A biophysical mechanism may control the collinearity of *Hoxd* genes during the early phase of limb development

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Abstract

A biophysical model has been proposed which deals with the observed collinearity of *Hox* gene expressions in developing vertebrate limbs. It is assumed that physical forces gradually dislocate the genes of the *Hoxd* cluster from inside the chromosome territory into the interchromosome domain, where the genes are activated. In particular, the action of Coulomb electric forces has been estimated in detail. Genetic engineering experiments (deletions, duplications and transpositions) were recently reported for *Hoxd* expression during limb development. Here, we analyse these results and show that the biophysical model explains them successfully.

Keywords: Hox *collinearity, gene deletion, duplication, transposition*

Introduction

The collinearity of *Hox* gene expressions is a surprising feature observed during animal development along the primary and secondary body axes.[1,2] These gene expressions follow in space and time the sequence of gene ordering from 3′ to 5′ in the *Hox* cluster.[2,3] In the *Hoxd* cluster, the genes are lined up in the 3′ to 5′ direction and their expression domains (*Hoxd1, Hoxd2, Hoxd3*, . . .) along the anteroposterior axis follow a peculiar pattern: the anterior border of expression for each gene is shifted in the anterior–posterior direction — for example, the anterior border of *Hoxd1* expression is more anterior than the anterior border of *Hoxd2* expression etc. Thus, a pattern of partially overlapping expression domains along the head to tail axis is formed ('spatial collinearity').[2] Furthermore, the genes of the *Hoxd* cluster are activated one after the other in the same 3′ to 5′ order ('temporal collinearity').[2,3] Tarchini and Duboule[4] have put forward a model in which, during early limb budding (first wave), two regulatory influences control the activation of *Hoxd* genes: one mechanism is located at the telomeric (3′) side of the cluster (early limb control regulation; ELCR) and the other relies on sequences centromeric to the cluster (POST). Thus, in order to describe the data in terms of biomolecular processes only, two opposing collinearity mechanisms have to be introduced.[4]

The biophysical model — Comparison with experiments

An alternative biophysical model was introduced, dealing with the initial stages of limb development.[5] According to this model, the inactive *Hox* cluster is anchored inside the chromosome territory (CT), where the gene regulatory regions are inaccessible for transcription. Physical forces may pull the *Hox* fibre towards the interchromosome domain (ICD). Just outside the CT, the genes co-localise both *in cis* and *in trans* with local concentrations of transcription factories, and gene

transcription is initiated (Figure 1). The intensity of a *Hox* gene transcription decreases when the gene moves in the ICD away from the CT boundary. Since the nature of the physical forces is unknown, we consider the electric Coulomb forces as plausible candidates.[5] Normally, the *Hox* cluster is negatively charged, and positive polar molecules are deposited at some distance from the cluster, as shown schematically in Figure 1. The positive charges, *P*, in every cell nucleus correspond to an extracellular morphogen, which forms a gradient along the anteroposterior axis;[5] *P* is high in the posterior cells and decreases gradually towards the anterior cells. A Coulomb force is created that pulls the *Hox* cluster; it decondenses the gene fibre and translocates the *Hox* genes in the ICD, following the 3′ to 5′ order (Figure 1).

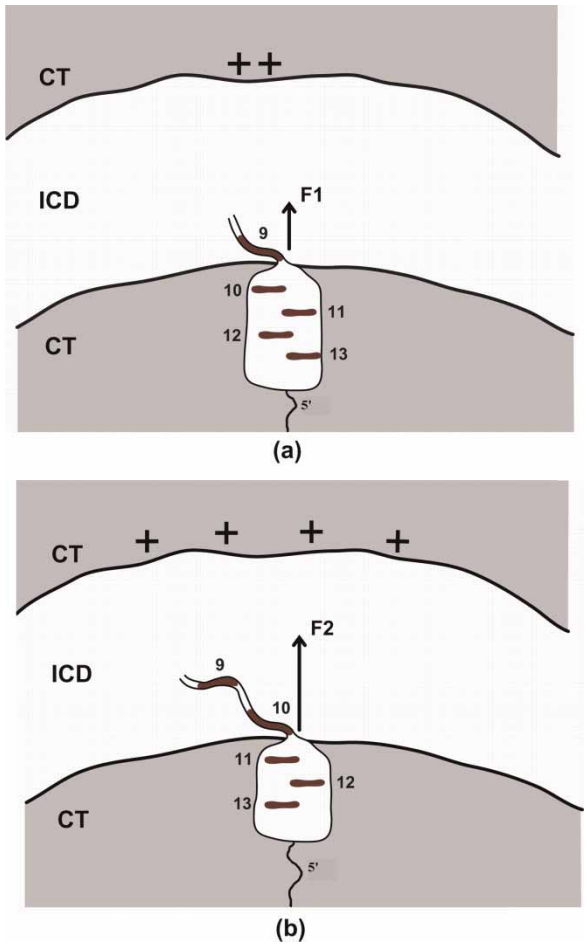

Figure 1. *Hoxd* extrusions. When no positive charges are deposited opposite the 5′ *Hoxd* subcluster, the subcluster remains inside the chromosome territory (CT). (a) For a low distribution of positive charges *P* (++), a small Coulomb force *F1* is created and one gene (*Hoxd9*) is extruded in the interchromosome domain (ICD). This occurs in domain D9 for cells in which the morphogen concentration is above the corresponding threshold T9. (b) For increased positive charges *P* (++++), the force *F2* is stronger and two consecutive genes (*Hoxd9* and *Hoxd10*) are extruded. This occurs in domain D10, where the morphogen is above threshold T10.

The biophysical model does not merely describe *Hox* gene collinearity. Its aim is to explain it and to provide the mechanism that causes the modes of *Hox* gene expression by integrating and coordinating actions at both the multicellular and subcellular levels. Of course, the control regions, such as ELCR and any other regulatory elements that have yet to be discovered, are essential tools of the biochemical machinery that participate in the realisation of *Hox* gene expression. We believe, however, that this essential biomolecular pathway is triggered and controlled by a more fundamental, single and unidirectional mechanism, such as the one presented here. The biophysical model reproduces spatial, temporal and quantitative collinearities well.[5]

Gene deletions, duplications and transpositions

Duboule and collaborators[4,6,7] have performed detailed and extensive experiments of *Hoxd* gene deletions and duplications in order to explore the features of collinearity during vertebrate limb development. Their findings for the early phase of limb development[4] are highly illuminating, and it is worth comparing them with the biophysical model predictions.

Suppose the subcluster of the 5′ *Hoxd* genes has an evenly distributed negative charge N ($N = 10$ in arbitrary units) and that every gene region (*Hoxd9, ... Hoxd13*) carries a charge $N/5 = 2$. Before activation of the 5′ *Hoxd* subcluster, all genes are embedded inside the CT. In this case, we can set $P = 0$ and the Coulomb force is 0. For activation of the first gene (*Hoxd9*), we set $P = 2$ (in arbitrary units) and $P = 10$ when all genes (*Hoxd9, ..., Hoxd13*) are extruded in the ICD.

The measure of the Coulomb force F acting on the subcluster is proportional to the positive and

negative charges. For our rough comparative estimates, we can assume:

$$F = P \times N$$

In this truncated Coulomb equation, we neglect the dependence of F on the distance between positive and negative charges, and the Coulomb's constant is set at 1 (in appropriate units). The Coulomb force causes gene fibre decondensation and extrusion of length L. We assume, in a first approximation, that this extrusion length L is proportional to F in the spirit of Hooke's law of elastic spring expansion. Deletion of one *Hoxd* gene region causes a reduction in the negative charge of the cluster; therefore, the Coulomb force will decrease and consequently the extrusion length of the *Hoxd* fibre will be shortened. The opposite will occur with a duplication of one *Hoxd* gene region in the cluster: the larger the duplication, the longer the gene fibre extrusion. In the supplementary material, we present some indicative experiments of gene deletions, duplications and transpositions[8] and compare them with the biophysical model predictions. The agreement is astonishing.

Discussion

In an analysis of the *Hoxd* locus in mouse development, activation along the primary anteroposterior axis is accompanied by fibre extrusion from the CT.[9] In the limb bud, at the same stage (E 9.5), however, no looping out is detected, even though the locus is still decondensed. A further confirmation of this difference is needed, taking into account that at that stage *Hoxd* expressions (eg *Hoxd4*) in the mouse limb bud are much weaker than in the tailbud.[9]

The biophysical model explains how collinearity phenomena occur during the early stages of limb development. At later stages, when all *Hoxd* genes are expressed, secondary regulations and gene interactions modulate their expressions and the complex landscape of digits starts emerging. Recently, Montavon *et al.*[7] analysed experimentally and theoretically the features of quantitative collinearity in developing digits. They used some of their essential findings to formulate a model for the differential importance of gene rank — the relative position of *Hoxd* genes with respect to the 5′ extremity of the cluster. For instance, they observed that when *Hoxd13* (the most strongly expressed gene) was deleted, the gene that substituted for it in the last 5′ position was expressed with an increased intensity. It is instructive to view this result in the framework of the biophysical model: as soon as *Hoxd13* is activated, the whole gene fibre is extruded and *Hoxd13* is located closest to the surface of the CT, and its expression is therefore strongest. (This occurs in the posterior domain of the limb bud.) When *Hoxd13* is deleted, the extruding Coulomb force F decreases and the extruded fibre retreats toward the interior of the CT. The consecutive gene (eg *Hoxd12*) is dislocated towards the CT surface and consequently its expression is strengthened. This is the essence of the observation by Montavon *et al.*[7] and it indicates, on the one hand, that this observation constitutes additional evidence in favour of the biophysical model. On the other hand, it shows that our interpretation of quantitative collinearity can be extended to all stages of limb development. Finally, the above authors have managed to establish a quasi-linear relationship between the total transcriptional activity at the locus and the number of transcription units — e.g. active genes in developing digits (Figure 4b in reference 7). This remarkable correlation supports our hypothesis of Coulomb force variations and the corresponding proportional changes in the extruded fibre lengths (reminiscent of Hooke's linear law of elastic expansions).

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Supplementary material

We can establish the following correlations between cell locations, Coulomb forces and subsequent *Hoxd* gene extrusions and subsequent activations.

1) For cells expressing only one gene (*Hoxd9*):

$P = 2, \quad N = 10, \quad F = 2 \times 10 = 20, \quad L = 1$

(Figure 1a), Domain D9

2) For cells expressing two genes (*Hoxd9* and *Hoxd10*):

$P = 4, \quad N = 10, \quad F = 4 \times 10 = 40, \quad L = 2$

(Figure 1b), Domain D10 < Domain D9

3) For cells expressing three genes (*Hoxd9*, *Hoxd10* and *Hoxd11*):

$P = 6, \quad N = 10, \quad F = 6 \times 10 = 60, \quad L = 3,$

Domain D11 < Domain D10 < Domain D9. . .etc.

In experiments where one gene of the subcluster is deleted, the negative charge of the cluster will decrease to $N = 8$ and the length of the extruded fibre will be shorter. Two gene deletions will produce an even bigger reduction in the length of the extruded fibre. By contrast, when one gene is duplicated, N will increase to 12 and the extruded fibre will be longer etc.

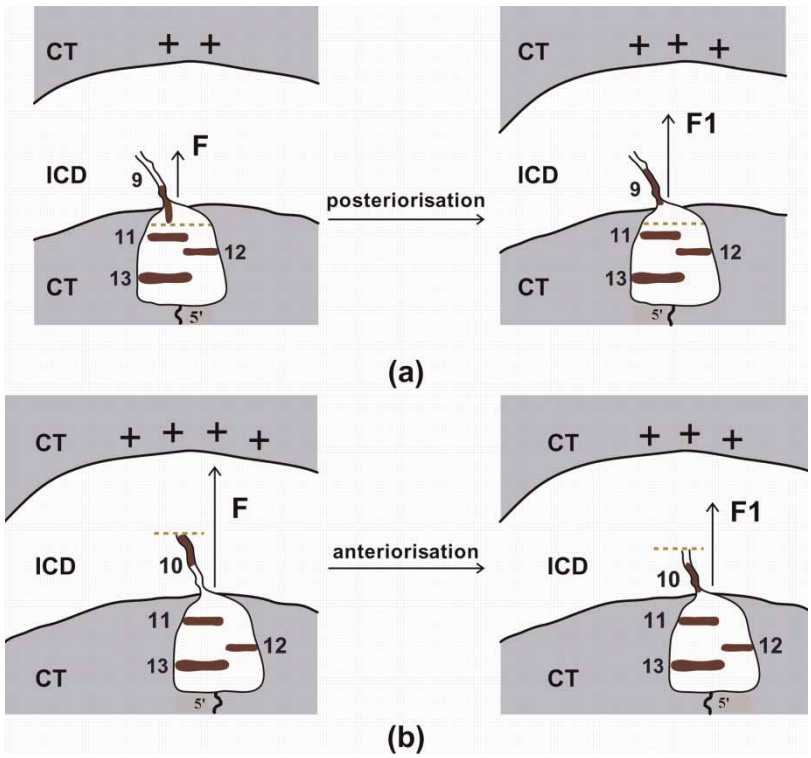

Figure 1S. *Hoxd* gene deletions. (a) Posterior gene deletions. Consider activation of *Hoxd9* when *Hoxd10* is deleted (left). *N* decreases and the force *F* is smaller than F1; therefore, *Hoxd9* retreats inside the CT. For the extrusion of *Hoxd9*, *P* has to increase to more posterior locations (right). (b) Anterior gene deletions. Consider activated gene *Hoxd10*. When anterior gene *Hoxd9* is deleted, *F* decreases (left) but is still higher than the force *F1* needed for the extrusion of the first gene (*Hoxd9*), hence, *Hoxd10* expression shifts to more anterior cells with lower *P* (right).

In the following, we estimate how deletions and duplications modify gene expressions, and particularly in domains D9, D10 and D11.

(a) Posterior gene deletions

Consider cells in which *Hoxd9* is normally activated (domain D9). The normal force acting on the fibre is (at least): $F1 = 2 \times 10 = 20$ (Figure 1a). If posterior gene *Hoxd10* is deleted, the negative charge N of the cluster decreases to 8 and the pulling force on the fibre becomes $F = 2 \times 8 = 16$, which is below the normal value of 20. Consequently, *Hoxd9* will retreat towards the interior of the CT (Figure 1Sa; left).

For the complete extrusion of *Hoxd9*, a stronger force is needed and this is achieved by a posterior shift to cells where the morphogen value is higher, as is the corresponding P value: P (++ → +++). If more than one posterior gene is deleted, the posterior shift of *Hoxd9* activation will increase. Tarchini and Duboule[4] designated this as an unexpected observation. For the biophysical model, this posteriorisation is an obvious outcome.

(b) Anterior gene deletions

This case is more involved because, besides the charge modification of the cluster, the intervention also affects the length of the extruded gene fibre (Figure 1Sb). Consider, for example, cells in which *Hoxd9* and *Hoxd10* are activated, where the normal extrusion force is (at least) $F2 = 40$. If *Hoxd9* is deleted, the extrusion force on the fibre is reduced to $F = 4 \times 8 = 32$, which is lower than 40. In this situation, *Hoxd10* is the first extruded gene. For this first gene, we estimated that the necessary extrusion force is $F1 = 20$ at least.

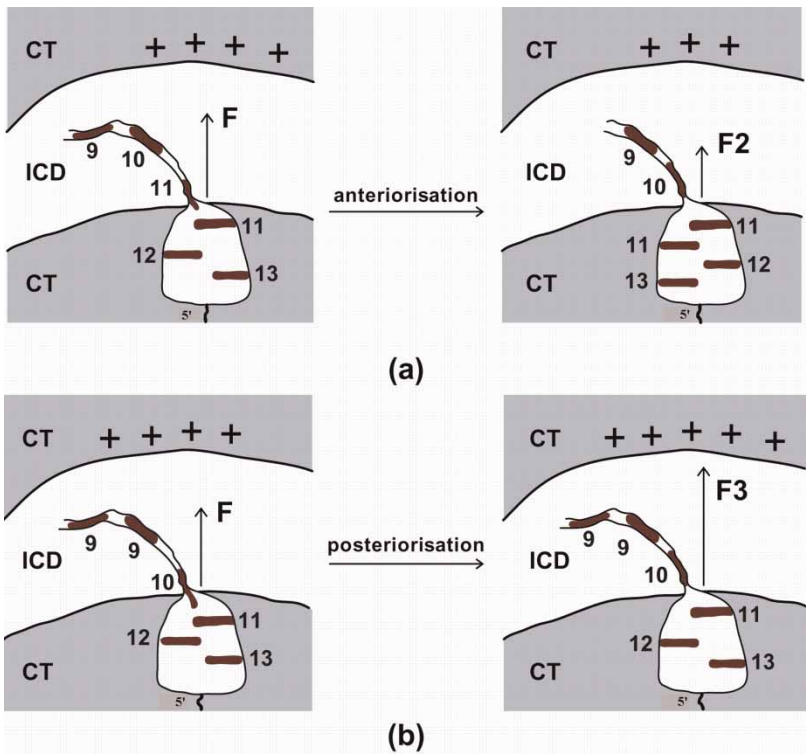

Figure 2S. *Hoxd* gene duplications. Consider cells expressing *Hoxd9* and *Hoxd10*. (a) Posterior gene duplications. If *Hoxd11* is duplicated (left), *F* increases above the minimum value *F2*; therefore, *Hoxd10* can be expressed ectopically in more anterior locations (right). (b) Anterior gene duplication. If *Hoxd9* is duplicated, the pulling force increases (left) but the increase is not sufficient to reach *F3* for the extrusion of three genes; therefore, *P* must increase for activation of the third gene (*Hoxd10*), whose expression is then shifted posteriorly (right).

For activation of *Hoxd10*, F can be reduced to $F1$, and this occurs at more anterior cell positions with a lower P value: P ($++++ \rightarrow +++$). An ectopic expression of *Hoxd10* in the anterior region of the limb bud will be observed, associated with a premature expression of the gene. This was verified by Tarchini and Duboule.[4]

(c) Posterior gene duplications

Compared to gene deletions, gene duplications have an opposite effect on *Hoxd* gene expressions. Consider, again, cells in which *Hoxd9* and *Hoxd10* are activated, where the pulling force is (at least) $F2 = 40$. If posterior gene *Hoxd11* is duplicated (Figure 2Sa), the charge of the cluster will increase to $N = 12$.

The pulling force on the fibre will become: $F = 4 \times 12 = 48$, which is higher than $F2$. Activation of *Hoxd10*, however, can start with $F2$; therefore, F can be reduced to this value. This is achieved by an anterior shift of D10; in this case, the morphogen decreases, and so does P ($++++ \rightarrow +++$) and $F \rightarrow F2$. The result is an anteriorisation and premature expression of *Hoxd10*.

Let us examine *Hoxd10* expression at a position and time without *Hoxd11* duplication. At this position, after *Hoxd11* duplication, *Hoxd10* will be abnormally translocated further inside the ICD, away from the CT border. A downregulation of *Hoxd10* expression is expected at this position (quantitative collinearity). This was verified by Kmita *et al.*[6]

(d) Anterior gene duplications

Consider the case of two activated genes (*Hoxd9* and *Hoxd10*), where $F2 = 40$. If anterior gene *Hoxd9* is duplicated, the pulling force on the fibre increases from $F2$ to $F = 48$. For activation of *Hoxd10*, however, this increase is not sufficient, since now three genes must be extruded. The corresponding pulling force for three genes is $F3 = 60$ (Figure 2Sb). For a stronger force $F3$, P must take a higher value: P ($++++ \rightarrow +++++$). Therefore, after an anterior gene duplication, the expression of *Hoxd10* will be shifted posteriorly, in agreement with the findings of Tarchini and Duboule.[4]

These calculations can be applied to extended gene deletions and duplications incorporating several genes from the entire *Hoxd* cluster. Following the above rules, the estimation of anteriorisations or posteriorisations is straightforward, and the results agree with the findings of Duboule and co-workers. The conclusion is that *Hoxd* gene deletions and duplications strongly support the present electric force mechanism of the biophysical model.

Hoxb1 transposition in *Hoxd*

Recently, the *Hoxb1* transgene was transposed at the 5′ end of the *Hoxd* cluster.[8] The results from this transposition are in agreement with the biophysical model. For example, in the wild-type mouse embryo at stage E7.5, *Hoxd13* is not expressed in the primitive streak tissue. By contrast, *Hoxd13* is activated when the *Hoxb1-LacZ* reporter is inserted at the 5′ end of the *Hoxd* cluster. This finding is explained by the biophysical model, since, in analogy with the *Hoxd* regions, the *Hoxb1* transgene carries a negative charge and the total N increases in the transgene embryo (as analysed in detail above). The pulling force on the gene fibre therefore increases, so that *Hoxd13* loops out of the CT and its activation is possible. In the wild-type limb bud at E9.5, the *Hoxd* region, although decondensed, does not extrude out of the CT.[8] In the *Hoxb1-LacZ* embryo, however, *Hoxd* does loop out and this can be attributed, again, to the increased force of attraction that pulls the gene fibre out of the CT.