An enhancer-titration effect induces digit-specific regulatory alleles of the HoxD cluster

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Abstract

Mice carrying transgenes targeted upstream the HoxD cluster display abnormal digits, with alterations resembling those obtained with loss of functions of Hoxd genes. Because the HoxD cluster remained entirely untouched by the insertional events, we investigated whether these phenotypes were induced by regulatory modifications at a distance. We report here that these targeted relocations behaved as hypomorphic alleles of the distantly located gene Hoxd13 and showed that posterior Hox genes located in cis with the integration site were down-regulated. Genetic analyses suggested that this down-regulation resulted from the titration of the activity of a remote located enhancer sequence. These results indicate that the transcriptional efficiency of Hoxd genes in digits could be modulated by the presence of other, unrelated, promoters, within the regulatory landscape of this enhancer. Modifications in these latter transcription units may thus impact upon digit morphology, through misregulation of Hoxd genes, thus illustrating the “buffering effect” that such a global regulatory element can exert upon a short genomic interval.

Keywords: Gene transposition; Enhancer modulation; Distal limb

Introduction

The development of the vertebrate limb requires complex gene regulatory interactions that direct its characteristic outgrowth and morphological patterning (Dudley and Tabin, 2000; Vogt and Duboule, 1999). Transcription factors encoded by the Hox gene family are key players in this process, as shown by combined gene inactivation experiments, which led to partial limb deficits. For example, the absence of both Hoxd11 and Hoxa11 functions induced a severe agenesis of the zeugopode (forearms and forelegs) (Davis et al., 1995), whereas fetuses lacking both Hoxa13 and Hoxd13 functions displayed no autopod (hands and feet) (Fromental-Ramain et al., 1996; Kondo et al., 1997). It is therefore of utmost importance that these genes be correctly regulated during fetal development.

In birds and mammals, the four contiguous genes located at the 5′ extremity of the HoxD cluster (from Hoxd10 to Hoxd13) are expressed in the developing autopod, i.e., in the presumptive domain for digit development (Dollé et al., 1989; Dollé 1993; Nelson et al., 1996). The similarity of their expression profiles suggested the existence of a single, shared enhancer sequence which would control the transcription of these four genes in distal limb cells. This proposal was subsequently validated by the localization of this global regulatory sequence, about 200 kb upstream the HoxD cluster (Spitz et al., submitted for publication). Furthermore, experiments involving a scanning deletion and duplication analysis of these genes revealed that the enhancer had little specificity for Hoxd gene promoters. Variations in the number of target promoters were accompanied by regulatory re-allocations, the enhancer effect being re-
distributed among the novel configuration of promoters (Kmita et al., 2002a). In particular, the Hoxd9 promoter, which was normally barely active in distal cells in contrast to those of Hoxd10 to Hoxd13, was activated there whenever these latter loci were deleted. This suggested that, in the normal situation, the weak expression of Hoxd9 in digits was not due to its inability to respond to the appropriate enhancer but, instead, to a titration effect of more 5′ located promoters such as those of Hoxd13 or Hoxd12. In the presence of these latter loci, the enhancer was mostly involved in interacting with them, at the expense of those transcription units located at a more remote position such as Hoxd9 (Kmita et al., 2002a).

These results indicated that expression of Hoxd13 to Hoxd10 in developing digits was the result of a general regulatory equilibrium between several transcription units. This balance did not only involve Hox target promoters, but also, on a larger scale, transcription units unrelated to Hox genes since at least one other locus was found to respond to this regulation within the same genomic landscape. The Evx2 gene is located about 8 kb upstream Hoxd13, and its expression in developing digits is identical to that of this latter gene (Dollé et al., 1994). Interestingly, while the product of this gene may not be, by itself, of critical importance for digit development (Hérault et al., 1996), the mere presence of this transcription unit likely shapes the proper development of these structures, through the involvement in the above mentioned equilibrium. In the absence of upstream promoters, one might anticipate the response of Hoxd genes to the enhancer to be different; hence, it is possible that modifications in a gene located far away from the cluster might readily impact upon digit morphology, through transcriptional modifications of Hoxd genes.

If the overall outcome of the digit enhancer regulation depends upon the number and distribution of promoters within a large genomic landscape, one should be able to modify this outcome by introducing supernumerary promoters. In this respect, engineered duplications of Hoxd loci, through targeted meiotic recombination (Kmita et al., 2002a), revealed that only those promoters located at the extremity of the cluster would efficiently respond to the enhancer, due to the presence of a DNA motive that helped the enhancer to position there. However, these experiments did not address the possible effect, on this regulatory equilibrium, of introducing a supernumerary promoter between the enhancer and the HoxD cluster, nor did they investigate the impact of a non-Hox, genuine TATAA box promoter, when recombined within this landscape. These latter questions are of interest both to understand the mechanism behind the action of this enhancer and its specificity of interaction as well as to trace back how, in the course of our evolution, such a regulatory equilibrium was reached.

To investigate this issue, we analyzed a set of mouse strains, either newly produced or that had been previously engineered, by targeting a particular transgene immediately upstream of, or at some distance from, the HoxD cluster. While some of these reporter transgene relocations were previously used to study another aspect of Hox gene regulation (Kondo and Duboule, 1999), they proved to be ideal tools to assess for a potential titration effect, as they had been inserted right in between the enhancer sequence and target Hox promoters. Because this reporter transgene contained two different promoters, we could also evaluate which of the Hox or non-Hox promoter was the most efficient in competing with native Hox promoters. Here, we show that positioning promoters between the digit enhancer and its Hox target(s) loci induced a strong decrease in the response of the native Hox promoters, suggesting that supernumerary promoters can titrate out the activity of the enhancer. We further demonstrate that this titration effect can also be triggered by a non-Hox promoter, indicating that whichever promoter is present within this digit regulatory landscape, it will fall under the control of this global enhancer sequence. The impact of this observation on our current views of Hox gene regulation is discussed.

**Materials and methods**

**Mouse lines and skeletal analysis**

The generation of the rel0, relI, and relII lines was previously described (Kondo and Duboule, 1999). For relIII, the same Hoxd9lacZ transgene was cloned, together with the PGKneo resistance cassette, into the KpnI site lying 10,853 bp upstream of the insertion site used for relII (phage AE3-5). Since rel homozygous males were sterile, stocks were kept by crossing homozygous females against heterozygous males. The Hoxd13lacZ allele used for complementation analysis was described in Kmita et al. (2000b). For the analysis of posterior Hoxd gene expression in mutant embryos, we crossed relIII against the following partial deletions of the complex: Hoxd13, TgHd11Del3 (Zakany and Duboule, 1996); Hoxd12, TgHd11Del17 (Zakany and Duboule, 1999); Hoxd11 and Hoxd10, TgHb1Del17 (Kmita et al., 2000a). Adult mice were processed for skeletal preparations according to Inouye (1976).

**Genotyping, in situ hybridization, and β-galactosidase staining**

Juvenile mice and embryos were typed either by PCR or by Southern blot on tail or placental DNA. In situ hybridizations were performed on whole-mount embryos according to the established procedures and using the conventional probes (Evx2, Hérault et al., 1996; Hoxd13, Kmita et al., 2000a; Hoxd12, Hoxd11, and Hoxd10, Kmita et al., 2000b). Expression from the Hoxd9lacZ transgene was monitored by standard detection of β-galactosidase activity.
Results

Transgene relocations upstream the HoxD cluster

To monitor the regulatory controls acting in the course of HoxD gene activation, we embarked on a systematic transgene insertion, upstream the HoxD cluster. We first engineered a reporter transgene consisting of the Hoxd9 gene, along with both 5' and 3' flanking regions, containing lacZ reporter sequences inserted in-frame. This transgene was fused to a selectable cassette consisting of the neomycin gene driven by the housekeeping PGK promoter, flanked by two loxP sites, to allow for subsequent excision of this cassette. The entire construct was flanked by genomic sequences necessary for homologous recombination in ES cells, at different locations upstream of the cluster. In a first attempt, it was positioned between the Evx2 and Hoxd13 genes, right at the 5' end of the cluster (rel0). A second site for recombination was selected ca. 10 kb upstream rel0 (rell) and a third site (relIII), again separated from relI by a ca. 15-kb large DNA interval. The production of mice carrying these three targeted insertions was reported in Kondo and Duboule (1999).

Due to the presence of interesting regulatory features observed in these configurations, we wanted to extend this series, i.e., to have the transgene inserted at a more remote distance from the cluster. Consequently, we engineered another relocation, whereby the same reporter DNA was recombined 40 kb upstream of the start codon of Hoxd13. The required homologous arms were isolated from lambda.
This conblastocyst injection, allowed us to produce mice carrying Duboule, 1999), and recombination in ES cells, followed by clones, obtained through a walk over this region (Kondo and Hoxd, 1999). Fig. 3. Expression of posterior Hoxd genes was down-regulated in digits of rel mutant embryos. Ventral views of dissected forelimbs from 11.5 dpc embryos. Schemes at the top and bottom represent a wild-type and a relIII mutant embryos, respectively. (A,C,E,G) Expression of posterior Hoxd genes in control +/+Df[HoxD] embryos. (B,D,F,H) Expression of the same genes from relIII/Df[HoxD] mutant embryos. Note that the autopod signal from relIII mutant embryos was clearly weaker, as compared to controls. Both Hoxd11 (E,F) and Hoxd10 (G,H) were prominently expressed in a more proximal, forearm region. In rel mutants, the ratio of distal over proximal signals was much lower.

Fig. 4. Both Hoxd9lacZ and PGKneo transgenes responded to the distal limb enhancer and contributed to the rel mutant phenotype. (A) Left: Scheme illustrating the relII configuration. The middle panels show the expression from either the Hoxd9lacZ promoter (top) as assessed by β-galactosidase staining, or the PGKneo promoter (bottom) using an antisense RNA probe. On the right is shown a forelimb skeletal preparation from a relII/Hoxd13lacZ trans-heterozygous animal. P2 was lost in digit II (arrow) and P1 was fused to P2 in digit III (arrowhead). A remnant of P2 was detected in digit V (asterisk). (B) Left: scheme of the relIIneo configuration obtained after deletion of the PGKneo transgene using the Cre recombinase treatment. No significant change was observed in the expression from the Hoxd9lacZ promoter (lacZ). The skeleton of relIIneo-Hoxd13lacZ (right) displayed a less severe phenotype, with a detectable P2 in digit II, though with a defective joint to P1. In the middle bottom panel, the expression from the endogenous Hoxd9 promoter in a wild-type embryo is shown to document the expression in a proximal domain, whereas signal was absent from the distal domain. The embryos are 11.5 days old and hemizygous for the transgene unless indicated.

rel mutations are allelic to Hoxd13

For complementation analyses, we used a loss-of-function allele of Hoxd13, containing lacZ sequences inserted into the first exon (Hoxd13lacZ) (Kmita et al., 2000b). Animals heterozygous for such a mutation showed no detectable alteration in limbs, in contrast to the homozygous specimen that displayed the well-characterized Hoxd13 phenotype (Dolle et al., 1993). On the other hand, animals

clones, obtained through a walk over this region (Kondo and Duboule, 1999), and recombination in ES cells, followed by blastocyst injection, allowed us to produce mice carrying this configuration. As for previous relocations, these animals (relIII) could be maintained as a line, by crossing homozygous females with heterozygous males. Homozygous males were sterile, though.

Relocation (rel) mice display ectrodactyly

Unexpectedly, we found that mice homozygous for the various transgene relocations displayed a limb phenotype with full penetrance. This phenotype was similar in all four rel mutant stocks and consisted of a significant shortening of the second and fifth digits in the forelimbs, as well as a thickening of the anterior hindpaw, at the basis of the first digit. A detailed analysis of adult skeletal preparations revealed that mutant forelimbs had a robust shortening of the second phalanges (P2) of digits II and V (Fig. 1). In digit V, this phalange was even lost, in some cases, whereas in digit II, the joint between P2 and P1 was frequently lost, or incompletely developed. In addition, we often observed the appearance of a supernumerary postaxial bony element. In rel mutant hindlimbs, the first metatarsal bone was abnormally thicker and shorter in size (Fig. 2D). A comparative analysis of homozygous animals of different rel stocks indicated a tendency of rel0 mutants to display a weaker phenotype than that of other, more upstream-located insertions sites.
hemizygous for the relIII condition were also devoid of alterations, unlike animals carrying two copies of the recombined relIII transgene. We crossed these two strains and mice carrying both mutant loci in trans displayed clear abnormalities in both fore- and hindlimbs (Fig. 2), resembling the Hoxd13 phenotype, though with a less severe aspect.

All animals of this trans-hetero/hemizygous condition had virtually lost their P2 in digit II, and the majority of specimen no longer had a P2 in digit V (Fig. 2E and F). The joint between P1 and P2, in digit III, was also lacking in some animals. A supernumerary postaxial bony element was scored in all analyzed forelimb skeletons, as for genuine Hoxd13 phenotype (not shown). In hindlimbs, the first metatarsal was dysmorphic, with an aberrantly thick and shorter aspect, and was occasionally fused to P1. Consequently, trans-complementation was not observed between the two mutations, suggesting that they were allelic to each other. This complementation analysis was extended to other rel mice, and all mice carrying a relocated transgene showed a similar inability to complement the Hoxd13 heterozygous loss-of-function allele, as in the case of relIII (Figs. 2, 4, and 6). From these results, we concluded that, even though the insertions were far away from the Hoxd13 transcription unit, they could all be genetically considered as hypomorphic alleles of this gene.

Relocations are regulatory alleles of Hoxd13

To understand this non-trans-complementation effect, we looked at the expression of the resident Hoxd genes, using in situ hybridization. We used a set of deficiencies covering either the posterior part of the cluster, or most of the HoxD complex, such that expression of Hoxd genes could be assessed from the chromosome carrying the recombined transgene in cis. We first assessed the expression of Hoxd genes in relIII mice and a comparison between animal heterozygous for a deficiency (Df(HoxD)/+) covering seven genes of the HoxD cluster (Zakany and Duboule, 1999) and littermate embryos carrying the same deficiency, but with the relIII allele on the other chromosome (Df(HoxD)/relIII), at day 11.5, revealed a striking decrease in the level of steady-state RNA, in embryos carrying the relIII allele (Fig. 3). This decrease in signal intensity was not only scored for Hoxd13, but also for the three other genes that are normally expressed in developing digits, i.e., Hoxd12, Hoxd11, and Hoxd10. In these latter two cases, the presence of the “forearm domain” could be used as an internal control to better visualize the importance of the decrease in the amount of transcripts in the digit domain (Fig. 3).

Comparable expression studies were carried out with other relocation alleles, including rel0, and, in all cases, a decrease in transcript level was readily detected in the digits of those Hoxd genes located in cis with the inserted transgene (not shown). Such a down-regulation in the transcription of this series of genes precisely accounted for the observed phenotype, which thus resulted from a combined weakening of Hoxd gene transcription in digits. Consequently, rel mutations are allelic to the posterior part of the HoxD cluster as a whole, rather than to Hoxd13 alone. The insertion of the transgene upstream of the cluster induced a concomitant decrease in the transcription of a set of Hox genes located as far as 40 kb away. Because the relocated transgenes were positioned in between the digit enhancer and its target promoters, such a transcriptional down-regulation could reflect either an interference with Hoxd promoters, created by the proximity, or a titration effect of the transgenic construct; these additional promoters interact with the enhancer at the expense of the genuine targets.

Promoter interference versus enhancer titration

We did not favor the former hypothesis, mostly because the observed effect was the same, regardless of which insertion site was chosen for the reporter transgene, from 5 to 40 kb upstream of the cluster. Second, the regulatory effect was observed exclusively in developing digits, and no other expression feature of any of these genes appeared affected by the transgene relocation. This was particularly striking with the forearm domain of both Hoxd10 and Hoxd11, which remained unchanged at the same time the digit domain was seriously affected (Fig. 3). Also, because all relocation alleles displayed a very similar phenotype, even though their insertion sites were different, it is unlikely that the effect was due to the direct disruption of a regulatory sequence necessary for normal posterior Hoxd gene function in developing digits.

Consequently, the observed transcriptional down-regulation caused by the relocations likely reflected a titration effect of the enhancer function, by the relocated piece of DNA. In the presence of the transgene, the enhancer was partly rerouted toward these ectopic promoters, at the expense of the usual target loci. In such a case, one would expect the reporter transgene to be expressed itself in developing digits, with the specificity of the normal target genes. Therefore, we looked at the expression of Hoxd9/lacZ, a gene that is normally barely transcribed in this domain (Fig. 4), but instead, in more proximal cells of the limb bud. β-Galactosidase staining revealed significant expression of the reporter transgene, with the exact same specificity as posterior Hoxd genes (Fig. 4), suggesting that the Hoxd9 promoter was indeed able to interact with the digit enhancer and to titrate out its activity.

Titration effect

Because the PGK promoter was also included in the relocated transgene, as part of the selection cassette, we could check whether the promoter of a gene that is unrelated to Hox genes would respond to this enhancer as well. We used a neomycin antisense RNA probe for in situ hybrid-
izations and detected a strong signal in the distal aspect of the limb bud, much as for Hoxd13 or Hoxd12 (Fig. 4), demonstrating that the enhancer sequence would also control the activity of the PGK promoter in distal limb cells. Consequently, the enhancer sequence not only could work with GC-rich promoters, as those of all Hoxd genes, but also with TATAA box containing promoters such as that of the PGK gene, even when located outside the cluster, i.e., far from genuine target promoters.

As both promoters were shown to respond to this regulation, we wanted to assess whether both also contributed to the titration effect seen on Hoxd target promoters. We used the two loxP sites flanking the PGKneo selection cassette to excise this piece of DNA in vitro through Cre recombinase treatment, to produce a line of mice carrying the Hoxd9 transgene while lacking the selection cassette (relH11002−). In this way, only the Hoxd9 promoter was left, which allowed us to directly evaluate the involvement of these two promoters in the titration effect. As homozygous relH11002− mutant mice did not survive to adulthood, we generated trans-heterozygous animals carrying both the relH11002− and the Hoxd13lacZ alleles on different chromosomes. The digit phenotype was analyzed either in the presence or in the absence of the PGKneo cassette in the relIl allele. In the absence of the neomycin cassette (relH11002−/Hoxd13lacZ), the analyzed specimen displayed obvious skeletal abnormalities, most evident in forelimbs. They showed a nearly complete loss of P2 in digit V and a frequent lack (or incomplete development) of the P1 to P2 joint in digit II (Fig. 4).

However, even when the articulation was lost, P2 was always recognizable in the latter digit. In marked contrast, the phenotype was distinctly stronger whenever the PGKneo fragment was present (relH11002−/Hoxd13lacZ animals). In this case, P2 was always absent from digit II, or reduced to a remnant that was fused to P1, and the P1 to P2 joint was lost from digit III in all samples observed. Other aspects clearly confirmed a more severe condition, in the presence of the PGK promoter, than with the Hoxd9 transgene alone, even though this latter construct clearly induced digital alterations on its own.

From these results, we concluded that both Hoxd9 and the PGK promoters contributed to the titration effect of the digit enhancer. The severity of the phenotypes seemed to be additive, as deletion of the PGK promoter decreased the titration effect. When the PGK promoter was excised, the enhancer was rerouted toward the Hoxd cluster, rather than toward the Hoxd9 relocated promoter, in which case the phenotype would have been the same in both conditions. We checked this issue by looking at the expression of the Hoxd9/lacZ either in the presence or in the absence of the PGK cassette and found no significant difference (Fig. 4), which confirmed that the enhancer sequence most likely interacted with both relocated promoters separately, as opposed to either a competition effect or a mechanism whereby one promoter would favor, or trigger, the interaction between the enhancer and the neighboring promoter.

### Position-dependent titration effect

We next investigated whether this titration effect was dependent upon the position of the supernumerary promoters, in particular, relative to the enhancer sequence and the native target Evx2 and Hoxd promoters. We compared the expression of the Evx2 gene in transgenic lines carrying the relocated transgene between either Evx2 and the enhancer (e.g. in the relIII allele) or downstream Evx2 using the rel0 allele. In this latter configuration, the topographic relationship between Evx2 and the enhancer remained unchanged (see Fig. 1).

Interestingly, a clear-cut difference was observed in the expression of Evx2, depending upon whether the relocated transgene was positioned upstream or downstream of it. When relocated upstream (i.e., in the relI to relIII alleles), Evx2 expression was severely down-regulated (Fig. 5), as was the case for posterior Hoxd genes (Fig. 3). However, the relocation of the transgene between Evx2 and Hoxd13, while affecting this latter transcription unit, had little impact on Evx2, suggesting that titration of the enhancer effect was position-dependent, with a directional component. Likewise, β-galactosidase staining revealed a slightly weaker staining of the reporter transgene when integrated at the rel0 position, i.e., when Evx2 was now in between the transgene and the enhancer, than when present between Evx2 and the enhancer, a position at which Hoxd9lacZ was strongly transcribed.

Accordingly, phenotypic analyses of trans-heterozygous combinations between various relocations and the Hoxd13lacZ allele revealed that rel0 mutants displayed alterations weaker than those induced by the other relocations. This was most evident when monitoring the presence of the second phalange of forelimb digit II, which was always present in rel0 mutants, whereas it was systematically lost in all specimens examined from the other relocation mutants (Fig. 6).

### Discussion

The four most 5′ located genes of the HoxD cluster are expressed similarly in the presumptive domain for digits, during mammalian development. Previous work has shown that this is due to the presence of a single remote enhancer sequence, which controls all four promoters (Spitz et al., submitted for publication). Experiments involving modifications, in complexo, of either the number, the position, or the order of these genes have revealed that the enhancer–promoter interaction was poorly specific, as regulatory reallocations were observed whenever the number of promoters was changed. For example, deletion of the Hoxd13 locus induced a strong up-regulation of the Hoxd12 gene in digits only, likely due to enhancer rerouting toward this latter promoter (Kmita et al., 2002a). Therefore, the overall expression profiles of these genes depend upon a mechanism...
of internal promoter competition, biased by the presence of DNA sequence that helps targeting the enhancer toward one end of the cluster.

To look at global gene regulation at the HoxD cluster, we had previously produced a set of alleles carrying a reporter transgene targeted at different positions upstream of the complex. In this work, we have completed this series of targeted transgene insertions, such that a total of four lines were produced, referred to as relocations (rel0 to relIII), rel0 being an insertion immediately upstream of Hoxd13 (the nearest one), whereas relIII (the more further away from Hoxd13) was located some 40 kb upstream. Interestingly, all these lines of mice displayed an abnormal phenotype in their digits, a feature that we were unable to account for at first. In this article, we use genetics to show that these alterations are allelic to Hoxd genes. We further demonstrate that every relocation constitutes a regulatory allele of the HoxD cluster, which specifically affects the digit domain: a tissue-specific regulatory allele. Finally we show that the alterations are induced by a combined loss-of-function of several Hoxd genes in digits, as a consequence of the titration of enhancer activity by the introduction of foreign promoters within this regulatory landscape.

**Enhancer titration**

Complementation tests indicated that the phenotypes of mice carrying a relocated transgene were likely allelic to the HoxD cluster, even for those where the transgene was targeted about 40 kb from the cluster. This was largely confirmed by expression analyses, which clearly revealed the reasons for such digital alterations: a general down-regulation of transcription in the digit domain of Hoxd13 to Hoxd10. This observation documented the nature of the allelism between these various configurations and posterior Hoxd genes. Because the effect was scored on multiple genes, we refer to an allelism with the HoxD cluster as a whole, rather than with genes in particular.

Several mechanisms can be proposed to account for such a down-regulation, at a distance, of multiple neighboring genes. First, transgenes could have been relocated right within a limb regulatory element, inducing a loss of efficiency in the process, leading to reduced transcription. Alternatively, the insertion of a transgene may have caused a transcriptional interference between the activity of this transgene and that of nearby located genes (Eszterhas et al., 2002). Finally, relocated transgenes may have titrated out the activity of an enhancer sequence through promoter competition (Sharpe et al., 1998). We do not favor the first two hypotheses. First, it is unlikely that four different insertion sites (rel0 to relIII), located within 40 kb, would disturb one and the same regulatory module. In addition, the regulatory element responsible for expression in digits was recently mapped at quite a distance upstream of this region (Spitz et al., submitted for publication). Second, one would also expect from a transcriptional interference to become weaker from the nearest to the most distant insertion. Yet the opposite observation was made, as the phenotype of rel0 was milder than that of relIII. Furthermore, the global expression of these four posterior Hoxd genes was not affected by the relocations, except for the digit pattern, making it difficult to reconcile with the second alternative.

Instead, the various observations described in this article suggest that the down-regulation of Hoxd13 to Hoxd10 in digits was due to the presence of competing promoters, located between the target genes and the enhancer, which could titrate out the activity of this latter regulatory element. First, both the Hoxd9 and the PGK promoters were found...
functional in developing digits, as judged by lacZ and neomycin expression, respectively, showing that both transgenes were now targets of the enhancer. This was further confirmed by the partial rescue of the phenotype, after excision of the PGKneo cassette, due to a concurrent decrease in the titration effect. Therefore, we concluded that, in the presence of foreign transcription units, the enhancer interacted with these latter transgenes, at the expense of genuine target promoters located further away. Interestingly, the TATAA box-containing PGK promoter is quite different from GC-rich regions routinely found upstream of Hox genes, illustrating the somewhat loose specificity of this digit enhancer, which would likely interact with whichever promoter would be localized within its realm of action.

**Position-dependent titration**

A detailed analysis of various rel phenotypes revealed two classes of digit skeletal alterations. A rather strong effect was seen with relI, relII, and relIII, whereas rel0 induced similar phenotypic alterations but significantly and repeatedly less pronounced. Because the exact same transgene was relocated in all these experiments, we concluded that this difference in phenotypes reflected the position and/or topology of particular insertion sites, or a distance effect. In this latter view, transgenes located at a distance upstream of the cluster would be in a more favorable position to catch the activity of the enhancer sequence, for example, as a result of either a scanning mechanism, the enhancer “progressing” toward the cluster (Blackwood and Kadonaga, 1998), or a more stochastic flip-flop-like mechanism, the enhancer contacting nearby promoters with a greater frequency than more remote located targets (Dillon et al., 1997). In such a case, one would have expected relI mice to be less affected than relIII, a situation we could not demonstrate. It is nevertheless possible that subtle differences were not scored or would not impact on the morphology of digits.

Another possibility is that the transgene would similarly interact with the enhancer sequence at all insertion sites, but the down-regulation of Hoxd genes at the rel0 site would not be as important as for the other rel sites. This may be the case, in particular, when considering the situation in wild-type mice, where the enhancer appears to mostly contact the Hoxd13 locus, but also to exert an action over the closely neighboring genes due to a “leakage effect,” reflecting the inaccuracy of the process (Kmita et al., 2002a). In this view, the enhancer would contact the rel0 transgene with the exact same frequency and/or probability than the other, but the mere position of the rel0 promoters right next to that of Hoxd13 would only slightly change the efficiency of the contact with this latter gene, whereby ensuring a morphology better than those obtained with other relocations. In these latter cases, the enhancer would be trapped at a distance from the Hoxd targets. Along the same line, it is also possible that the Evx2 gene, a gene that may have a weak function in digits (Hérault et al., 1996), would contribute and rescue some of the morphological defects induced by the effect of rel0 upon Hoxd13 transcription, due to its increased transcription, when compared to other rel alleles.

These results illustrate the potential problem in assigning particular enhancer sequences to a determined (group of) gene(s). In the case described here, not only can this regulatory sequence control a whole set of promoters, of different kinds, within a particular genomic landscape, but it is the presence of every single promoter which may determine the transcriptional efficiency of the others, through a competition/titration mechanism. Modifications affecting the number of promoters, their accessibility or their topography, may thus impact upon the regulation of the entire landscape.

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