Biophysics Precedes Biochemistry In Hox Gene Collinearity

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Abstract

Background: Hox gene collinearity is a universal phenomenon most emphatically observed during the development of vertebrate embryos. The last decade or so, a series of genetic engineering experiments have been performed including Hox gene deletions, duplications or transpositions. These experiments are illuminating and helpful to understand the mechanisms responsible for Hox gene collinearity. Until now several models have been proposed based on well established biomolecular mechanisms. Even though these models, and in particular the 'two-waves model', efficiently describe many features of the phenomenon, they leave several other findings unexplained.

Results: A 'biophysical model' has been proposed in a series of publications, based on quite a different hypothesis, namely the generation of physical forces. These forces act on the Hox clusters and translocate the genes step by step from inside the chromatin territory, where they remain inactive, toward the transcription factory domain where they can be transcribed. This model feature was recently confirmed. The corpus of data can be explained by the biophysical model, even in parts where the two-waves model is not successful. Furthermore, the biophysical model can reproduce the wide class of experimental findings after implanting signalling molecules (e.g. FGF or SHH) in the developing vertebrate limb bud.

Conclusion: Hox gene transcription proceeds in two steps: firstly, a physical force is generated that moves the genes gradually and in a timely manner to a location where activation is possible. Subsequently, the bio-molecular machinery (e.g. enhancers, repressors etc) takes over and completes the transcription of Hox genes. Experiments are proposed to test this hypothesis.

Introduction

Hox genes are essential tools for the development and function of numerous animal and plant species. Evolutionary considerations indicate that chromatin organization in clusters increases the functionality of Hox genes manifested, for instance, in the relatively recent genomes of vertebrates [1]. Their crucial role in the normal (and abnormal) development of human embryos is evident.

In evolutionaty terms, the emerging property of collinearity is related to the conserved clustering of the Hox complexes [2]. In the developing limbs of mice the Hox genes belonging to the HoxA cluster are activated one after the other along the proximal-distal axis of the limb following the same sequence as they are located along the 3' to 5' direction on the chromosome 2 (*spatial collinearity*). This surprising property of Hox genes was first observed in 1978 by E. B. Lewis in the genes of the bithorax complex (*BX-C*) of the *Drosophila* embryo [3]. In the HoxD cluster the genes are numbered Hoxd1, Hoxd2, Hoxd3,… starting from the anterior side of the cluster. Hoxd1 is expressed first followed by Hoxd2 and subsequently by Hoxd3 etc. This sequential expression in time is coined *temporal collinearity* [4]. A third kind of collinearity was observed in the vertebrates: when at a given position in the limb bud several Hox genes are expressed, the expression of the most posterior gene in the cluster is stronger than the other gene expressions (*quantitative collinearity*) [5, 6].

Genetic engineering experiments can illuminate the mechanism responsible for the collinearity of Ho*x* genes. Manipulations like Hox gene deletions or duplications are helpful to formulate explanatory models [7-9]. Accordingly in a 'two-waves model', it is proposed that enhancers located in a 'landscape' telomeric to the HoxD cluster are acting on the Hoxd genes of the cluster [7-9]. A cooperating centromeric landscape with a negative effect balances the influence of the telomeric enhancers [9] (Fig.1a).
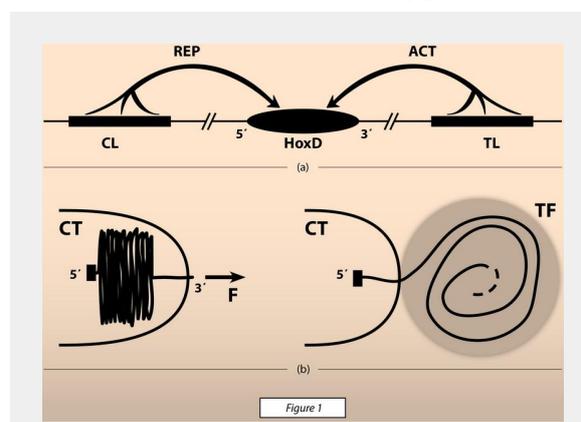

Figure 1

Figure 1: The two-waves model vs the biophysical model (schematically) (a) In the two-waves model the enhancers of the telomeric landscape (TL) activate the Hoxd genes in the early phase of limb bud development (ACT). Activation is balanced by a repression (REP) originating from the centromeric landscape (CL).

(b) In the biophysical model the inactive Hox cluster is condensed and anchored at the 5' end inside the chromatin territory (CT) (left). Variable pulling forces F applied at the 3' end of the cluster translocate the Hox genes step by step toward the transcription factory domain (TF) (right). The dashed line represents the telomeric end of the Hox cluster.

The model distinguishes an early and a late wave of Hox gene activation [10,11]. In the early phase, the Hoxd enhancers were recently located in a telomeric landscape [11] (Fig.1a). Deletion experiments in the region of the telomeric desert were performed and the action of the enhancers on the Hoxd gene expressions was analyzed in detail [11]. The two-waves model was extended to a late phase of limb development in order to describe the complex pattern of digit formation [11]. The above two-waves model, based only on biomolecular mechanisms, can explain many of the experimental findings. However several other results remain unexplained as, for instance, in the following cases: a) in mutant mouse embryos, the DNA regions posterior to the probe Hoxd genes were deleted. According to the two-waves model the expressions in these mutant limbs were not expected to differ from the wild type expressions since the gene distances from their telomeric enhancers remained unchanged. Unexpectedly the expressions in the mutant limbs were systematically posteriorized – the anterior border of the expressions was shifted posteriorily [7]. b) in similar deletion experiments the deleted gene regions were located anteriorily to the probe Hoxd genes. In this case some of the observed expressions were impossible to anticipate [8]. The above findings that the two-waves model cannot reproduce are explained in the next Section.

The DNA string is tangled with different proteins and particularly with histones so that a characteristic chromatin structure is formed. During Hox gene activation a dynamic transition of the chromatin architecture is observed. This dynamic 3D chromatin reorganization has been intensively studied in recent years (see e.g. [10]). It is thus established that the chromatin of an inactive Hox cluster is packed in a condensed configuration. When Hox gene activation is switched on a decondensation of the chromatin fiber

occurs.

An Alternative Approach

In order to explain Hox gene collinearity a 'biophysical model' was proposed based on quite a different premise. According to this model a macroscopic morphogen gradient is established along the limb bud (see next Section). The peak of the gradient is located at the distal tip of the bud (Fig.2). Threshold T defines the region (0A) where the morphogen concentration is higher than T. The Hox gene is expressed in this region (Fig.2a).
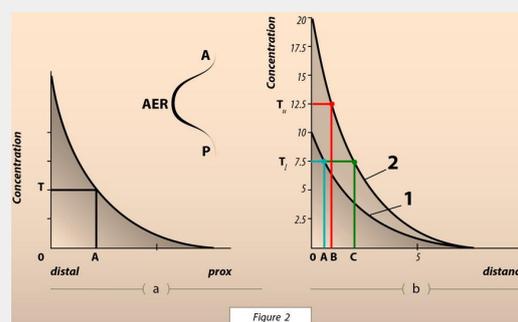
Figure 2

**Figure 2: Morphogen gradient in the proximal-distal axis of the limb bud**
(a) The morphogen source is located in the apical ectodermal ridge (AER) at the distal tip of the bud. A and B are the anterior and posterior sides of the bud respectively
(b) The source strength is doubled and the normal gradient (1) increases to (2). Tl and Tu are the lower and upper thresholds respectively. For normal limbs and gradient (1) the expression of Hoxa13 extends to (0A). For gradient (2) the normal expression domain (0A) shifts to (BC). In region (0B) the Hoxa13 expression disappears.

Signal molecules are transduced inside every cell and they are allocated in specific domains in the cell nucleus. Evidence for the above processes is presented in several papers [12,13]. It is assumed that such molecules are deposited outside the cluster in the Interchromosome Domain (ICD) on a nuclear 'envelope' of the cluster assigning a property 'P' to the envelope (Fig.3). The graded P- property consists of a molecular signal entailing information on the cell position along the proximal-distal axis of the bud (Fig.3). When no molecules are deposited on the cluster envelope, P is zero and the cluster is condensed and inactive inside the Chromatin Territory (CT) (Fig.3a). The posterior end of the Hox cluster is fixed inside the CT while its anterior end is loose and

close to the surface of the CT. The Hox cluster itself is endowed with an associated property 'N' which increases according to the size of the DNA fiber. The two properties, P and N, can create a pulling force acting on the 3' end of the cluster (Fig.3). The measure of this force F depends linearly and concurrently on both properties P and N:

$$F = P * N \quad ---------------->(1)$$
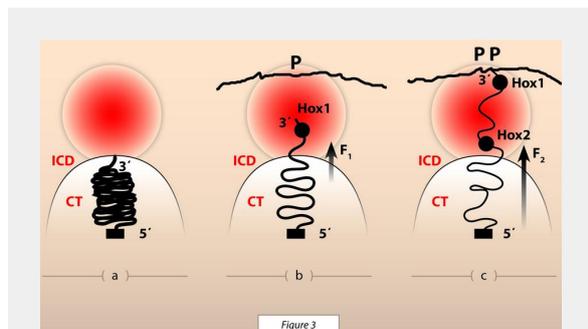
Figure 3: Hox gene translocations - elastic spring analogue

(a) The inactive Hox cluster is condensed inside the chromatin territory (CT). The 5'end is fixed while the loose 3'end can move toward the transcription factory (TF) (pink disc) lying in the interchromosome domain (ICD).

(b) Molecules P are deposited in the envelope located in the ICD and surrounding the Hox cluster. A pulling force F1 shifts Hox1 in the TF disc. (c) According to Eq.1, more molecules P create a stronger force F2 translocating both Hox1 and Hox2 in the TF domain.

One can think of possible mechanisms generating forces on gene clusters [14]. Heuristically, a possible interpretation of the abstract Eq.1 could be the following: the deposited molecules are polar and the P-factor represents the electrically positive charge of the enveloping environment of the cluster. In a complementary assumption, the N-factor can represent the negative electric charge of the cluster reminiscent of the negative charge of the DNA backbone. The following arguments could justify the above hypothesis: a) the introduction of P and N and the force F generation do not contradict any First Principle hence their elaboration is legitimate [6,14,15]. b) the gene translocation from place to place in space is a consequence of the axiom of Physics that the movement of an object is caused by some physical force. c) however strange or unconventional a hypothesis may be, its acceptance and vindication depend solely on its ability to successfully reproduce the data (see below). In many cases controversial or even counter-intuitive assumptions were finally

experimentally confirmed.

At this stage it is worth stressing the following points: i) The expression for F resembles a quenched Coulomb force where the dependence on the distance between the two charge distributions P and N is ignored. The force F on Hox genes increases for cells in the proximal-distal (or anterior-posterior) direction and it pulls sequentially the genes of the Hox cluster toward the (ICD) (Fig.3b,c). ii) The unidirectional force F (Fig.3) is an oversimmplification due to our ignorance of the detailed distributions of the electric charges on both the Hox cluster envelope and the cluster itself. A realistic approach should take into account these distributions and the dependence of the constituent forces on the relative distances between the electric charges. In reality the exact calculation of the meandering movement of the decondensing DNA fiber is an extremely difficult task (see Section 4). iii) The action of F on the Hox cluster should be compared to the expansion of an elastic spring [6]. According to this picture the 'spring' is fixed at the 5' end and the force F decondenses the DNA fiber and pulls gradually the 3' end toward the transcription factory domain (TF) (Fig. 3).

The genes are transferred step by step (Hox1, Hox2, Hox3,...) toward the TF domain where they are activated (Fig.1b). The above predicted Hox gene movements were observed by Duboule and coworkers but no explanation of the phenomenon was given [10]. In contrast, the biophysical model predicts these gene movements and it attributes this phenomenon to the action of pulling forces F [14-16]. Note that the above interpretation of Eq.1 is not unique. However, the present version of the biophysical model can explain the results as derived from Hox gene manipulations (deletions, duplications or transpositions). The details are presented elsewhere [6, 15-17].

Signaling molecules activating the HoxD cluster are established along both the proximal-distal and the anterior-posterior axes of the bud (see next Section). The direction of the resultant force acting on the HoxD cluster lies between these two axes of the bud. Analyzed here are only the two cases that the two-waves model fails to describe as mentioned in the previous Section. a) In deletions posterior to a probe gene, the factor N is reduced and, according to Eq.1, F is weaker and the probe gene retreats from ICD into CT where the gene is not activated (Fig.3a). In order to balance this back shift, P must increase so that a stronger force F will pull the DNA fiber into ICD in the TF domain where the probe gene is activated. The increase of P occurs in more posterior (distal) cells (Fig.2a) where the anterior border of the probe gene

expression is shifted posteriorily [6,15]. This natural consequence following the biophysical model is unexpected according to the two-waves model [7]. b) For deletions anterior to the probe gene the situation is more complicated: firstly, F decreases (because of the N reduction). Secondly, because of the force reduction the extruded DNA fiber is shortened. However, the deleted DNA region is not necessarily equal to the reduction of the extruded fiber. This is due to the variable local properties (elastic, electric etc) of Hox gene regions along the DNA fiber. A detailed analysis has been performed elsewhere [17]. Some particular cases correspond to the impossible to anticipate expression variations of the probe genes [8]. Furthermore, some predictions based on this analysis were formulated concerning the expressions of probe genes Hoxd11 and Hoxd13 when specific anterior gene regions are deleted [17].

It has been independently observed by Cook and collaborators that, for their activation, the genes approach a transcription factory [18, 19]. From a series of experiments this team concluded that for gene transcription it is the DNA fiber that moves toward the immobile polymerases inside the TF and not the opposite way around [18]. This conclusion supports the pulling forces hypothesis while the existing results of Hox gene manipulations are successfully reproduced by the biophysical model. Nonetheless additional experiments are necessary to further confirm this model. Such experiments have been proposed elsewhere [6, 17].

An important difference between the biophysical model and the biomolecular two-waves model is related to their scale difference. Whereas the two-wave model deals with genetic phenomena referring strictly to the sub-nuclear domain, the biophysical model is multiscalar incorporating both the chromosome microscale dimension (factor N) and the multicellular macroscale domain reflected in factor P of Eq.1. As a result, the two-waves model can refer to experiments involving only genetic manipulations. In contrast, the biophysical model can describe not only genetic engineering interventions but, via the P-factor, it reproduces successfully the results from the vast area of (extra)cellular signaling experiments in the morphogenetic field (see next Section).

Macroscale Regulation

In eq.1, the P-factor transfers the positional information from the macroscale dimension of the morphogenetic field to the microscale of the gene cluster. At the level of the morphogenetic field a set of signaling molecules (RA, SHH, FGFs...) have been found to regulate and control *Hox* gene expression in development [20-22]. Growing evidence indicates that graded signaling along the three limb axes control the pattern of the developing limb [22]. In particular, an FGF gradient is involved in the pattern formation along the proximal-distal axis (Fig.2).

Several mechanisms such as graded morphogens or time spent in the progress zone have been proposed for the positional signaling in the limb [22, 23]. Recent findings support a mechanism according to which a morphogen source is located at the distal tip of the bud and the produced signal (FGF) at the apical ectodermal ridge (AER) is spread mainly by passive diffusion along the distal-proximal dimension [23, 24].

A one-dimensional mathematical model was formulated to estimate the progressive spreading in space and time of a morphogen concentration due to diffusion. In this analysis it was further assumed that the morphogen is decomposed by first order chemical kinetics while the morphogen source is incorporated in the boundary condition at the margin of the morphogenetic field [24]. The diffusion-plus-degradation equation for the morphogen concentration $C(x,t)$ is:

$$\partial C/\partial t = D\, \partial^2 C/\partial x^2 - KC \quad (2)$$

where D and k are the diffusion and decomposition constants respectively. Eq.2 was solved exactly for all spatial points x and time t [24]. In the case the morphogenetic field is semiinfinite ($0 \leq x \leq \infty$) we assume that at time t = 0, C = 0 for the whole field. The morphogen source is located at the point x = 0 where the concentration is kept constant at the level Co for all time. The explicit solution of Eq.2 tends asymptotically to a steady-state which is a simple exponential function [24]:

$$C(x) = C_o e^{-(\sqrt{k/D})x} \quad (3)$$

Such decreasing exponential functions represent the morphogenetic gradients depicted in Fig. 2a,b.

It is instructive to consider the experimental case of implantation in the chick limb bud of beads soaked in FGF4. These experiments can be well described by a morphogen gradient model where the boundaries of Hoxa gene expressions are determined by morphogen thresholds along the proximal-distal axis of the bud [23] (Fig.2). Around the distally implanted FGF bead the Hoxa13 expression unexpectedly switches off. This surprising finding can be explained by assuming that every gene is activated by a morphogen concentration in a range between an upper and a lower threshold [23] (Fig.2b). For example, in Fig. 2b and for the normal gradient (1), Hoxa13 is activated in

an area (0A) of the bud where the morphogen concentration exceeds T_l and is lower than T_u. When the morphogen concentration is either lower than Tl or exceeds Tu the gene expression fades out (Fig.2b). In an experiment where a bead soaked in FGF is transplanted distally in the bud, the normal gradient (1) increases to (2) and the Hoxa13 expression disappears around the origin since in this bud area, according to gradient (2), the concentration exceeds T_u.
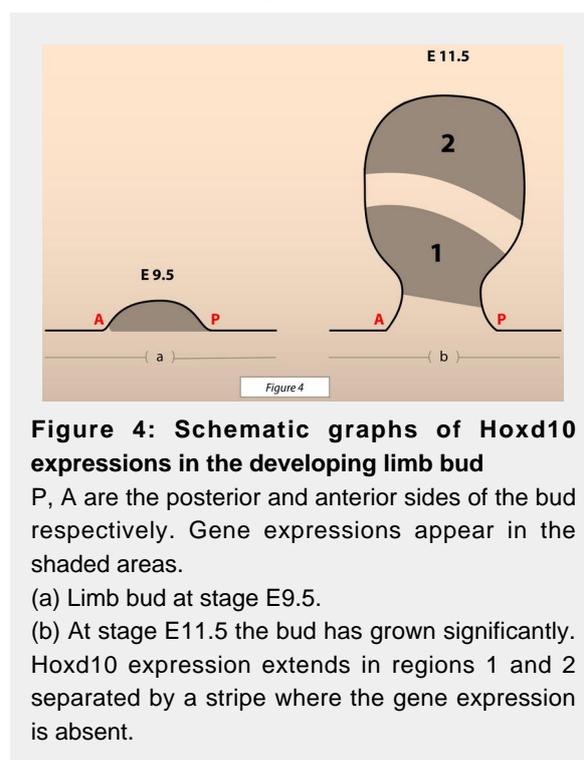
If a single molecular signal were the only regulator of Hox expression, an FGF4 increase would cause an ectopic expression proximally. However, such an ectopic expression was not observed and this indicates that FGF4 is necessary but not sufficient for Hoxa expression [23]. This is in agreement with the belief that Hox gene expression is a multifactorial process where several signaling mechanisms are involved [21, 25, 26]. The biophysical model is compatible with the gradient model and, furthermore, it explains the origin of the range of gene expressions between upper and lower morphogen thresholds: an excessive force F (due to a big P-value in Eq.1) shifts the genes outside the TF domain where the genes are switched off. The force F in Eq.1 depends on both the TF microdomain and the range between thresholds of the macroscale morphogen gradient (Fig,2b). This constitutes a multiscale interrelation which is a characteristic feature of Systems Biology (27).

In the early stages of development the biophysical model is associated with an entanglement of space and time [15]: early activation comes along with anterior expression whereas posterior expression is coupled with late gene activation. Such an interlocking was observed in the Hoxb cluster of the chick embryo [28]. At later stages the space-time interlocking relaxes [8]. Note that temporal collinearity is fundamental for understanding Hox cluster organization in evolutionary terms [29]. Furthermore, temporal collinearity is related to the ordered and compacted Hox clustering of vertebrates. These evolutionary implications have been recently examined in the framework of the biophysical model [30]. Stochastic modeling used to demonstrate that white noise perturbing Hox gene expression, favors the relatively compact clustering as a tool to develop more complex body structures. The mouse and amphioxus genomes were used for this modeling [30].

Generation of Complex patterns

As stressed above, the biophysical model is applicable

to the early stages of development generating simply connected areas of gene expression. At later stages, secondary mechanisms interfere and complicated spatial shapes of expression emerge, leading to the final digit forms. At this point it is worth analyzing a mechanism creating pattern complexity that could supplement the transcription process. Consider the recent experiment on the action of Hoxd gene enhancers located in the telomeric desert [11]. At stage E9.5 the expression of Hoxd10 is simply connected, extending almost in the entire limb bud (Fig.4a). At a later stage E11.5 the bud has grown significantly and the expression of Hoxd10 is split into two domains separated by a 'ditch' zone where the gene expression is absent (Fig.4b). This expression absence in the intermediate region separates the Hoxd10 expression in the proximal and distal regions of the bud. In the framework of the biophysical model one could think of a secondary mechanism for gene silencing in the ditch stripe.

Figure 4: Schematic graphs of Hoxd10 expressions in the developing limb bud
P, A are the posterior and anterior sides of the bud respectively. Gene expressions appear in the shaded areas.
(a) Limb bud at stage E9.5.
(b) At stage E11.5 the bud has grown significantly. Hoxd10 expression extends in regions 1 and 2 separated by a stripe where the gene expression is absent.

In a distant cell the pulling force on the DNA fiber is stronger than the force in a proximal cell. Accordingly, the extruded DNA fiber in a distal cell is longer than the extruded fiber of a proximal cell. In a proximal cell, the fiber segment [Hoxd10 –Hoxd12] is embedded inside the transcription factory domain TF (Fig.5a). The force is not strong enough to pull Hoxd13 inside the TF. For a cell in the intermediate region, the pulling force is stronger and a segment of the longer meandering DNA fiber can 'crawl' outside the TF domain (Fig.5b). As a result, Hoxd10 and Hoxd11 are

switched off in agreement with their observed expression suppression. The stronger force pulls Hoxd13 inside the TF and this gene is therefore expressed. For a distant cell the pulling local forces increase even more and the fiber may be bent further and compacted inside the TF domain (Fig.5c). The above explanation should be compared to the description incorporated in a 'regulatory model for Hoxd gene collinearity' [11].
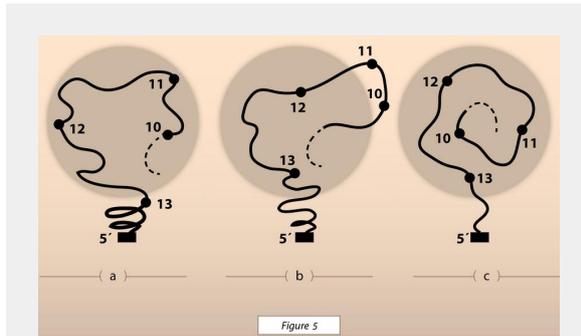
Figure 5

Figure 5: The meandering DNA fiber of the posterior Hoxd genes at stage E11.5

(a) In proximal cells the relatively weak pulling forces leave Hoxd13 outside the TF domain (grey disc). Hoxd10-Hoxd12 are expressed in the proximal region of the bud.

(b) In intermediate cells (higher P-values), the pulling forces increase decondensing the DNA fiber and shifting Hoxd13 inside the TF domain. However, a fiber segment entailing Hoxd10 and Hoxd11 is pulled outside the TF region where these gene expressions fade out

(c) In distal (posterior) cells, the even stronger forces bend further the DNA fiber and compact all Hoxd genes inside the TF domain

Predictions and Conclusions

1. Besides the morphogen diffusion mechanism presented above, an alternative relay signaling has been observed in many other cases of embryonic development. See for instance the patterning along the dorsal-ventral axis in echinoderms [31]. It is therefore important to distinguish which long range signaling is applied in each case. The relay mechanism implies a constant endogenous time needed for a response to the received signal and for its further transmission. Therefore the velocity of long range signal propagation is constant- independent of the source strength [24]. In contrast the velocity of a diffused signal varies and, for a given time interval, the distance covered by the signal depends on the strength of the source [24]. Accordingly, the observed

Hoxa13 expressions in the limb bud after a constant time interval and for variable strengths of the FGF source clearly favor the morphogen diffusion model [23].

2. Following the argumentation of the previous Section, the posterior region to probe Hoxd10 gene consists of the fiber segment [Hoxd13 –Hoxd12-Hoxd11]. According to the two-waves model, the deletion of this posterior DNA segment (or part thereof) will not affect the Hoxd10 expression since, the distance of Hoxd10 from the telomeric enhancers will not be affected. Therefore all Hoxd10 expressions presented in Fig.3 of ref. [11] will remain unchanged in mutant limbs where some posterior region is deleted. In contrast, the biophysical model predicts that any such posterior deletion will cause a posterior shift of the Hoxd10 expression as explained in Section 2. Consequently in Fig.4a the Hoxd10 expression will fade away anteriorily while in Fig.4b the expression will be limited in region 2. Obviously the two models differ in their predictions. It would be interesting to experimentally test these diverging expectations.

3. In the framework of the two-waves model it remains to be demonstrated whether the gene translocation 'underlies collinear activation or is consequence of it' [10]. The biophysical model answers this dilemma by putting it in a different context: Hox gene collinearity depends on two inseparable and indispensable prerequisites: firstly on the ordered gene location in the cluster and secondly on the step by step gene translocation. If either of these two requirements is missing collinearity is impossible

4. The action of telomeric and centromeric landscapes are important for the integrated process of Hoxd gene transcription (Fig.1a). However, the underlying cause of Hoxd activation is the timely gene shift while the landscape effects are a syndrome depending on the initial cause. In the spirit of synthesis, a model in two steps can be formulated for the explanation of Hox gene collinearity: 1) physical forces move stepwise the Hox genes at the appropriate position at the right time and 2) subsequently, the biochemical machinery of gene regulators (enhancers, repressors etc) accomplishes gene transcription. It is amazing that a simple mathematical expression like Eq.1 for the pulling forces could meet the 'major challenge to integrate Genetics with Molecular and Cellular Biology' [32].

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Abbreviations

ICD: interchromosome domain

CT: chromatin territory

TF: transcription factory

Addendum

After the submission of the present work, an article appeared in eLIFE written by D. Duboule and coworkers (D. Noordermeer et al. eLIFE 2014;3:e02557. DOI: 10.7554/eLife.02557). In this article the 3D chromatin architecture in the locus of the HoxD cluster is analyzed. In addition experiments are proposed at the cellular level (e.g. RA treatment) in order to explore the involvement of physical forces in the collinear gene activation. Note that such an experiment with implanted FGF4 beads in the limb bud is presented in detail here in Section 3. Accordingly, the generated physical forces of the biophysical model can naturally explain the surprising disappearance of the Hoxa13 expression around the inserted bead.