but their borders lie between the segment boundaries. The initial parasegment organization of the embryo gives rise to the overt segmental organization shown by the larva and later by the adult. The gene system formed by the segmentation genes is responsible for the formation of (para)segments (reviewed in Ingham and Martínez Arias, 1992; Pankratz and Jäckle, 1993).

Segmentation originates from the combined action of maternal organizers on the zygotic genes (reviewed in St. Johnston and Nüsslein-Volhard, 1992; Pankratz and Jäckle, 1993; Sprenger and Nüsslein-Volhard, 1993; Rivera-Pomar and Jäckle, 1996). These zygotic *segmentation genes* can be classified into three categories depending on the number of segments affected by their mutations (Nüsslein-Volhard and Wieschaus, 1980). The *gap* genes affect several contiguous segments while the *pair-rule* genes affect complete alternate segments. Finally, the *segment polarity* genes affect each segment. From the point of view of genetic interactions, each of these three sets of genes forms a *cross-regulatory module*

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doi:10.1016/S0022-5193(03)00201-7

where all genes are involved in intertwined feedback circuits (for a discussion on *genetic cross-regulatory modules*, see Thieffry and Sánchez, in press). These modules are further organized into a *hierarchical system* in which interactions take place in a *temporal order*. The maternal products involved in segmentation act upon the zygotic genome, resulting in the activation of *gap* genes. The combined action of maternal and gap gene products determines the activation of *pair-rule* genes. These are expressed as seven stripes. Some *pair-rule* genes are needed for the formation of even-numbered segments and others for the formation of odd-numbered segments. Finally, the *pair-rule* genes control the activity of *segment polarity* genes, and ultimately determine which cells will express the *segment polarity* gene *engrailed* (*en*) in juxtaposition to cells that express the *segment polarity* gene *wingless* (*wg*) along the anterior–posterior axis of the embryo trunk. The juxtaposition of these two types of cells determines the formation of the (para) segment boundaries.

In a previous paper, we proposed and analysed a qualitative model of the *gap* cross-regulatory module, while taking into account inputs from maternal products (Sánchez and Thieffry, 2001). This model accounts for the delineation of four distinct domains along the trunk of the embryo. These domains define a pre-pattern for the expression of the next segmental module, namely the *pair-rule* genes, through different combinations of expression levels of the *gap* genes. The present manuscript focuses on the analysis of the network of cross-regulations among *pair-rule* genes. More specifically, it aims at better understanding how these genes control the formation of alternate juxtaposed En- and Wg-stripes that form the (para)segmental boundaries. It is important to keep in mind that we are dealing with the initial activation of *en* and *wg* by the *pair-rule* genes, i.e. with the initial formation of the (para)segment boundaries, and not with the maintenance of these boundaries, which is due to interactions between the *segment polarity* genes (for a model of the *segment-polarity* network, see Von Dassow et al., 2000; Von Dassow and Odell, 2002). The analysis presented here on the *pair-rule* cross-regulatory module complements that performed by Reinitz et al. (1998), who studied the mechanism of Eve-stripe formation as a response to particular combinations of maternal and gap products.

2. The pair-rule cross-regulatory module

On the basis of the temporal expression pattern, two types of *pair-rule* genes are distinguished. The maternal and gap proteins predominantly regulate the initial periodic expression pattern of the "primary" *pair-rule* genes. The primary *pair-rule* genes considered here are *even-skipped* (*eve*), *runt* (*run*), *hairy* (*h*), and *fushi-tarazu* (*ftz*). For the sake of simplicity, we also consider *ftz* as a primary *pair-rule* gene as its initial uniform expression does not depend on the other primary *pair-rule* genes, even though *hairy* is transiently required for the maintenance of the Ftz-stripes (Howard and Ingham, 1986; Carroll and Scott, 1986; Ingham and Gergen, 1988; Carroll and Vavra, 1989; Yu and Pick, 1995). The periodic expression pattern of the "secondary" *pair-rule* genes depends largely on that of the primary *pair-rule* genes (see below). The secondary *pair-rule* genes considered here are *odd-skipped* (*odd*), *paired* (*prd*), *sloppy-paired* (*slp*) and *partner-of-paired* (*ppa*).

The seven stripes of primary *pair-rule* genes develop on a stripe-by-stripe basis as a response to the non-periodic distribution of maternal and gap products. Particular combinations of these products bind to the *cis*-acting region-specific enhancers of the primary *pair-rule* genes, which control the expression of these genes to form different stripes. In the case of the secondary *pair-rule* genes, the seven stripes are formed in unison as a response to the maternal and gap products in the pre-set periodic pattern of primary *pair-rule* gene products. This response involves a single *cis*-acting regulatory enhancer.

The initial expression patterns of *pair-rule* genes form bell-shaped domains, which are later refined into the final narrow stripes, providing the positional information for the generation of Engrailed- and Wingless-stripes (see Fig. 1). This refinement process results from interactions between the *pair-rule* genes (see Fig. 2). These interactions are common to all locations along the anterior–posterior axis where the (para)segment boundaries (i.e. juxtaposition of En- and Wg-expressing cells) form. As the final role of the *pair-rule* module is to determine the formation of alternate En- and Wg-stripes along the anterior–posterior axis of the embryo, a subset of *pair-rule* genes that play the key role in regulating the spatial location of these stripes was selected. Other *pair-rule* genes, such as *odd-paired* (*opa*), are further required for the activation of both *en* and *wg* genes, but not for setting spatial expression pattern of these genes (Benedyk et al., 1994).

The *pair-rule* genes *eve* and *ftz* are expressed in seven roughly complementary stripes overlapping at their edges along the anterior–posterior axis of the embryo trunk. They play a key role in the formation of parasegments by determining the state of activity of the segment polarity genes *en* and *wg*, which are expressed in 14 stripes (reviewed in Ingham and Martínez Arias, 1992; Pankratz and Jäckle, 1993). Both Eve and Ftz products act as activators of *en*: Eve is responsible for the odd-numbered En-stripes and Ftz for the even-numbered En-stripes. Moreover, both Eve and Ftz act as repressors of *wg*. Cells expressing *en* mark the anterior (posterior) border of parasegments (segments) and cells expressing *wg* mark the posterior (anterior) border of parasegments (segments).

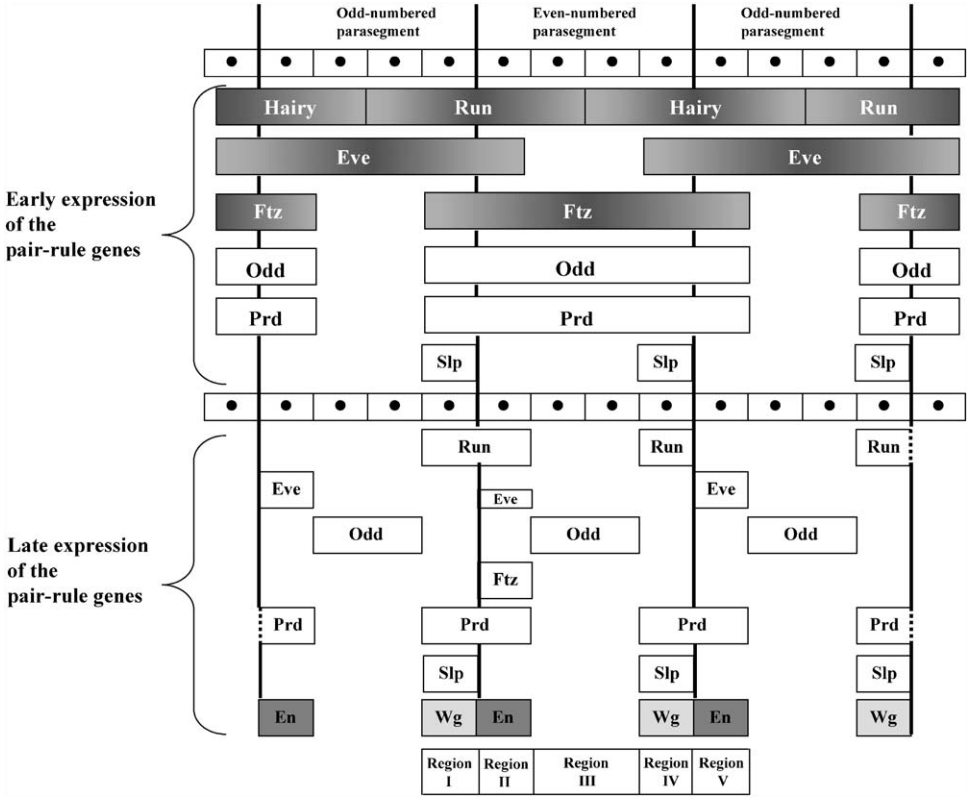

Fig. 1. Schematic representation of the main *pair-rule* gene expression domains. The initial expression patterns of *pair-rule* genes form seven bell-shaped domains (top), which are later refined into final narrow stripes (bottom). Gene *hairy* is shown on the top of the figure because this gene ceases its expression earlier than its target genes *ftz* and *run*, before the final refinement process leading to the narrow stripes of the *pair-rule* genes directly involved in *en* and *wg* activation. Five different regions (I–V) defining two parasegment borders are indicated. The interactions between the *pair-rule* genes are the same in all locations along the anterior–posterior axis where the parasegment boundaries, represented by thick lines. These are flanked by two regions expressing *wg* (region I or IV) and *en* (region II or V) respectively. Regions IIIa and IIIb correspond to the middle of the parasegment, each representing a region adjacent to the *en* or *wg* expressing cells within the parasegment. As a whole, region III is characterized by the lack of expression of both *en* and *wg* and by the expression of *odd*. The primary and secondary *pair-rule* genes are shown in graded shadow and empty boxes, respectively. Genes symbols: *runt* (Run), *even-skipped* (Eve), *fushi-tarazu* (Ftz), *odd-skipped* (Odd), *paired* (Prd), *sloppy-paired* (Slp), *engrailed* (En) and *wingless* (Wg).

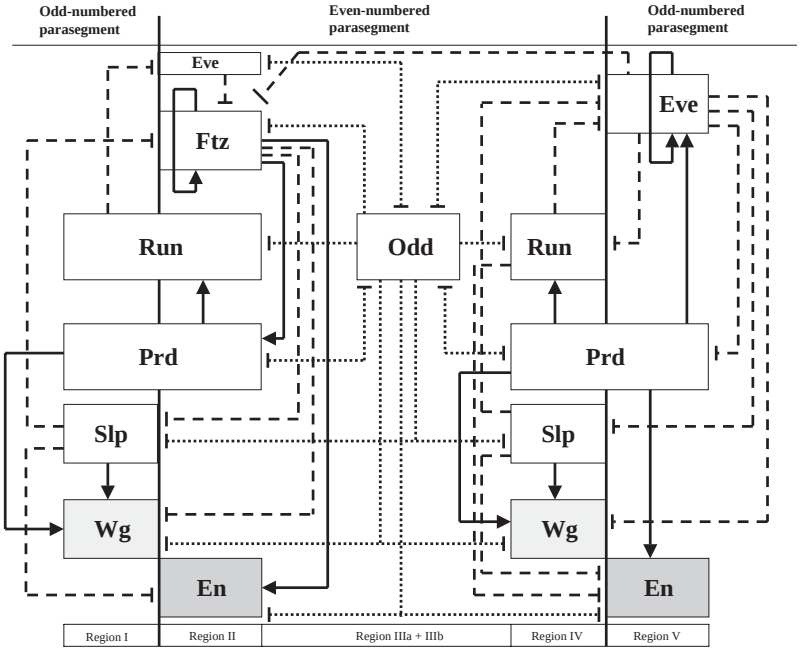

Fig. 2. Final *pair-rule* expression domains after the refinement process (represented in bottom of Fig. 1) and interactions between these genes. Solid arrows stand for positive interactions, dotted blunt arrows indicate negative interactions. For other symbols, see legend of Fig. 1.

The final expression domains of the *pair-rule* genes resulting from the refinement process are schematically represented in Fig. 2, together with those of *en* and *wg* (references cited in Table 1 and Fig. 7A). Numbered from I to V, these regions are inferred from experimental data on the expression domains of the corresponding genes:

(1) In regions I and IV, *wg* is activated in the presence of significant expression of *prd*, *ppa*, *run* and *slp*, but in the absence of significant expression of *eve*, *ftz* and *odd*.
(2) In region II, *en* is activated by high expression of *ftz*, in the presence of significant *eve*, *prd*, *ppa* and *run* expression, but in the absence of *slp* and *odd*.
(3) In region III, neither *en* nor *wg* are expressed because of the presence of the repressing product of *odd*. Only *ppa* and *odd* are significantly expressed.
(4) In region V, *en* is activated only in the presence of high *eve* and *prd* expressions.

Fig. 3 shows a diagrammatic description of the network responsible for the final refinement process. This network is formed by cross-regulations between the *pair-rule* genes *eve, ftz, odd, run, prd, ppa* and *slp*, which constitute the *pair-rule cross-regulatory module* directly involved in the activation of *en* and *wg* in adjacent cells.

Table 1
Interactions between *pair-rule* and *en* and *wg* genes

Interaction	Main experimental observations	Ref.
h —|*ftz*	In loss-of-function *h* mutants, *ftz* is ectopically expressed. Ectopic *h* expression suppresses *ftz* expression	Howard and Ingham (1986), Carroll and Scott (1986), Ish-Horowicz and Pinchin (1987), Ingham and Gergen (1988), Carroll and Vavra (1989)
h —|*run*	In loss-of-function *h* mutants, *run* is ectopically expressed.	Ingham and Gergen (1988), Carroll and Vavra (1989)
eve → *eve*	The *eve* promoter contains Eve-binding sites that when mutated dramatically affect expression of gene *eve*	Frasch et al. (1988), Goto et al. (1989), Harding et al. (1989), Jiang et al. (1991)
eve —|*ftz*	Ectopic *eve* expression represses gene *ftz*	Manoukian and Krause (1992)
eve —|*ppa*	In loss-of-function *eve* mutants, *ppa* is ectopically expressed	Raj et al. (2000)
eve —|*prd*	Peak concentrations of early Eve protein restrict the posterior borders of Prd-stripes, and maintain repression of *prd* between early Prd-stripes	Gutjahr et al. (1993), Fujioka et al. (1995)
eve —|*run*	Ectopic *eve* expression represses gene *run*	Manoukian and Krause (1992)
eve —|*slp*	In embryos carrying an *eve*-transgene whose expression is locally restricted, ectopic *slp* expression occurs in Eve-deficient regions	Fujioka et al. (1995)
eve —|*odd*	Eve "clears" *odd*-expression from the anterior-most cells of each Ftz-stripe, but in loss-of-function *eve* mutants the expression of odd remains.	Manoukian and Krause (1992), Fujioka et al. (1995)
ftz → *ftz*	Ftz protein directly binds and regulates expression of its own gene	Hiromi and Gehring (1987), Ish-Horowicz et al., (1989), Pick et al. (1990), Schier and Gehring (1992)
ftz → *prd*	In loss-of-function *ftz* mutants, the Prd-stripes are narrower than in wild type embryos	Baumgartner and Noll (1991), Kilchherr et al. (1986), Gutjahr et al. (1994)
ftz —|*slp*	In loss-of-function *ftz* mutants, the Slp-stripes broadened	Nasiadka and Krause (1999)
odd —|*eve*	Ectopic *odd* expression represses gene *eve*	Saulier Le-Dréan et al. (1998)
odd —|*ftz*	Ectopic *odd* expression represses gene *ftz*	Saulier Le-Dréan et al. (1998)
odd —|*slp*	Ectopic *odd* expression represses gene *slp*	Saulier Le-Dréan et al. (1998)
odd —|*prd*	Ectopic *odd* expression represses gene *prd*	Saulier Le-Dréan et al. (1998)
odd —|*run*	Ectopic *odd* expression represses gene *run*	Saulier Le-Dréan et al. (1998)
prd → *eve*	Binding of the Prd protein to the regulatory L element of *eve* promoter is required to activate the formation of late Eve-stripes	Fujioka et al. (1996)
run —|*eve*	Ectopic *run* expression represses gene *eve*	Manoukian and Krause (1993)
slp —|*eve*	In loss-of-function *slp* mutants, gene *eve* is ectopically expressed	Cadigan et al. (1994)
prd —|*odd*	*prd* prevents *odd* expression in the posterior-most cells of the parasegments, where gene *wg* will be activated	Mullen and DiNardo (1995)
ppa —|*prd*	Ectopic *ppa* expression reduces the levels of Prd protein	Raj et al. (2000)
slp —|*ftz*	In loss-of-function *slp* mutants, gene *ftz* is ectopically expressed	Cadigan et al. (1994)
prd → *run*	In loss-of-function *prd* mutants, the seven additional Run-stripes are not formed, and the seven original Prd-stripes become weaken	Klingler and Gergen (1993)

In the first column, normal arrows (→) represent positive interactions, blunt arrows (—|) represent negative interactions. Experimental evidences supporting each of these interactions are summarized in the middle column, and documented by bibliographical references in the last column.

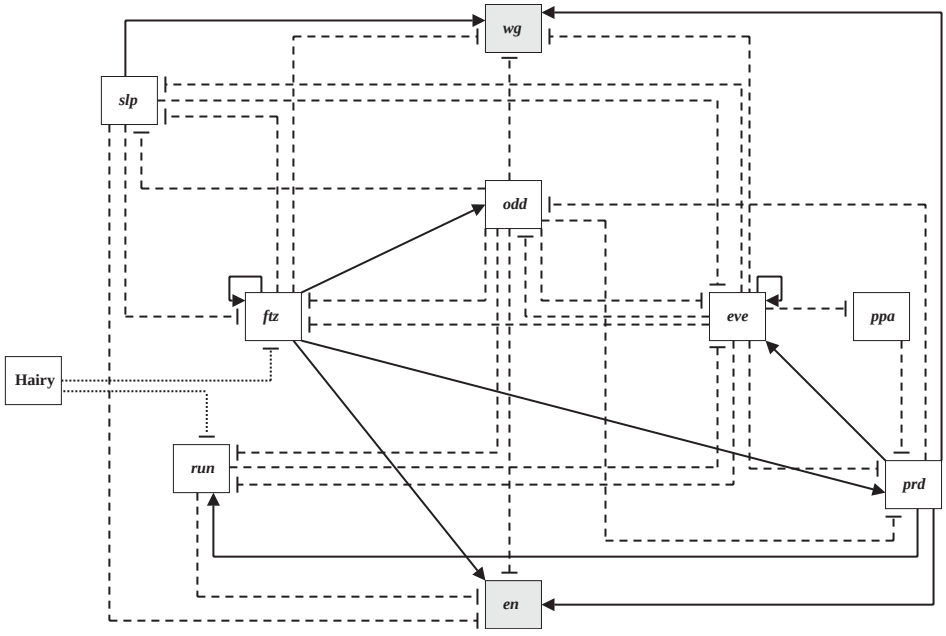

Fig. 3. Graph of interactions for the *pair-rule* cross-regulatory module made up of genes *eve, ftz, odd, run, prd, ppa* and *slp*. Gene *hairy* is considered here as an input affecting *ftz* and *run* early expression. Segment-polarity genes *en* and *wg* are outputs of this module. Solid arrows stand for positive interactions; dotted blunt arrows indicate negative interactions.

Note that the *pair-rule* gene *hairy* (*h*) is considered here as an input gene. Indeed, *hairy* ceases to be expressed earlier than the other *pair-rule* genes, in particular well before its two target genes *ftz* and *run*, and also before the final *pair-rule* refinement process starts (Ish-Horowicz et al., 1985; Ingham et al., 1985; Ingham and Gergen, 1988; Klingler and Gergen, 1993). Consequently, *hairy* shows only the typical early *pair-rule* expression pattern, with no further refinement. We do not take into consideration the repression effect of *run* upon *hairy* because this effect appears to be stripe-specific, with the Hairy-stripe 1 the most notably affected. Indeed, in the absence of *run* activity (Ingham and Gergen, 1988; Carroll and Vavra, 1989), or in case of ectopic expression of *run* (Tsai and Gergen, 1994; Tracy et al., 2000), the Hairy pattern remains fairly periodic. Furthermore, this effect occurs at the beginning of activation of *pair-rule* genes by the maternal-gap inputs but disappears during the refinement process. Finally, the effect of *run* upon *hairy* appears to be indirect (Manoukian and Krause, 1993).

This graph encompasses all direct interactions known to occur between the *pair-rule* genes considered and between these and the *segment-polarity* genes *en* and *wg*. These last genes are also included as they constitute the main outputs of the *pair-rule* module. This set of direct interactions was derived from the phenotype of *pair-rule* mutants and from results describing the effect of ectopic *pair-rule* expression on the expression of other *pair-rule* genes. Fig. 3 is therefore based on experimental data plus assumptions. These refer to cases where a direct interaction was supposed whenever the function of a given gene is required for either activation or repression of its target gene, in the absence of experimental data definitively establishing the direct character of the interaction in question. A summary of the experimental evidence backing this graph of interactions is provided in Table 1.

Extensive analysis of this graph leads to the recognition of 51 intertwined, distinct feedback circuits (28 positive and 23 negative), each involving from one to seven elements. Such feedback circuits have already been shown to play crucial roles in the dynamics of gene expression. Indeed, positive circuits (involving an even number of negative interactions) are instrumental in the generation of differentiation pathways, whereas negative circuits (involving an odd number of negative interactions) may generate homeostatic dynamics or oscillatory gene expression (Thomas et al., 1995). The network is thus very complex, but it is clear that only a subset of the 51 circuits of the system can really be instrumental. We shall see that our logical approach allows the delineation of the roles of the most relevant circuits.

3. Logical formalization of the pair-rule cross-regulatory module

A *generalized logical formalism* was used to analyse the *pair-rule* cross-regulatory module shown in Fig. 3. For an introduction to this formalism, see Thomas (1991) and Thomas et al. (1995); for applications of this

Gene product	Thresholds and corresponding order relationships
Eve	$(\theta_{Eve \rightarrow eve} = \theta_{Eve \dashv odd}) < \theta_{Eve \dashv \{ppa, run, slp, ftz\}} < \theta_{Eve \dashv prd}$
Prd	$\theta_{Prd \rightarrow eve} = \theta_{Prd \rightarrow run} = \theta_{Prd \dashv odd}$
Ppa	$\theta_{Ppa \dashv prd}$
Run	$\theta_{Run \dashv eve}$
Slp	$\theta_{Slp \dashv eve} = \theta_{Slp \dashv ftz}$
Ftz	$\theta_{Ftz \rightarrow \{ftz, prd, odd\}} = \theta_{Ftz \dashv slp}$
Odd	$\theta_{Odd \dashv eve, prd, run, slp, ftz}$

(A)

$$V = d_v(k_v + k_{v,v}v^{(1)} + k_{v,p}p^{(1)} + k_{v,r}\bar{r} + k_{v,s}\bar{s} + k_{v,d}\bar{d})$$
$$P = d_p(k_p + k_{p,z}z^{(1)} + k_{p,v}\overline{v^{(3)}} + k_{p,a}\bar{a} + k_{p,d}\bar{d})$$
$$A = d_a(k_a + k_{a,v}\overline{v^{(2)}})$$
$$R = d_r(k_r + k_{r,p}p^{(1)} + k_{r,v}\overline{v^{(2)}} + k_{r,d}\bar{d})$$
$$S = d_s(k_s + k_{s,v}\overline{v^{(2)}} + k_{s,z}\overline{z^{(1)}} + k_{s,d}\bar{d})$$
$$Z = d_z(k_z + k_{z,z}z^{(1)} + k_{z,v}\overline{v^{(2)}} + k_{z,s}\bar{s} + k_{z,d}\bar{d})$$
$$D = d_d(k_d + k_{d,z}z^{(1)} + k_{d,v}\overline{v^{(1)}} + k_{d,p}\overline{p^{(1)}})$$

$$V = eve,\ P = prd,\ A = ppa,\ R = run,\ S = slp,\ Z = ftz,\ D = odd$$

(B)

Fig. 4. (A) Functional threshold levels for the pair-rule gene products. Eve is assumed to have three functional threshold values on the basis of the effects of *eve* ectopic expression on the other *pair-rule* genes. The gene *eve* was placed under the control of the heat-shock promoter, and different heat-shock pulses were performed. Short pulses repress only *odd*, whereas longer pulses are required to repress gene *run, ftz* and *prd* (Manoukian and Krause, 1992). Moreover, peak concentrations of early Eve restrict the posterior borders of Prd-stripes and maintain repression of *prd* between early Prd-stripes (Fujioka et al., 1995). Consequently, we propose that Eve can activate itself, and repress *odd* at low concentrations ($\mathbf{v} \geqslant 1$); repression of *run, slp* and *ftz* by Eve would occur at middle concentrations ($\mathbf{v} \geqslant 2$); finally, *prd* would be repressed only by still higher concentration of Eve ($\mathbf{v} = 3$). In the absence of similar functional data for the other *pair-rule* genes and for parsimony reasons, a single threshold has been considered for all the corresponding cross-regulations. (B) Generalized logical equations for the *pair-rule* cross-regulatory module. A logical variable is associated with the level of each pair-rule product. The logical variables associated with the pair-rule proteins Eve, Prd, Ppa, Run, Slp, Ftz and Odd are **v**, **p**, **a**, **r**, **s**, **z** and **d**, respectively. In addition, a logical function qualitatively represents the level of transcription of the corresponding gene; **V**, **P**, **A**, **R**, **S**, **Z** and **D** thus stand for the logical functions corresponding to the genes *eve, prd, ppa, run, slp, ftz* and *odd*, respectively. The *k*'s stand for real parameters, which quantify the weight of each interaction. The terms k_v, k_p, k_a, k_r, k_s, k_z and k_d represent the basal expression levels of the genes *eve, prd, ppa, run, slp, ftz* and *odd*, respectively (since these genes show no basal expression in wild-type conditions, these basal parameters all take the value zero). The *d*'s are scaling operators, which transform the bracketed terms into logical parameters, denoted by (capital letter) *K*'s. Whenever a superscript is appended to a variable in the equations, it refers to one specific threshold, leading to the definition of a specific Boolean variable. The bar on top of the variables stands for "logical NOT". For a more detailed description of this formalism see (Thomas, 1991). Inputs such as maternal and gap products or *hairy* are not explicitly represented in these equations. For the sake of simplicity, the differential effects of these inputs are taken into account through the formulation of three conditions (C1–C3) enabling the resolution of competitive situations involving specific pairs of *pair-rule* genes.

formalism to the analysis of developmental gene systems see Sánchez et al. (1997) and Sánchez and Thieffry (2001).

Briefly, a *logical variable* is associated with each pair-rule product so that one specific integer value **(0, 1, 2, ...)** is assigned to each functional level (i.e. functional product concentration or activity). Whenever needed, *multilevel* logical variables are used to represent situations where distinct functional concentrations of the same regulatory product are involved. Furthermore, a *logical function* is associated with each *pair-rule* gene to represent its current level of expression (e.g. transcription level). For a given gene, the value of this function depends on that of relevant regulatory input variables (see Fig. 4 for a full description of the *pair-rule* equations).

In Fig. 4A, the functional *threshold* levels of the pair-rule gene products and their corresponding order relationships are shown. It was assumed that Eve product has three functional thresholds (see the first

equation in Fig. 4B). A central idea to the model developed here is that the initial bell-shaped expression of gene *eve* behaves as a morphogen, with different concentrations of Eve protein having different effects on its target genes (Manoukian and Krause, 1992; Fujioka et al., 1995). For the rest of the *pair-rule* genes, it was assumed for parsimony that their products have a single functional threshold for all interactions with the other *pair-rule* genes (see legend of Fig. 4 for a justification of this threshold configuration).

The set of equations corresponding to the graph of Fig. 3 is shown in Fig. 4B. None of the maternal-gap products or *hairy* input are explicitly considered in these equations. On the basis of these equations, a *general state table* (not shown) can be built. This table describes the expression state of the *pair-rule* genes (*logical functions*) in terms of *logical parameters* for each qualitatively distinct combination of pair-rule product concentrations (*logical variables*). The values of the logical parameters correspond to a qualitative evaluation of the effect of each interaction or combination of interactions controlling the expression of a given gene. Depending on the values of the logical parameters, the general state table encompasses a large but finite number of dynamical behaviours. For more information on the derivation of state tables and of logical parameters from generalized logical equations, see the appendix of Sánchez and Thieffry (2001).

As a first step, we have analysed the capacity of the *pair-rule* cross-regulatory module to adopt the four different cellular states required to set the right expression pattern for both *en* and *wg*. A set of minimal values was selected for the logical parameters (K's), enabling the generation of the four specific cellular states found in regions I–V (see Table 2).

In Fig. 5, a compact representation of the asynchronous logical dynamics of the *pair-rule* cross-regulatory module is presented (*logical dynamical graph*). What is stressed here is the capacity of this module to reach alternative stable states depending exclusively on the interactions between the seven constitutive genes, i.e. for the system in isolation. The initial state thus corresponds to a situation where all seven pair-rule products are absent, which formally translates into a value "**0000000**" for the *logical state vector* "**vparszd**", where "**v**" stands for *eve*, "**p**" for *prd*, "**a**" for *ppa*, "**r**" for *run*, "**s**" for *slp*, "**z**" for *ftz*, and "**d**" for *odd* activity (or product level). In this state, for proper parameter values, the lack of repressing activities lead to the activation of genes *prd*, *ppa*, *slp*, *ftz* and *odd* ("+" superscripts on **p**, **a**, **s**, **z** and **d** variables in the *virgin state* **0000000**), and the system is driven through one of five alternative pathways, ultimately leading to the selection of one of the four *stable* expression states **[3200000]**, **[1111020]**, **[0111100]** and **[0010001]**.

Table 2
Logical parameter (K's) values for the *pair-rule* module

Gene	Value 1	Value 2	Value 3
eve	$K_{v.vrs}$		$K_{v.vprsd}$
	$K_{v.psd}$		
	$K_{v.vsd}$		
	$K_{v.prsd}$		
	$K_{v.vpsd}$		
	$K_{v.vprs}$		
	$K_{v.vrsd}$		
prd	$K_{p.d}$	$K_{p.ad}$	
	$K_{p.vd}$	$K_{p.vad}$	
	$K_{p.zd}$	$K_{p.azd}$	
	$K_{p.zvd}$	$K_{p.zvad}$	
ppa	$K_{a.v}$		
run	$K_{r.pvd}$		
slp	$K_{s.vzd}$		
ftz	$K_{z.vsd}$	$K_{z.zvsd}$	
odd	$K_{d.vp}$		
	$K_{d.zvp}$		

Only the non-zero parameter are specified. The first subscript identifies the gene to which the parameter is attached. The other subscripts identify the positive contributions of other gene on the level of expression of the regulated gene (i.e. a regulatory level above the relevant threshold in the case of an activation, below in the opposite case). See Thomas (1991) for further explanations.

The first two stable states correspond to the situation where *en* is activated, resulting from the action of *eve* (odd-numbered En-stripe) and *ftz* (even-numbered En-stripe) respectively. The third stable state corresponds to the activation of *wg* (in both odd- and even-numbered Wg-stripes). Finally, the fourth stable state corresponds to a region where *odd* is expressed, thereby preventing the expression of both *en* and *wg*. Our logical model for the *pair-rule* cross-regulatory module thus has the intrinsic capacity of generating four stable states corresponding to the four different patterns of *pair-rule* gene expression, ultimately leading to the exclusive activation of *en*, *wg*, or *odd*.

The dynamical pathway effectively followed by the *pair-rule* system, from the naïve state towards one of its final states, further depends on the time delays associated with each change of logical value. In general, it is assumed that these delays are different. All possible transitions have been considered changing one variable value at a time. For example, in Fig. 5, increasing the value of variable **d** first (Odd concentration or activity level), the system reaches the transient state **0000001**, which ultimately leads to the stable state **[0010001]**. If variable **a** (Ppa level) is first commuted, the system reaches the transient state **0010000**; if variable **p** (Prd level) is then commuted, the system moves to the transient state **1110000**. From there, it might go through different pathways: (a) if variable **v** (Eve level) commutes first to its value **2**, the system moves to the transient

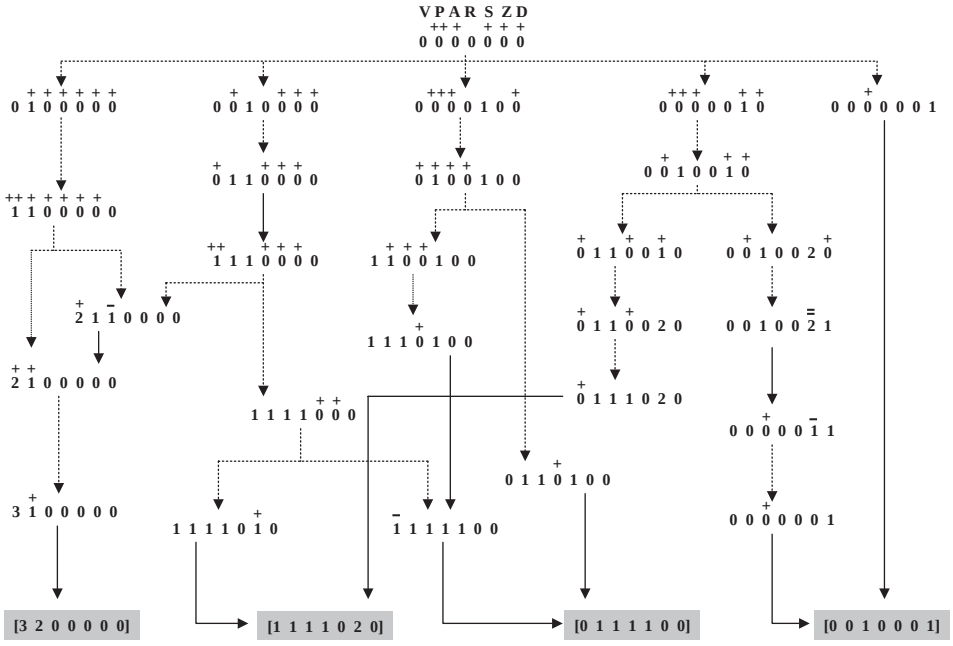

Fig. 5. Simulation of the intrinsic dynamics of the *pair-rule* cross-regulatory module in the absence of any maternal or gap inputs. A compact notation is used consisting of writing the calls for value changes as variable superscripts in all transient logical states. A "+" means that the value of the variable is called to increase (i.e. the value of the function is greater than that of the corresponding variable), whereas a "−" means that the value of the variable is called to decrease (i.e. the value of the function is smaller than that of the corresponding variable). Whenever all variable and function values are equal, the corresponding state is stable, which is denoted with brackets (e.g. **[3200000]**). Starting from the naïve state **vparszd = 0000000**, some of the pathways followed by the system are indicated by arrows. Solid arrows represent single transitions, whereas dotted arrows represent multiple (successive) transitions.

state **2100000** and from there to the stable state **[3200000]**; (b) if variable **r** (Run level) is commuted before **v**, the system goes to the transient state **1111000**, and from there to states **[1111020]** or **[0111100]**, according to whether **z** (Ftz level) or **s** (Slp level) is commuted first.

On the basis of the analysis of this dynamical graph, two main conclusions can be drawn. First, the qualitative evaluation of the interactions between the genes (values of the logical parameter or *K*'s) endows the system with the capacity to generate the expression states corresponding to the activation of genes *en* and *wg* in juxtaposed cells. Second, the asymptotic behaviour of the system depends on which of the *pair-rule* gene become activated first. However, from a wider point of view, the "stable states" ultimately reached by the *pair-rule* cross-regulatory module correspond in reality to transient expression patterns, which determine the activation of the segment polarity genes *en* and *wg*.

In any case, the occurrence of multiple expression states would be due to the action of some of the positive circuits defined by cross-interactions among *pair-rule* genes. In the context of the generalized logical formalism, it is possible to compute parameter constraints for each of the 51 circuits of the *pair-rule* cross-regulatory module to be functional (i.e. to generate differentiation in the case of a positive circuit, or homeostasis in the case of a negative circuit). The comparison of the parameter values selected with the parameter constraints computed for each circuit leads to the identification of only seven (partially) functional circuits (six positive and one negative circuit involving from one to three genes; see first row of Table 3). The respective roles of these seven circuits will become clearer below, in the context of the analysis of mutant simulations.

4. Dynamics of the pair-rule cross-regulatory module

From a general viewpoint two important properties characterize developmental regulatory systems. First, they typically have the intrinsic capacity to generate multiple expression states. Second, they are always embedded within a field of morphogenetic signals (*positional information*), which provides decisive inputs for the system (for the notion of positional information, see Wolpert, 1969). Depending on morphogenetic signals, a developmental regulatory system will reach one specific stable state.

As mentioned above, the combined action of maternal and gap gene products determines the activation of *pair-rule* genes. As a consequence, the final state reached by the *pair-rule* module in a given region in the embryo will depend on the initial *pair-rule* genes activated by the local combination of maternal and gap products. In this respect, it must be remembered that maternal and gap inputs may activate a given *pair-rule* gene at different

Table 3
Results of logical simulations of various types of perturbation of the expression of the *pair-rule* module: loss-of-function mutations (A), ectopic gene expression (B), and *cis*-regulatory mutations (C)

Genetic background	Stable states **v p a r s z d**	Embryo regions	EN/WG expression	(partially) Functional circuits	Comments
(A) Loss-of-function mutations					
wild type	**0 0 1 0 0 0 1**	III	—	eve (+), eve/run (+),	
	0 1 1 1 1 0 0	I, IV	Wg	eve/slp (+), prd/odd (+),	
	1 1 1 1 0 2 0	II	En	slp/ftz (+), eve-ftz-slp	
	3 2 0 0 0 0 0	V	En	(−), prd/odd/ftz (+)	
eve	**0 0 1 0 0 0 1**	I–V	—	prd/odd (+), slp/ftz (+),	Loss of all En- and Wg-stripes
	0 1 1 1 1 0 0	Not reached	(Wg)	prd/odd/ftz (+)	
	0 1 1 1 0 2 0	Not reached	(En)		
prd	**0 0 1 0 0 0 1**	I, III–V	—	eve (+), slp/ftz (+)	Loss of odd-numbered En-stripes and all Wg-stripes
	1 0 1 0 0 2 0	II	En		
ppa	**0 0 0 0 0 0 1**	III	—	eve (+), eve/run (+),	En- and Wg-stripes are normally formed (though three of the wild-type stable states are modified)
	0 2 0 1 1 0 0	I, IV	Wg	eve/slp (+), prd/odd (+),	
	1 2 0 1 0 2 0	II	En	slp/ftz (+), eve-ftz-slp	
	3 2 0 0 0 0 0	V	En	(−), prd/odd/ftz (+)	
run	**0 0 1 0 0 0 1**	III	—	eve (+), eve/slp (+),	Loss of odd-numbered Wg-stripes
	0 1 1 0 1 0 0	IV	Wg	prd/odd (+), slp/ftz (+),	
	3 2 0 0 0 0 0	I, II,V	En	eve-ftz-slp (−), prd/odd/ftz (+)	
slp	**0 0 1 0 0 0 1**	III	—	eve (+), eve/run (+),	Replacement of Wg-stripes by En-stripes
	1 1 1 1 0 2 0	I, II, (IV)	En	prd/odd (+), prd/odd/ftz	
	3 2 0 0 0 0 0	(IV), V	En	(+)	
ftz	**0 1 1 1 1 0 0**	I–IV	Wg	eve (+), eve/run (+),	Loss of even-numbered En-stripes and expansion of Wg-stripes
	3 2 0 0 0 0 0	V	En	eve/slp (+), prd/odd (+)	
odd	**0 1 1 1 1 0 0**	I, IIIb, IV	Wg	eve (+), eve/run (+),	Even-numbered En- and Wg-stripes are expanded posteriorly and anteriorly into the parasegment, respectively
	1 1 1 1 0 2 0	II, IIIa	En	eve/slp (+), slp/ftz (+),	
	3 2 0 0 0 0 0	V	En	eve-ftz-slp (–)	
eve;odd	**0 1 1 1 0 2 0**	I, II, IIIa	En	slp/ftz (+)	The odd Wg-stripes are replaced by En-stripes; the even En-stripes expand posteriorly; the odd En-stripes are replaced by Wg-stripes; the even Wg-stripes expands anteriorly
	0 1 1 1 1 0 0	IIIb, IV, V	Wg		
ftz;odd	**0 1 1 1 1 0 0**	I–IV	Wg	eve (+), eve/run (+),	The even En-stripes are lost and the Wg-stripes are expanded
	3 2 0 0 0 0 0	V	En	eve/slp (+)	
prd;slp	**0 0 1 0 0 0 1**	III	—	eve (+)	Replacement of Wg-stripes by En-stripes
	1 0 1 0 0 2 0	I, II, IV, V	En		
(B) Ectopic pair-rule expressions					
wild type	**0 0 1 0 0 0 1**	III	—	eve (+), eve/run (+),	
	0 1 1 1 1 0 0	I, IV	Wg	eve/slp (+), prd/odd (+),	
	1 1 1 1 0 2 0	II	En	slp/ftz (+), eve-ftz-slp	
	3 2 0 0 0 0 0	V	En	(−), prd/odd/ftz (+)	
eve	**3 2 0 0 0 0 0**	I–V	En	None!	The Wg-stripes are lost and the En-stripes are expanded
prd	**0 2 1 1 1 0 0**	I, IIIb, IV	Wg	eve (+), eve/run (+),	Anterior expansion of Wg-stripes
	1 2 1 1 0 2 0	II, IIIa	En	eve/slp (+), slp/ftz (+),	
	3 2 0 0 0 0 0	V	En	eve-ftz-slp (−),	

Table 3 (*continued*)

Genetic background	Stable states **v p a r s z d**	Embryo regions	EN/WG expression	(partially) Functional circuits	Comments
ppa	**0 1 1 1 1 0 0**	I, IV	Wg	eve (+), eve/run (+),	Loss of odd En-stripes
	1 1 1 1 0 2 0	II	En	eve/slp (+), prd/odd (+),	
	0 0 1 0 0 0 1	III	—	slp/ftz (+), eve-ftz-slp	
	3 1 1 0 0 0 0	V	—	(−), prd/odd/ftz (+)	
run	**0 0 1 1 0 0 1**	III	—	eve (+), prd/odd (+),	Replacement of odd En-stripes
	0 1 1 1 1 0 0	I, IV, V	Wg	slp/ftz (+), prd/odd/ftz	by Wg-stripes
	1 1 1 1 0 2 0	II	En	(+)	
slp	**0 0 1 0 1 0 1**	III	—	prd/odd (+)	Replacement of En-stripes by
	0 1 1 1 1 0 0	I, II, IV, V	Wg		Wg-stripes
ftz	**0 0 1 0 0 2 1**	III	—	eve (+), eve/run (+),	Replacement of Wg-stripes by
	1 1 1 1 0 2 0	I, II, IV	En	prd/odd (+)	En-stripes
	3 2 0 0 0 2 0	V	En		
odd	**0 0 1 0 0 0 1**	I–V	—	eve (+)	Loss of En- and Wg-stripes
	1 0 1 0 0 0 1	Not reached	(−)		
(C) Cis auto-regulatory pair-rule mutations					
wild type	**0 0 1 0 0 0 1**	III	—	eve (+), eve/run (+),	
	0 1 1 1 1 0 0	I, IV	Wg	eve/slp (+), prd/odd (+),	
	1 1 1 1 0 2 0	II	En	slp/ftz (+), eve-ftz-slp	
	3 2 0 0 0 0 0	V	En	(−), prd/odd/ftz (+)	
eve	**0 0 1 0 0 0 1**	III	—	prd/odd (+), slp/ftz (+),	Possible replacement of odd
	0 1 1 1 1 0 0	I, IV, (V)	Wg	prd/odd/ftz (+)	En-stripes by Wg-stripes
	1 1 1 1 0 2 0	II, (V)	En		
ftz	**0 0 1 0 0 0 1**	III	—	eve (+), eve/run (+),	Loss of even En-stripes
	0 1 1 1 1 0 0	I, IV	Wg	eve/slp (+), prd/odd (+),	
	1 1 1 1 0 1 0	II	—	slp/ftz (+), eve-ftz-slp	
	3 2 0 0 0 0 0	V	En	(−), prd/odd/ftz (+)	

In all cases, the first column specifies the genetic background simulated. The second column gives all the stable states found. The third column indicates the embryonic regions in which these states are found. The fourth column mentions which of *en* or *wg* is expressed under the action of these *pair-rule* expression states. The fifth column lists the feedback circuits, which are found to be functional at least in a part of the (seven-dimensional) logical space. Finally, the last column summarizes the most salient phenotypic changes expected on the basis of the results of the corresponding simulation.

levels, leading to different amounts of the pair-rule product. Secondly, more than one single *pair-rule* gene can be activated within the same nuclei. Note that at the time when *pair-rule* genes are activated, their transcripts remain localized in the apical compartment of the cytoplasm, close to the periphery of the egg, so that diffusion of the pair-rule proteins between the incipient cells is largely impeded (Davis and Ish-Horowicz, 1991). Hence, we consider here that the *pair-rule* genes act cell-autonomously. Therefore, the final result attained by the *pair-rule* module at a given location depends on the initial levels of activation of the *pair-rule* genes plus their cross-regulatory interactions. As a result, different situations occur in contiguous regions along the trunk of the embryo, leading to the cellular states characterized by the exclusive activation of *en*, *wg*, or *odd*.

The behaviour of the *pair-rule* module as a whole entity is the same at all locations along the anterior–posterior axis where the (para)segmental boundaries are formed. However, we still need to specify how the maternal-gap signals and *pair-rule* cross-interactions together specify the correct En and Wg expression patterns. These conditions can be derived from the qualitative analysis of the dynamical behaviour of the *pair-rule* module in response to maternal and gap inputs. They correspond to competitive situations between regulatory products with opposite effects within the same cell, or to situations where two mutually repressing genes become simultaneously activated. For the sake of explanation, let us start by stating the conditions derived before discussing the dynamics of the *pair-rule* cross-regulatory module in response to maternal-gap inputs:

Condition C1. The repressor effect of *eve* on *odd* prevails over the repressor effect of *odd* upon *eve*.

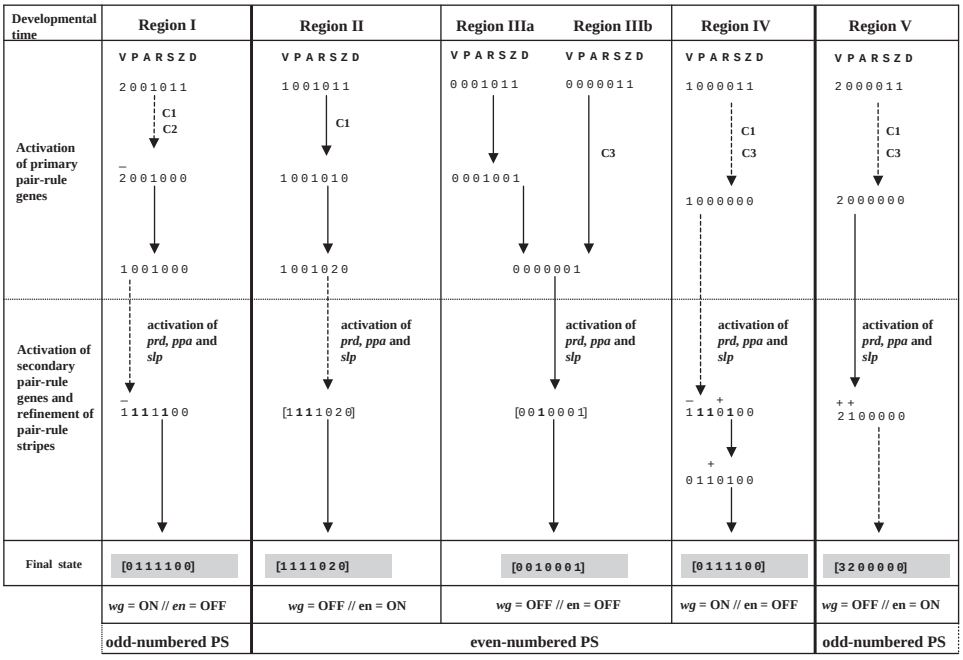

Fig. 6. Simulation of the dynamics of the *pair-rule* cross-regulatory module under the action of maternal and gap inputs, in the different regions I–V. For each of these regions, the final expression state of the *pair-rule* module is indicated, as well as the transcription state of genes *en* and *wg*, (bottom). Conditions C1–C3 are used to reach a decision in specific *pair-rule* competition states (see text for explanation; symbols are defined in the legends of Figs. 1 and 3).

Condition C2. The maternal-gap signal that initiates the activation of *run* overcomes the repressing effect *eve* upon *run*.

Condition C3. Initially, *hairy* expression is able to repress *run* and *ftz* (Howard and Ingham, 1986; Carroll and Scott, 1986; Ish-Horowicz and Pinchin, 1987; Ingham and Gergen, 1988; Carroll and Vavra, 1989). This condition defines the role of the input gene *hairy* on the other *pair-rule* genes. This input is crucial for the refinement of both initial Run- and Ftz-stripes.

These three conditions represent the effects of particular combinations of maternal, gap, and Hairy inputs along the trunk of the embryo, resulting in competitive expressions of *pair-rule* genes. For the sake of simplicity, since these conditions apply to all initial signals acting on the *pair-rule* genes, we have chosen to formulate them in biological terms instead of adding them explicitly to the equations. In Fig. 6, the resulting dynamics of the *pair-rule* module in each region of the embryo (I–V) is shown using the logical parameter values of Table 2. The development time elapsing between the initial action of the maternal-gap input up to the final *pair-rule* states has been divided into two broad periods. Indeed, the *pair-rule* genes are not all activated at the same time, but rather follow a temporal order. The primary *pair-rule genes* first respond to the maternal-gap input. These are later followed by the secondary *pair-rule genes*, the activation of which depends on the presence of primary pair-rule products. Recall that *ftz* is considered here as primary *pair-rule* gene, and although it is known that Ftz product activates *odd*, it is assumed here for the sake of simplicity that both *ftz* and *odd* are simultaneously activated.

4.1. Activation of the primary pair-rule genes

Initially, maternal-gap input activates the first *pair-rule* genes and their cross-interactions drives the *pair-rule* module to specific states in the different regions I–V (see Fig. 6, upper part):

(a) In region I, the maternal-gap signal activates *eve*, *run*, *ftz* and *odd*, leading to the state **2001011**. Eve can thus repress *ftz* and *odd* (Condition C1) whereas Run reduces *eve* expression (Condition C2). As a consequence, the system moves to the state **1001000**.

(b) In region II, *eve*, *run*, *ftz* and *odd* are activated, leading to the state **1001011**. Eve can then repress *odd* but not *ftz*. The auto-activation of *ftz* further leads to its maximal expression state **1001020**.

(c) In the anterior part of region III (IIIa), *run*, *ftz* and *odd* are activated, leading to the state **0001011**; *odd* can thus repress *run* and *ftz*, ultimately leading to the state **0000001**. In the posterior part of region III (IIIb), *ftz*, *odd*, as well as *hairy* are initially activated. Hairy then prevents *run* expression in

this region and represses *ftz* (Condition C3). Odd could also participate in *ftz* repression, so that the system moves to the state **0000001**.

(d) In region IV, *eve*, *ftz* and *odd* are initially activated, leading to the state **1000011**, *hairy* is also expressed and represses *ftz* (Condition C3), while Eve represses *odd* (Condition C1). The system is consequently driven to the state **1000000**.

(e) In region V, *eve*, *ftz* and *odd* are expressed together with *hairy*, leading to the state **2000011**. The gene *ftz* is repressed by Hairy (Condition C3), whereas Eve represses *odd* (Condition C1), and eventually also represses *ftz*, leading the system to the state **2000000**.

4.2. Activation of the secondary pair-rule genes and refinement of the pair-rule expression stripes

The secondary *pair-rule* genes now start to be expressed and the refinement process that determines the final formation of narrow pair-rule stripes occurs:

(a) In region I, *prd, ppa* and *slp* are activated and the system moves from **1001000** to **1111100**. However, as Slp and Run repress *eve*, the system end up in the stable state **[0111100]** and consequently *wg* is activated.

(b) In region II, *prd* and *ppa* are activated while *slp* remains silent due to the presence of Ftz. The system thus moves from **1001020** to finally reach **[1111020]**, and *en* becomes activated.

(c) In region III, *ppa* is activated, whereas *prd* and *slp* are ultimately shut off due to the presence of Odd. The system goes from **0000001** to final state **[0010001]**, impeding the expression of both *en* and *wg*.

(d) In region IV, *prd, ppa* and *slp* are further activated, leading to state **1110100**. Prd then activates *run*, whereas Slp and Run repress *eve*, so that the system finally reaches the state **[0111100]**, ultimately leading to *wg* activation.

(e) In region V, *prd* is activated but the presence of Eve (over its second threshold) ultimately impedes the expression of *ppa* and *slp*. The system thus moves to state **2100000**. The combined action of early Eve and Prd allows a still higher expression of *eve*, whereas *prd* expression also increase in the absence of Ppa protein, ultimately leading to the state **[3200000]** and to *en* activation.

In summary, once embedded in the maternal-gap prepattern, the *pair-rule* cross-regulatory module always selects a specific expression state corresponding to the activation of either *en* or *wg* in alternate juxtaposed cells, or to a situation where neither of these two genes is activated in the presence of Odd. Note that the dynamical pathways followed by the *pair-rule* cross-regulatory module in regions I–V correspond to subsets of the transitions intrinsically allowed for the *pair-rule* module taken in isolation (see Fig. 5).

5. The activation of genes engrailed and wingless by the pair-rule cross-regulatory module

In the preceding section, *en* and *wg* expression patterns were derived from our knowledge of the corresponding wild-type *pair-rule* expression in the five alternating different regions along the embryo trunk. In order to simulate the effect of *pair-rule* mutants, however, one must specify how *en* and *wg* would respond to any combination of *pair-rule* expression. This amounts to the formulation of logical equations for the expression of these genes, including the specification of interaction thresholds and of a consistent set of logical parameter values.

The interactions of *pair-rule* genes upon *en* and *wg* were derived from the analysis of the phenotypes of *pair-rule* loss-of-function mutants and those resulting from ectopic expression *pair-rule* genes. The experimental results backing these interactions are summarized in Fig. 7A, whereas the resulting set of equations is shown in Fig. 7B. Note that the gene *opa* is included in these equations since it is required to activate both *en* and *wg*. However *opa* plays no role in spatial patterning per se. With respect to the threshold and parameter values, we relied upon the following observations:

(a) Ppa interacts with Prd, promoting its partial degradation. Indeed, in *hs-ppa* embryos (*hs* standing for a heat shock promoter), a decrease in Prd concentration of about 50% leads to a depletion of *en* expression in region V, whereas *wg* remains normally activated in region IV (Raj et al., 2000). This indicates that *en* and *wg* need different amounts of Prd protein to be activated. As a consequence, two functional thresholds are associated with the Prd product, the lowest allowing the activation gene *wg* and interactions with other *pair-rule* genes, the highest needed to activate *en*. In addition, in prd^{-} mutants, the even-numbered Wg-stripes are lost and the odd-numbered Wg-stripes are weaker than normal (Benedyk et al., 1994). For the sake of simplicity, it is assumed here that all the Wg-stripes are lost in these mutants.

(b) Similarly, two thresholds are associated with *ftz* product: the highest for *en* activation, the lowest for interaction with other *pair-rule* genes. High amounts of Ftz might be necessary to activate gene *en* because *run*, a repressor of *en*, is also expressed in region II (see Discussion below).

(c) The activation of *en* in region V requires the expression of *prd* and the repression of *run, slp* and *odd*.

(A) Interactions between pair-rule genes and *engrailed* and *wingless*

Interaction	Main experimental observations	Ref.
prd → *en*	Loss-of-function *prd* mutants fail to form odd-numbered En-stripes	DiNardo & O'Farrell (1987), Ingham *et al.* (1988), Morrissey *et al.* (1991)
ftz → *en*	Loss-of-function *ftz* mutants fail to form even-numbered En-stripes	DiNardo & O'Farrell (1987), Howard & Ingham (1986)
run —| *en*	Ectopic *run* expression represses formation of odd-numbered En-stripes	Manoukian & Krause (1993)
slp —| *en*	In loss-of-function *slp* mutants, gene *en* is ectopically expressed. Ectopic *slp* expression represses gene *en*	Cadigan *et al.* (1994)
odd —| *en*	Ectopic *odd* expression represses gene *en*	Saulier-Le Dréan *et al.* (1998)
prd → *wg*	In loss-of-function *prd* mutants, the even-numbered Wg-stripes are lost and the odd-numbered Wg-stripes are weakened	Ingham & Hidalgo (1993), Benedyk *et al.* (1994)
slp → *wg*	All the cells expressing *wg* in loss-of-function *ptc* mutants require functional *slp* for *wg* expression	Cadigan *et al.* (1994)
eve —| *wg*	In loss-of-function *eve* mutants, gene *wg* is ectopically expressed	Ingham *et al.* (1988)
ftz —| *wg*	Broadening of Wg-stripes in loss-of-function *ftz* mutants, and ectopic *ftz* expression represses gene *wg*	Ingham *et al.* (1988), Ish-Horowicz *et al.* (1989)
odd —| *wg*	Ectopic *odd* expression represses gene *wg*	Saulier-Le Dréan *et al.* (1998)

(B) Generalized logical equations for activation of gene *engrailed* and *wingless* by the pair-rule cross-regulatory module

$$E = d_e(k_e + k_{e.y}y + k_{e.p}p^{(2)} + k_{e.r}\bar{r} + k_{e.s}\bar{s} + k_{e.z}z^{(2)} + k_{e.d}\bar{d})$$

$$W = d_w(k_w + k_{w.y}y + k_{w.v}\overline{v^{(1)}} + k_{w.p}p^{(1)} + k_{w.s}s + k_{w.z}\overline{z^{(1)}} + k_{w.d}\bar{d})$$

$E = en$, $W = wg$, $Y = opa$, $V = eve$, $P = prd$, $R = run$, $S = slp$, $Z = ftz$, $D = odd$

(C) Parameter values for *engrailed* and *wingless*

***engrailed* parameters:** $K_{e.yzsd} = K_{e.yprsd} = K_{e.yzrsd} = K_{e.ypzsd} = K_{e.ypzrsd} = 1$

***wingless* parameters:** $K_{w.ypsvzd} = 1$

Fig. 7. (A) Interactions between the *pair-rule* genes, *en* and *wg*. Solid arrows indicate positive interactions, dotted blunt arrows indicate negative interactions. (B) Generalized logical equations for gene *en* and *wg* (see legend to Fig. 4B for further explanation). (C) Selected parameter values. Only the non-zero parameters are mentioned.

(d) The activation of *en* in region II requires the expression of *ftz* and the repression of *slp* and *odd*.
(e) The activation of *wg* in region II and IV requires the expression of both *prd* and *slp*, as well as the repression of *eve, ftz* and *odd*.
(f) Finally, the expression of *odd* in region III causes repression of both *en* and *wg*.

Altogether, these observations lead to the unique set of parameter values for genes *en* and *wg* shown in Fig. 7C.

6. Analysis of mutations at the pair-rule genes

One way to test the qualitative robustness and consistency of this *pair-rule* model is to check the effect of various types of perturbations. Below, the phenotypes predicted from the simulation of different *pair-rule* mutations and ectopic expressions are discussed. Some of these simulations correspond to situations already experimentally analysed (see the references cited in Table 1 and Fig. 7), whereas other simulations still await experimental support. Three main classes of mutations were simulated: loss-of-function mutations and ectopic expressions of all *pair-rule* genes, and *cis*-acting mutations affecting the autoregulatory function of *eve* or *ftz*. For a fuller description of our formal treatment of these perturbations, see Sánchez and Thieffry (2001) and Thieffry and Sánchez (2003). In brief, a loss-of-function mutation at a given gene implies that this gene produces a non-functional product, which amounts to assign the value zero to the corresponding logical variable (regulatory product concentration) and logical function (gene expression level). The ectopic expression of a gene implies that this gene is expressed in an unregulated manner beyond its normal spatial-temporal expression domain. Accordingly, to simulate ectopic expression, the corresponding variable and function are forced to take a

higher value. Finally, a *cis*-acting mutation affecting the autoregulatory function of a gene implies that this gene remains normally controlled by all its regulators excepting itself. This amounts to eliminate the term corresponding to autoregulation from the equation that defines the expression state of the gene.

Table 3 summarizes the results of the simulations performed. In this table, each row describes the effect of a specific perturbation on the dynamics of the *pair-rule* module in the five embryonic regions shown in Fig. 6, i.e. the final state attained by the *pair-rule* module in each region, and the resulting presence/absence of En- and Wg-stripes. A straightforward comparison with available experimental results can therefore be made.

Each of these mutations affects the feedback circuits involving the corresponding genes (fifth column of Fig. 3). As most of these circuits are positive, their inactivation invariably leads to the loss of some of the steady states, often accompanied by a displacement of (some of) the remaining stable states.

6.1. Simulation of loss-of-function mutants

6.1.1. Loss-of-function eve mutations

The *pair-rule* module reaches the state **vparszd = [0010001]** in all regions. Consequently, all En-stripes are lost, in agreement with experimental results (Harding et al., 1986; Macdonald and Struhl, 1986; DiNardo and O'Farrell, 1987; Ingham et al., 1988). According to our simulation, the Wg-stripes are also lost. Notwithstanding, it has been reported that, in *eve* loss-of-function mutants, there is a reduction in the number of Wg-stripes, but those that remain are wider than normal (Ingham et al., 1988). We have no explanation for the origin of the remaining Wg-stripes reported in eve^{-} mutants. However, these results appear to be at odds with the phenotypes reported in the case of losses of other *pair-rule* functions. Indeed, *eve* acts as a direct repressor of *odd* (Manoukian and Krause, 1992; Fujioka et al., 1995). On the other hand, *odd* acts as a direct repressor of *wg* (Saulier-Le Dréan et al., 1998). Therefore, the loss of *eve* function would be expected to cause a de-repression of *odd*, which in turn would cause a repression of *wg*. This is in agreement with published data on the phenotype of double *eve; odd* mutants, where *wg* is not repressed in spite of the absence of *eve* activity because there is no *odd* activity. Note that the system encompasses two additional stable states, **[0111100]** and **[0111020]** (corresponding to Wg and En expression, respectively) which cannot be reached from normal maternal-gap initial conditions.

Among the seven wild-type functional feedback circuits, only three (positive) remain functional, yet they allow the coexistence of three different stable states. We can thus infer that the three positive circuits involving *eve* (*eve*, *eve/run*, and *eve/slp*) cooperate to form a unique additional attractor corresponding to the highest *eve* expression levels. We suggest that such an interplay of three positive circuits involving *eve* would allow the generation of a highly co-operative expression of this crucial morphogenetic factor (see also Lewis et al., 1977; Meinhardt, 1978; Kerszberg and Changeux, 1994; Sánchez and Thieffry, 2001).

6.1.2. Loss-of-function prd mutations

In regions I, III, IV and V, the *pair-rule* module reaches the state **[0010001]**, which corresponds to the lack of activation of both *en* and *wg*. In region II, it attains the state **[1010020]**, leading to *en* activation by Ftz. As a consequence, the odd-numbered En-stripes and all Wg-stripes are lost, in agreement with published data (DiNardo and O'Farrell, 1987; Ingham et al., 1988; Morrissey et al., 1991; Ingham and Hidalgo, 1993; Benedyk et al., 1994).

As Prd is needed for high *eve* expression, it is also needed for the functionality of the circuit involving high Eve levels. As a result, in *prd* loss-of-function mutants, the functionality of most circuits is lost, except that of *eve* auto-regulation and *slp/ftz*. This depletion of functional positive circuits results in the loss of two stable states, one corresponding to the activation of *en*, the other to the activation of *wg*.

6.1.3. Loss-of-function ppa mutations

In regions I and IV, the *pair-rule* module reaches the state **[0201100]** and *wg* is activated by Prd and Slp. In region II, the system attains the state **[1201020]**, leading to *en* activation by Ftz. In region III, the state **[0000001]** is reached, where the presence of Odd and the absence of both Prd and Slp impede the expression of both *en* and *wg*. Finally, in region V, the module reaches the state **[3200000]** and *en* is activated. All En- and Wg-stripes are thus formed normally, in agreement with available experimental data (Raj et al., 2000).

6.1.4. Loss-of-function run mutations

The *pair-rule* module reaches the state **[3200000]** in regions I, II and V, leading to the activation of *en*. In region IV, the state **[0110100]** is attained, and *wg* becomes activated because of the presence of Prd and Slp. In region III, the system reaches the state **[0010001]**, so that neither *en* nor *wg* is activated. Odd-numbered Wg-stripes are thus replaced by En-stripes, which expand in the absence of Ftz. This agrees with experimental data (Ingham and Gergen, 1988). Consequently, posterior compartments (En-expressing cells) flank odd-numbered parasegments (Martínez-Arias and White, 1988).

The loss of functionality of the sole positive circuit *eve/run* results in the disappearance of the stable state that corresponds to the activation of *en* by Ftz, indicating that the repression exerted by *run* upon *eve* is necessary for a proper activation of *ftz*.

6.1.5. Loss-of-function slp mutations

In regions I and II, the *pair-rule* **module** reaches the state **[1111020]**, leading to the activation of *en*. In region V, the state **[3200000]** is attained and *en* becomes activated. In region III, the system reaches the state **[0010001]** and neither *en* nor *wg* become activated. In region IV, our simulations suggest that the system can reach either the state **[111020]** or the state **[3200000]**, depending on which of Run or Eve first reaches its functional threshold level. In any case, the gene *en* is activated in this region. Consequently, Wg-stripes are replaced by En-stripes, in agreement with published results (Cadigan et al., 1994).

The inactivation of the two positive circuit *eve/slp* and *slp/ftz* results in the loss of the stable state corresponding to the activation of *wg*. In addition, this result emphasizes the crucial role of Slp in the setting of the anterior border of both even- and odd-numbered En-stripes.

6.1.6. Loss-of-function ftz mutations

The *pair-rule* module reaches the state **[0111100]** corresponding to the activation of *wg* in all regions but region V, where it attains the state **[3200000]**, which corresponds to the activation of *en*. The even-numbered En-stripes (region II) are thus replaced by posterior-expanding Wg-stripes in agreement with experimental data (Nasiadka and Krause, 1999). The odd-numbered En-stripes (region V) are normally formed in agreement with published data (DiNardo and O'Farrell, 1987; Ingham et al., 1988).

The inactivation of two positive circuits (*slp/ftz*, *prd/odd/ftz*) results in the loss of two stable states, one corresponding to the activation of *odd* and the other to the activation of *en*. This result highlights the role played by Odd in positioning En- and Wg-stripes.

6.1.7. Loss-of-function odd mutations

The *pair-rule* module behaves normally in regions I, II, IV and V. In region IIIa it attains the state **[1111020]** and *en* is activated by the posterior expansion of Ftz. In region IIIb it attains the state **[0111100]** and Prd and Slp activate *wg*. All the En- and Wg-stripes are thus formed normally, excepting the even-numbered En-stripes, which are expanded posteriorly, and the even-numbered Wg-stripes, which expand anteriorly. This is again supported by published data (DiNardo and O'Farrell, 1987; Mullen and DiNardo, 1995).

The inactivation of the two positive circuits involving *odd* leads to the loss of the stable state corresponding to a lack of *en* and *wg* expression. Here also, the role of Odd in the positioning of En- and Wg-stripes is emphasized.

6.1.8. Double loss-of-function eve;odd mutations

The simulation of double *eve;odd* mutants leads to the state **[0111020]** in regions I, II and IIIa, and thus to *en* activation by Ftz. In regions IIIb, IV and V, the state **[0111100]** is reached and *wg* becomes activated. Even-numbered En-stripes (lost in *eve* loss-of-function mutants, confer 6.1.1) are thus recovered and additional En-stripes appear, in agreement with experimental results (DiNardo and O'Farrell, 1987). En-stripes are activated as a consequence of the posterior expansion of *ftz* expression in these double mutants, in agreement with published observations (Coulter and Wieschaus, 1988; Mullen and DiNardo, 1995).

In this double mutant, only one (positive) circuit remains functional, *slp/ftz*, which is responsible for the coexistence of the two remaining stable states, corresponding to the activation of *en* by Ftz, and to the activation of *wg*, respectively.

6.1.9. Double loss-of-function ftz;odd mutations

The simulation of double *ftz;odd* mutants leads to the state **[0111100]** in regions I, II, III and IV, so that *wg* is activated. In region V, the state **[3200000]** is reached and *en* becomes activated. The even-numbered En-stripes (region II) are consequently replaced by Wg-stripes. In addition, *wg* expression expands within the parasegment. Note that *ftz* and double *ftz;odd* loss-of-function mutants show the same phenotype, in agreement with published results (Mullen and DiNardo, 1995).

In this double loss-of-function mutant,only three functional positive circuits are conserved, all involving *eve* and together defining a unique separating (hyper) plan between the basins of attractions of the two remaining stable states, which correspond to the activation of *wg* and to the activation of *en* at high Eve levels, respectively.

6.1.10. Double loss-of-function prd;slp mutations

In region III, the system reaches the state **[0010001]**, as in the wild type. In all other regions I, II, IV and V, the system reaches the state **[1010020]**, leading to *en* activation by Ftz. Consequently, Wg stripes are replaced by En stripes. This double mutant has not been yet reported.

The only remaining functional circuit is the *eve* autoregulatory circuit, which is still able to define a separatrix between two attracting domains corresponding to the activation of *en* by Ftz and to the lack of *en* and *wg* activation in the presence of Odd. This result emphasizes the role played by the minor Eve-stripes (*eve* autoregulatory circuit) in the activation of Ftz-stripes (see also Discussion).

6.2. Simulation of ectopic pair-rule gene expression

6.2.1. Ectopic expression of eve

The *pair-rule* module attains the state **[3200000]** in all regions so that *en* is activated in all five domains I–V (i.e. in the whole trunk). All Wg-stripes are consequently lost and all En-stripes expanded in agreement with experimental data (Manoukian and Krause, 1992). Note that

none of the 51 feedback circuits of the system is functional, which is consistent with the dynamical behaviour encompassing a unique stable state.

6.2.2. Ectopic expression of prd

The *pair-rule* module reaches the state **[0211100]** in regions I, IIIb and IV, and *wg* is activated by Prd and Slp. In region II and IIIa, the state **[1211020]** is attained and *en* becomes activated by Ftz. In region V the system goes to the state **[3200000]** and Prd activates *en*. Consequently, the Wg-stripes are widened anteriorly, which is consistent with the reported phenotype (Copeland et al., 1996).

The loss of functionality of the two positive circuits involving *odd* amounts to the disappearance of the stable state corresponding to the lack of *en* and *wg* activation. This result emphasizes the role played by Odd in preventing the activation of these two genes in the middle of the parasegment.

6.2.3. Ectopic expression of ppa

In all regions but V, the *pair-rule* module reaches the wild-type state. In region V, the state **[3110000]** is reached, so that neither *en* nor *wg* becomes activated, in the presence of low Prd concentration. Such a low Prd concentration cannot activate *en*, nor *wg* in the absence of Slp. Consequently, the odd-numbered En-stripes are lost, in agreement with published data (Raj et al., 2000).

6.2.4. Ectopic expression of run

The *pair-rule* module reaches the state **[0111100]** in regions I, IV and V, so that *wg* is activated. In region II, it attains the state **[1111020]** and *en* becomes activated. In region III, the state **[0011001]** is reached, and neither *en* nor *wg* can be expressed because of the presence of Odd, in combination with a low level of Prd (for gene *en*) and a lack of Slp (for gene *wg*). Consequently, odd-numbered En-stripes are replaced by Wg-stripes, whereas the even-numbered En-stripes remain normal in agreement with the available experimental data (Manoukian and Krause, 1993).

Among the seven functional feedback circuits, those involving *eve* are all inactivated, leading to the loss of the stable state corresponding to high Eve levels.

6.2.5. Ectopic expression of slp

The *pair-rule* module reaches the state **[0111100]** in regions I, II, IV and V, so that *wg* is activated. In region III, it attains the state **[0010101]** and neither *en* nor *wg* are activated. Consequently, the En-stripes will be replaced by Wg-stripes (a prediction still awaiting experimental support).

Only one (positive) circuit (*prd*/*odd*) remains functional: that at the origin of the separatrix between the basins of the two attractors just mentioned. The two stable states corresponding to the activation of *en* are lost.

6.2.6. Ectopic expression of ftz

In regions I, II and IV, the state **[1111020]** is reached and *en* is thus activated. In region III the system attains the state **[0010021]**, in which neither *en* nor *wg* can be expressed due to the lack of Prd and Slp and to the presence of Odd. In region V, the system reaches the state **[3200020]** and *en* is activated. Note that, in this latter region, *odd* becomes repressed by Eve (condition C1). Consequently, all Wg-stripes are lost and En-stripes are widened in agreement with published data (Nasiadka and Krause, 1999).

The feedback circuits involving *ftz* or *slp* (repressed by Ftz) are lost, resulting in the disappearance of the stable state corresponding to *wg* activation.

6.2.7. Ectopic expression of odd

The *pair-rule* module reaches the state **[0010001]** in all regions, so that neither *en* nor *wg* can be activated. All En- and Wg-stripes are thus lost in agreement with experimental data (Saulier-Le Dréan et al., 1998). Note that the system encompasses a second stable state, **[1010001]**, which would also give rise to the loss of both En and Wg expression, but which cannot be reached from normal maternal-gap initial conditions.

The ectopic expression of *odd* has a deleterious effect on all feedback circuits but *eve* autoregulation, amounting to the loss of three stable states corresponding to the activation of *en* and *wg*.

6.3. Simulation of cis-regulatory mutants

6.3.1. cis-acting mutations impeding eve autoregulation

In all regions but region V, the *pair-rule* module reaches the wild type expression states. In region V, the state **[0111100]** is reached, so that *wg* is activated instead of *en*. Alternatively, the state **[1111020]** could also be reached in region V, leading to the expression of *en*. Which of these states will be selected depends on which of Slp or Ftz reaches its functional threshold level first (**s** or $\mathbf{z}=1$). This indicates that *eve* autoregulatory function is instrumental for this gene to reach its highest level of expression, which is required for the activation of *en* by Prd. We therefore expect that odd-numbered En-stripes will be replaced by Wg-stripes, a prediction awaiting experimental support.

Formally, the loss of *eve* auto-regulatory circuit leads to the loss of the high Eve level stable state.

6.3.2. cis-acting mutations impeding ftz autoregulation

In all regions but II the *pair-rule* module reaches the wild type states. In region II, the system attains the state **[1111010]**. Here also, the autoregulatory function of *ftz* is needed for this gene to reach a high expression level,

which is in turn required to activate *en* and prevent *en* repression by Run (see further comments in the Discussion). The model thus predicts the loss of even-numbered En-stripes (region II). Such a mutation has not yet been reported.

Note that the loss of *ftz* autoregulation does not affect the number of stable states. This is consistent with the finding that this one-element circuit is not functional for the parameter values selected.

7. Discussion

The *pair-rule* cross-regulatory module is a cohesive part of the segmentation genetic system. In this temporal hierarchical system, the maternal and gap products act upon the *pair-rule* module, which in turn determine the cells expressing the segment polarity gene *en* in juxtaposition to cells expressing the segment polarity gene *wg* along the anterior–posterior axis of the embryo trunk. The juxtaposition of these two types of cells determines the formation of (para)segmental boundaries. In this paper, we propose a formal qualitative model allowing a proper delineation of the role of the *pair-rule* cross-regulatory module in the segmentation process in *Drosophila* embryo.

Two main conclusions arise from our dynamical model analysis of the *pair-rule* module. First, the delineation of the interactions between the genes (values of *K*'s in Table 2) endows the module with the capacity to generate four distinct *pair-rule* expression states, ultimately enabling the activation of *en* and *wg* in juxtaposed cells. Second, the selection of a specific cellular state depends on the *pair-rule* genes that become initially activated under the action of maternal and gap inputs. Indeed, the pre-pattern of maternal-gap inputs along the anterior–posterior axis of *Drosophila* embryo is instrumental in determining the final state attained by the *pair-rule* module. In this respect, three specific conditions for the action of the maternal-gap signals upon the *pair-rule* module and for the interactions between these were derived. Together with the selected parameter values (Table 2), these conditions are sufficient to specify a dynamical behaviour consistent with wild type data. Importantly, it was found that not only the maternal-gap inputs play a key role in the specification of the *pair-rule* pattern but that *pair-rule* cross-regulations also play a crucial role (see also Reinitz et al., 1998). In other words, the *pair-rule* cross-regulatory interactions participate in the interpretation of the maternal-gap pre-pattern, ultimately leading to four different *expression modes*. Indeed, our analysis of the feedback circuits reveals that only seven among the 51 intertwined circuits found in the *pair-rule* cross-regulatory module actually play a decision-taking role in differentiation. All mutations or expression alterations leading to a perturbation of these crucial feedback circuits lead to the loss of the capacity of the *pair-rule* module to generate certain stable states, enabling the activation of only one or none of the genes *en* and *wg*. In addition, the remaining states could be miss-localized.

7.1. The pair-rule module generates four main expression modes

At the onset of gastrulation, a repetitive series of juxtaposed En and Wg stripes are formed in the trunk of the *Drosophila* embryo, where the repeated pattern spans a whole parasegment plus the flanking nuclei. This spatial domain can be further subdivided into five regions endowed with specific expression patterns: regions I and IV have the same expression pattern , whereas regions III, II and V each have their own specific pattern. Each of these patterns involves a particular combination of active *pair-rule* genes, which corresponds to one of the four alternative stable states of our logical *pair-rule* model. A particular *pair-rule* gene dominates each of these *expression modes* (leading to the stable states shown in Table 3, in the row corresponding to the wild-type situation), while *pair-rule* cross-regulations ensure their relative stabilization.

7.1.1. The eve-expression mode

This mode is characterized by *eve* expression, which is responsible for the formation of odd-numbered En-stripes in region V. The expression of *run* and *slp* in neighbouring region IV set up the anterior border of *eve* expression in region V. Note that *odd*, the other repressor of *eve*, is not expressed in region IV. Although *run* is not initially activated by the maternal-gap inputs, secondary Run-stripes appear later in this region. Our model accounts for the formation of these via an activation by Prd ($K_{r.pvd} = 1$). Indeed, these secondary Run-stripes are not formed in *prd* loss-of-function mutants, even when *eve* and *odd* are not expressed ($K_{r.vd} = 0$). This activation of *run* in region IV is possible because the local amount of Eve ($\mathbf{v} = 1$) remains under the functional threshold associated with *run* repression. Similarly, *slp* becomes activated in region IV and its expression is sufficient to completely repress *eve*, even in the presence of its two activators, early Eve and Prd ($K_{v.vprd} = 0$). However, in the absence of Slp and in the presence of Run, *eve* expression remains low in presence of its activators ($K_{v.vpsd} = 1$). Finally, the posterior border of odd-numbered Eve-stripes is set up by *odd*, which is expressed in the middle of the (para)segment.

7.1.2. The ftz-expression mode

This mode is characterized by *ftz* expression, which is responsible for the formation of even-numbered En-stripes in region II. The expression of *slp* in region I set the anterior border of the *ftz* expression

($K_{z.zvd} = 0$). The posterior border of this domain is set up by *odd* expression in region III ($K_{z.zvs} = 0$). The minor Eve-stripe overlapping with Ftz in region II is limited to a low functional level of *eve* (**v** = **1**) because of the presence of Run ($K_{v.psd} = 1$), which is in turn maintained thanks to the presence of Prd ($K_{r.pvd} = 1$). We propose that the expression of *run* in this region would prevent *eve* to reach its higher expression level as a result of its autoregulation and because of the presence of Prd. The model thus accounts for the crucial role of *run* expression on that of *ftz* in region II. As a result, Ftz can activate *en*, leading to the formation of the even-numbered En-stripes.

7.1.3. The prd/slp expression mode

This mode is characterized by *prd* and *slp* expressions, leading to the formation of odd-numbered and even-numbered Wg-stripes in regions I and IV, respectively. Expressed at its higher level (**z** = **2**) in region II, *ftz* sets the posterior border of Slp in region I ($K_{s.vd} = 0$). In region IV, the Slp posterior border is set by the high *eve* expression in region V ($K_{s.vd} = 0$). Finally, in both regions I and IV, the anterior border of the Slp stripe is set by *odd* expression in the middle of the parasegment ($K_{s.vz} = 0$).

7.1.4. The odd expression mode

This mode is characterized by *odd* expression, which is responsible for the repression of both *en* and *wg* in the middle region of the parasegment (region III). The anterior border of the Odd-stripe is set by the presence of Eve in region II ($K_{d.zp} = 0$). Early in development, the presence of Eve in region IV also determines the Odd-stripe posterior border, but Eve cannot maintain this border since it soon disappears from region IV. Maintenance is ensured, however, by *prd*, which becomes expressed in region IV ($K_{d.zv} = 0$). As mentioned above, in regions I and II, Eve represses *odd*, and this repression is maintained by the minor Eve-stripe in region II, thereby fixing the position of the anterior border of Odd-stripe.

Note that *odd* is the repressor of its activator *ftz* (Saulier-Le Dréan et al., 1998). The initial expression of gene *ftz* in region III is thus eliminated by *odd* ($K_{z.zvs} = 0$). According to our model, once *odd* is activated in region III in the absence of its repressors Eve and Prd, it remains active in the absence of any activator. This could suggest autoregulation of *odd*, something that has not yet been reported. If this were the case, our model predicts that *odd* autoregulation would work only in the absence of its two repressors Eve and Prd.

7.2. General properties of the pair-rule cross-regulatory module

One of the assumptions underlying our model is that the initial bell-shaped expression of *eve* behaves as a morphogen, with different concentrations of the Eve protein having distinct effects upon target *pair-rule* genes. Manoukian and Krause (1992) and Fujioka et al. (1995) provide experimental data supporting this contention. This idea led both groups to propose that gene *eve* is needed not only for the activation of odd-numbered En-stripes but that it also plays a role in the activation of even-numbered En-stripes, even though these are directly activated by Ftz. They further proposed that *eve* is responsible for repressing *odd* at the anterior part of Odd-stripe, whereas *ftz* is not repressed in this region (recall that *ftz* and *odd* expressions initially overlap). Thanks to autoregulation, high Ftz level is maintained, activating *en* and giving rise to the even-numbered En-stripes in region II. However, the two groups disagree on the origin of Eve's repressing effect on *odd* in the anterior region of its initial expression domain. Manoukian and Krause (1992) assumed that this effect involves de novo minor Eve-stripes appearing in the anterior region of the *odd* expression domain. In contrast, Fujioka et al. (1995) proposed that early expression of *eve* is responsible for this *odd* repression. In this respect, our model analysis provides a formal demonstration of the requirement of *eve* for the formation of even-numbered En-stripes, in agreement with both groups, while further supporting the contention of Fujioka et al. (1995). In addition, our model accounts for the formation of minor Eve-stripes in the anterior region of even-numbered parasegments derived from early *eve* expression. More specifically, these minor stripes derive from the lower part of the right-hand side of the early bell-shape *eve* expression domain (Eve originally present in region II). This low amount of Eve in region II would suffice to repress *odd* but not *ftz*. At the same time, the expression of *run* constrains *eve* expression around its first functional level, enabling the appearance of minor Eve-stripes, which in turn maintain the repression of *odd*. In brief, the role of the minor Eve-stripes would thus be similar to that of the early bell-shaped Eve stripes.

Early Eve product is needed for the activation of late *eve*, since even in the absence of its three repressors Run, Slp and Odd, and in the presence of its activator Prd, *eve* cannot be fully activated ($K_{v.prsd} = 1$). Moreover, early Eve protein alone can activate late *eve* in the absence of its repressors, although the presence of Prd is also required for *eve* to reach its highest functional level ($K_{v.vprsd} = 3$). Consequently, the formation of late Eve-stripes requires the concerted action of both early Eve and Prd. This agrees with published data (Fujioka et al., 1996).

Other experimental results indicate that the peak concentrations in the early bell-shaped Eve-stripe can repress *prd*, whereas this gene becomes resistant to later repression by Eve during Eve-stripe refinement process. Our model accounts for this phenomenon through the

selection of specific parameter values ($K_{p.ad} = 2$). This selection reflects the *insensitivity* of *prd* with respect to late Eve, as long as its two other repressors Ppa and Odd remain absent.

The *pair-rule* cross-regulatory module appears partly *redundant*. For example, *run* and *slp* are both repressors of *eve* and both are expressed in region IV leading to the specification of the anterior border of the odd-numbered Eve-stripes. Similarly, *eve* and *prd* behave as repressors of *odd*, whereas *hairy* and *eve* are repressors of *ftz*. Our model can be used to provide some prospective explanation of this *redundancy* in terms of evolutionary by-products of *constrictions* between cross-regulating *pair-rule* genes. On several occasions in the course of the evolution of the *pair-rule* module, partly redundant genes would have been recruited in order to progressively specify the striking *pair-rule* expression pattern, while preserving pre-existing *pair-rule* regulatory relationships.

For example, according to our model *eve* and *run* cross-regulation evolved in such a way that the former can completely repress the latter at the appropriate functional threshold ($\mathbf{v} = \mathbf{2}$), whereas *run* cannot fully repress *eve*. This asymmetric relationship constitutes a *constriction* with respect to the interactions of these two genes with the other *pair-rule* genes, as well as with *en* and *wg*. As mentioned above, *run* is activated by gene *prd* in region IV (secondary Run-stripes) in the presence of low concentration of Eve ($\mathbf{v} = \mathbf{1}$). Thus, expression of *run* could coexist with a low expression of *eve* in this region. However, complete repression of *eve* is absolutely necessary to allow *wg* expression in region IV. In order to preserve pre-existing regulatory relationships, a solution would have been found in the recruitment of *slp* to prevent *eve* expression in this region. Alternatively, we can envision the evolutionary process the other away around: the pre-existence of a repressor of gene *eve,* Slp*,* would allow the configuration of *eve-run, prd-run* and *eve-wg* relationships. In any case, the involvement of *slp* would result in some redundancy between *run* and *slp* functions, as both genes act as repressors of *eve* in the same region.

A similar situation is encountered with the presence of the minor Eve-stripe in region II. Indeed, the activation of *run* by Prd in region II still enables low *eve* expression, sufficient to repress *odd*, a repressor of *ftz*. Consequently, *ftz* can be expressed and the even-numbered En-stripes are formed.

This *eve-run* relationship might also explain the recruitment of both *eve* and *ftz* to activate *en*. Indeed, if Run pre-existed in region II, *en* expression would have been enabled thanks to the recruitment of a new activator, *ftz*.

In other cases, some redundancy appears to be a "necessity" related to the temporal expression of the *pair-rule* genes. For example, in region IV, *eve* initially represses *odd*, but later *eve* is itself also repressed in this region by *slp*. The repression of *odd* is then taken over by *prd*. In other cases, the redundancy is related to specific spatial expression domains. This happens with the repression of gene *ftz* by *hairy* and *eve*. For example, in region IV, *hairy* repressing effect on *ftz* is needed because the local concentration of Eve ($\mathbf{v} = \mathbf{1}$) is too low to repress *ftz*. In region I, however, higher *eve* expression ($\mathbf{v} = \mathbf{2}$) suffices to repress *ftz* in the absence of Hairy.

8. Concluding remarks

Our logical model for the *pair-rule* cross-regulatory module accounts for its striking capacity to determine the formation of alternate juxtaposed En- and Wg-expressing cells at the origin of the (para)segment boundaries. This translates into the generation of four distinct expression states, each characterized by a particular combination of pair-rule products or *expression modes*, ultimately resulting in the exclusive activation of *en*, *wg*, or *odd*. The setting of these expression modes depends on the maternal-gap inputs, but also crucially on cross-regulations among *pair-rule* genes, which prove to be instrumental in the interpretation of the maternal-gap pre-pattern.

Furthermore, our logical analysis allows the identification of the most important feedback structures at the origin of the striking patterning properties of the *pair-rule* module. In this respect, a limited set of *positive regulatory circuits* (seven out of 51 different circuits) has been found at the core of the developmental decisions made in the different embryonic regions. The role of these circuits is further confirmed by the systematic simulation of various types of perturbations, including loss-of-function mutations, ectopic *pair-rule* expression, and (autoregulation) *cis*-regulatory mutations. Whenever published data are available, these are found to support our simulation results. Some simulations lead to predictions still awaiting experimental support (e.g. the predicted phenotypes in the case of *slp* ectopic expression, of the double *prd;slp* loss-of-function mutant, or of the specific inactivation of *eve* autoregulation). This extensive but far from exhaustive set of simulations demonstrates how and to what extent our logical model can serve as a basis for *in silico* mutant screenings, in particular multiple mutants which are often difficult to perform experimentally.

Finally, our model provides new insights into the redundancies in the regulatory architecture of the *pair-rule* module. On the basis of our logical simulations and analyses, we propose that these redundancies constitute by-products of *constrictions* due to pre-existing *pair-rule* cross-regulations during the evolution of the *pair-rule*

module. More specifically, these constrictions might be at the basis of the recruitment of genes *eve* and *ftz* for the activation of *en*.

The available data on the expression patterns of genes homologous to *Drosophila* segmentation genes in other insects strongly suggest that the segmentation mechanisms at the end of the segmentation process in *Drosophila* are more conserved than those of earlier steps (reviewed in Nagy and Carroll, 1994; Patel, 1994; French, 2001; Patel et al., 2001). There exists a fundamental difference in the segmentation process between long germ-band insects, in which segmentation occurs initially in a syncytium, and short germ-band insects, in which segmentation proceeds almost entirely in a cellular environment. Notwithstanding, the properties (or at least some of them) found for the *pair-rule* module controlling segmentation in *Drosophila*, a long germ-band insect, might also operate in the short-germ band insects. This is because the *pair-rule* module acts cell-autonomously to form the *pair-rule* pattern, whereas the module formed by the *gap* genes acts in a syncytium to form the *gap*-pattern.

Acknowledgements

This work was supported by grant PB98-0466 to L. Sánchez from DGICYT, Ministerio de Ciencia y Tecnología of Spain. D. Thieffry acknowledges the financial support from the *Action inter-EPST Bioinformatique*, France.

References

Baumgartner, S., Noll, M., 1991. Network of interactions among *pair-rule* genes regulating *paired* expression during primordial segmentation of *Drosophila*. Mech. Dev. 33, 1–18.

Benedyk, M., Mullen, J., DiNardo, S., 1994. *Odd-paired*: a zinc finger pair-rule protein required for the timely activation of *engrailed* and *wingless* in *Drosophila* embryos. Genes Dev. 8, 105–117.

Cadigan, K.M., Grossniklaus, U., Gehring, W.J., 1994. Localized expression of *sloppy paired* protein maintains the polarity of *Drosophila* parasegments. Genes Dev. 8, 899–913.

Carroll, S.B., Scott, M.P., 1986. Zygotically active genes that affect the spatial expression of the *fushi-tarazu* segmentation gene during early *Drosophila* embryogenesis. Cell 45, 113–126.

Carroll, S.B., Vavra, S.H., 1989. The zygotic control of *Drosophila pair-rule* gene expression II. Spatial repression by gap and pair-rule gene products. Development 107, 673–683.

Copeland, J., Nasiadka, A., Dietrich, B., Krause, H., 1996. Patterning of the *Drosophila* embryo by a homeodomain-deleted Ftz polypeptide. Nature 379, 162–165.

Coulter, D., Wieschaus, E., 1988. Gene activities and segmental patterning in *Drosophila*: Analysis of *odd-skipped* and *pair-rule* double mutants. Genes Dev. 2, 1812–1823.

Davis, I., Ish-Horowicz, D., 1991. Apical localization of *pair-rule* transcripts requires 3′ sequences and limits protein diffusion in the *Drosophila* blastoderm embryo. Cell 67, 927–940.

DiNardo, S., O'Farrell, P., 1987. Establishment and refinement of segmental pattern in the *Drosophila* embryo: spatial control of *engrailed* expression by *pair-rule* genes. Genes Dev. 1, 1212–1225.

Frasch, M., Warrior, R., Tugwood, J., Levine, M., 1988. Molecular analysis of *even-skipped* mutants in *Drosophila* development. Genes Dev. 2, 1824–1838.

French, V., 2001. Insect segmentation: genes, stripes and segments in 'Hoppers'. Curr. Biol. 11, R910–R913.

Fujioka, M., Jaynes, J.B., Goto, T., 1995. Early *even-skipped* stripes act as morphogenetic gradients at the single cell level to establish *engrailed* expression. Development 121, 4371–4382.

Fujioka, M., Miskiewicz, P., Raj, L., Gulledge, A.A., Weir, M., Goto, T., 1996. *Drosophila* paired regulates late even-skipped expression through a composite binding site for the paired domain and the homeodomain. Development 122, 2697–2707.

Goto, T., Macdonald, P., Maniatis, T., 1989. Early and late periodic patterns of *even skipped* expression are controlled by distinct regulatory elements that respond to different spatial cues. Cell 57, 413–422.

Gutjahr, T., Frei, E., Noll, M., 1993. Complex regulation of early *paired* expression: initial activation by gap genes and pattern modulation by *pair-rule* genes. Development 117, 609–623.

Gutjahr, T., Vanario-Alonso, C., Pick, L., Noll, M., 1994. Multiple regulatory elements direct the complex expression pattern of the *Drosophila* segmentation gene *paired*. Mech. Dev. 48, 119–128.

Harding, K., Rushlow, C., Doyle, H., Hoey, T., Levine, M., 1986. Cross-regulatory interactions among *pair-rule* genes in *Drosophila*. Science 233, 953–959.

Harding, K., Hoey, T., Warrior, R., Levine, M., 1989. Autoregulatory and gap gene response elements of the *even-skipped* promoter of *Drosophila*. EMBO J. 8, 1205–1212.

Hiromi, Y., Gehring, W., 1987. Regulation and function of the *Drosophila* segmentation gene *fushi tarazu*. Cell 50, 963–974.

Howard, K., Ingham, P., 1986. Regulatory interactions between the segmentation genes *fushi tarazu, hairy,* and *engrailed* in the *Drosophila* blastoderm. Cell 44, 949–957.

Ingham, P., Gergen, J., 1988. Interactions between the *pair-rule* genes *runt, hairy, even-skipped* and *fushi tarazu* and the establishment of periodic pattern in the *Drosophila* embryo. Development 104 (Suppl.), 51–60.

Ingham, P., Hidalgo, A., 1993. Regulation of *wingless* transcription in the *Drosophila* embryo. Development 117, 283–291.

Ingham, P.W., Martínez Arias, A., 1992. Boundaries and fields in early embryos. Cell 68, 221–235.

Ingham, P., Howard, K.R., Ish-Horowicz, D., 1985. Transcription pattern of the *Drosophila* segmentation gene *hairy*. Nature 318, 439–445.

Ingham, P., Baker, N., Martínez-Arias, A., 1988. Regulation of segment polarity genes in the *Drosophila* blastoderm by *fushi tarazu* and *even skipped*. Nature 331, 73–76.

Ish-Horowicz, D., Pinchin, S.M., 1987. Pattern abnormalities induced by ectopic expression of the *Drosophila* gene *hairy* are associated with repression of *ftz* transcription. Cell 51, 405–415.

Ish-Horowicz, D., Howrad, K.R., Pinchin, S.M., Ingham, P.W., 1985. Molecular and genetic analysis of the *hairy* locus in *Drosophila*. Cold Spring Harbor Symp. Quant. Biol. 50, 135–144.

Ish-Horowicz, D., Pinchin, S.M., Ingham, P., Gyurkovics, H., 1989. Autocatalytic *ftz* activation and metameric instability induced by ectopic *ftz* expression. Cell 57, 223–232.

Jiang, J., Hoey, T., Levine, M., 1991. Autoregulation of a segmentation gene in *Drosophila*: combinatorial interaction of the *even-skipped* homeo box protein with a distal enhancer element. Genes Dev. 5, 265–277.

Kerszberg, M., Changeux, J.P., 1994. A model for reading morphogenetic gradients: autocatalysis and competition at the gene level. Proc. Natl. Acad. Sci. USA 91, 5823–5827.

Kilchherr, F., Baumgartner, S., Bopp, D., Frei, E., Noll, M., 1986. Isolation of the *paired* gene of *Drosophila* and its spatial expression during early embryogenesis. Nature 321, 493–499.

Klingler, M., Gergen, J., 1993. Regulation of *runt* transcription in *Drosophila* segmentation genes. Mech. Dev. 43, 3–19.

Lewis, J., Slack, J.M., Wolpert, L.J., 1977. Thresholds in development. J. Theor. Biol. 65, 579–590.

Macdonald, P.M., Struhl, G., 1986. A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. Nature 324, 537–545.

Manoukian, A.S., Krause, H., 1992. Concentration-dependent activities of the *even-skipped* protein in *Drosophila* embryos. Genes Dev. 6, 1740–1751.

Manoukian, A.S., Krause, H.M., 1993. Control of segmental asymmetry in *Drosophila* embryos. Development 118, 785–796.

Martínez Arias, A., Lawrence, P., 1985. Parasegments and compartments in the *Drosophila* embryo. Nature 313, 639–642.

Martínez-Arias, A., White, R., 1988. *Ultrabithorax* and *engrailed* expression in *Drosophila* embryos mutant for segmentation genes of the *pair-rule* class. Development 102, 325–338.

Meinhardt, H., 1978. Space-dependent cell determination under the control of morphogen gradient. J. Theor. Biol. 74, 307–321.

Morrissey, D., Askew, D., Lakshmi, R., Weir, M., 1991. Functional dissection of the *paired* segmentation gene in *Drosophila* embryos. Genes Dev. 5, 1684–1696.

Mullen, J.R., DiNardo, S., 1995. Establishing parasegments in *Drosophila* embryos: roles of the *odd-skipped* and *naked* genes. Dev. Biol. 169, 295–308.

Nagy, L.M., Carroll, S., 1994. Conservation of *wingless* patterning functions in the short-germ embryos of *tribolium castaneum*. Nature 367, 460–463.

Nasiadka, A., Krause, H.M., 1999. Kinetic analysis of segmentation gene interactions in *Drosophila* embryos. Development 126, 1515–1526.

Nüsslein-Volhard, C., Wieschaus, E., 1980. Mutations affecting segment number and polarity in *Drosophila*. Nature 287, 795–801.

Pankratz, M.J., Jäckle, H., 1993. Blastoderm segmentation. In: Bate, M., Martínez Arias, A. (Eds.), The Development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory, New York, pp. 467–516.

Patel, N.H., 1994. Developmental evolution: insights from studies of insect segmentation. Science 266, 581–590.

Patel, N.H., Hayward, D.C., Lall, S., Pirkl, N.R., DiPietro, D., Ball, E.E., 2001. Grasshopper hunchback expression reveals conserved and novel aspects of axis formation and segmentation. Development 128, 3459–3472.

Pick, L., Schier, A., Affolter, M., Schmidt-Glenewinkel, T., Gehring, W., 1990. Analysis of the *ftz* upstream element: germ layer-specific enhancers are independently autoregulated. Genes Dev. 4, 1224–1239.

Raj, L., Vivekanand, P., Das, T.K., Badam, E., Fernandes, M., Finley, R.L., Brent, R., Appel, L.F., Hanes, S.D., Weir, M., 2000. Targeted localized degradation of paired protein in *Drosophila* development. Curr. Biol. 10, 1265–1272.

Reinitz, J., Kosman, D., Vanario Alonso, C.E., Sharp, D.H., 1998. Stripe forming architecture of the gap gene system. Dev. Genet. 23, 11–27.

Rivera-Pomar, R., Jäckle, H., 1996. From gradients to stripes in *Drosophila embryogenesis*: filling in the gaps. Trends Genet. 12, 478–483.

Sánchez, L., Thieffry, D., 2001. A logical analysis of the *Drosophila* gap-gene system. J. Theor. Biol. 211, 115–141.

Sánchez, L., VanHelden, J., Thieffry, D., 1997. Establishment of the dorso-ventral pattern during embryonic development of *Drosophila melanogaster*: a logical analysis. J. Theor. Biol. 189, 377–389.

Saulier-Le Dréan, B., Nasiadka, A., Dong, J., Krause, H., 1998. Dynamic changes in the functions of Odd-skipped during early *Drosophila* embryogenesis. Development 125, 4851–4861.

Schier, A., Gehring, W., 1992. Direct homeodomain-DNA interaction in the autoregulation of the *fushi tarazu* gene. Nature 356, 804–807.

Sprenger, F., Nüsslein-Volhard, C., 1993. The terminal system of axis determination in the *Drosophila* embryo. In: Bate, M., Martínez Arias, A. (Eds.), The Development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory, New York, pp. 365–386.

St. Johnston, D., Nüsslein-Volhard, C., 1992. The origin of pattern and polarity in the *Drosophila* embryo. Cell 68, 201–220.

Thieffry, D., Sanchez, L., 2003. Alternative epigenetic states understood in terms of specific regulatory structures. Ann. NY Acad. Sci. 981, 135–153.

Thieffry, D., Sánchez, L. (in press). Qualitative analysis of gene networks: towards the delineation of trans-regulatory modules. In: Schlosser, G., Wagner, G. (Eds.), Modularity in Development and Evolution. University of Chicago Press, Chicago.

Thomas, R., 1991. Regulatory networks seen as asynchronous automata: a logical description. J. Theor. Biol. 153, 1–23.

Thomas, R., Thieffry, D., Kaufman, M., 1995. Dynamical behaviour of biological regulatory networks. I. Biological role of feedback loops and practical use of the concept of the loop-characteristic state. Bul. Math. Biol. 57, 247–276.

Tracy Jr., W.D., Ning, X., Klingler, M., Kramer, S.G., Gergen, J.P., 2000. Quantitative analysis of gene function in the *Drosophila* embryo. Genetics 154, 273–284.

Tsai, Ch., Gergen, J.P., 1994. Gap gene properties of the *pair-rule* gene *runt* during *Drosophila* segmentation. Development 120, 1671–1683.

Von Dassow, G., Odell, G.M., 2002. Design and constraints of the *Drosophila* segment polarity module: robust spatial patterning emerges from intertwined cell state switches. J. Exp. Zool. 294, 179–215.

Von Dassow, G., Meir, E., Munro, E.M, Odell, G.M., 2000. The segment polarity network is a robust developmental module. Nature 406, 188–192.

Wolpert, L., 1969. Positional information and the spacial pattern of cellular differentiation. J. Theor. Biol. 25, 1–47.

Yu, Y., Pick, L., 1995. Non-periodic cues generate seven *ftz* stripes in the *Drosophila* embryo. Mech. Dev. 50, 163–175.