

# A nested deletion approach to generate *Cre* deleter mice with progressive *Hox* profiles

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**ABSTRACT** In mice, the *loxP/Cre* recombinase-dependent system of recombination offers powerful possibilities for engineering genetic configurations of interest. This system can also be advantageously used for conditional mutagenesis *in vivo*, whenever such an approach is required due to deleterious effects of either one mutation, or a combination thereof. Here, we report on the production of an allelic series of insertions of a *Hoxd11/Cre* fusion transgene at different positions within the *HoxD* complex, in order to produce the CRE recombinase with a 'Hox profile' progressively more extended. We used the *R26R* (R26R) reporter mouse line to functionally assess the distribution and efficiency of the CRE enzyme and discuss the usefulness of these various lines as deleter strains.

**KEY WORDS:** *Cre deleter mice, Hox D, TAMERE*

## Introduction

Mouse genetics disposes of a set of technologies, which have made this animal a valuable model for human development, physiology and diseases. Of particular interest is the *loxP/Cre* recombinase system, which allows for site- or time-specific recombination of particular target sequences, hence for a conditional approach of targeted genetic modifications (Sauer, 1998; Nagy, 2000). Using this system, it is for instance possible to activate a given transgene at the desired time and position, or alternatively, to inactivate a gene, or group of genes, only in those organs where one would like to study the phenotype. Therefore, this system helps to overcome the difficulties often created by early lethal conditions, due either to particular mutations, or, even more of a concern for the future, to the combination of various mutations.

Murine *Hox* genes provide an interesting example of the interest of this system. Mammals have 39 *Hox* genes encoding transcription factors, whose function is to organize the body plan (see e.g. Krumlauf, 1994). These genes are grouped into four large genomic clusters; *HoxA*, *B*, *C* and *D*, and loss-of-function mutations usually generate phenotypes, which however, are rarely drastic. This is due, in part, to the high level of functional redundancy that exists amongst these genes (e.g. Duboule and Morata, 1994). For instance, the inactivation of a given *Hox* gene may lead to compensatory effects either from its neighbouring genes, or from orthologous partners, i.e. genes located at the same positions on the other complexes (Rijli and Chambon, 1997; Zakany *et al.*, 1997). Therefore, it is necessary to produce combined loss-of-functions to better

evaluate the roles of these genes. This has proved to be a difficult task, as such multiple inactivations tend to decrease either the mating performance of the animals, or their fertility, such that it becomes increasingly difficult to obtain the required configuration. Furthermore, some of the alleles under scrutiny might be early lethal.

In order to study *Hox* gene regulation, we had previously targeted a *Hoxd11/lacZ* transgene encoding a fusion protein HOXD11/ $\beta$ -gal, to different positions within the *HoxD* complex. We noticed that the same fusion transgene was expressed with various profiles, which grossly corresponded to the expression profiles of the immediately neighbouring genes (van der Hoeven *et al.*, 1996; Zakany *et al.*, 1997). This observation suggested to us the possibility to produce a series of *HoxD* alleles expressing the CRE recombinase under the control of the *Hoxd11* promoter at various sites in the complex, in order to obtain mice expressing the enzyme in a progressively more extended 'Hox-like' pattern.

Here, we report the production and analysis of three such lines of mice, using a set of *loxP* site-carrying strains and our Targeted MEiotic REcombination (TAMERE) method (Hérault *et al.*, 1998). These various lines carry the same *Hoxd11/Cre* fusion transgene, but located at three different positions within *HoxD*. The first line had recombined the transgene immediately upstream *Hoxd13*, near the 5' end of the cluster. The second line carried the transgene between *Hoxd11* and *Hoxd10*, along with a deletion of the three

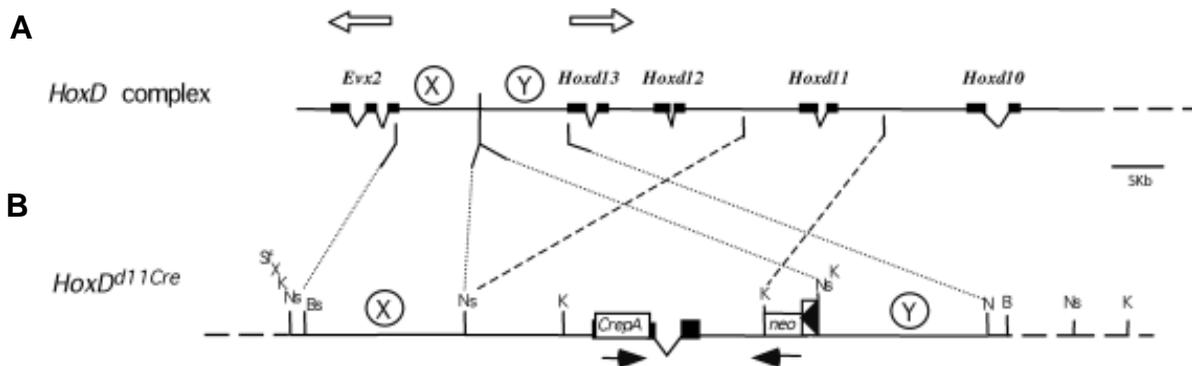
*Abbreviations used in this paper:* TAMERE, Targeted meiotic recombination.

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**Fig. 1. Targeted recombination of the *Hoxd11/Cre* fusion gene upstream the *Hoxd13* gene. (A)** The *HoxD* complex with the insertion site located between both homologous arms (X and Y). **(B)** The targeting vector was made out a large piece of DNA covering the *Hoxd11* locus, flanked by both homologous arms. The *Cre* coding sequences were introduced within the first exon of *Hoxd11* along with a polyadenylation signal (*Cre*pA). The PGKneo selection cassette, flanked by a single loxP site in 5', was introduced between the 3' end of the *Hoxd11* sequences and the 3' located homologous arm (*neo*), in the reverse transcriptional orientation (arrows). This construct was electroporated into ES cells and positive cells were used to produce targeted mice by transfer to the germ line.

posterior genes (*Hoxd13* to *Hoxd11*), whereas the third line had recombined the transgene upstream *Hoxd3*, along with a deletion of seven genes, from *Hoxd13* to *Hoxd4*. These lines were analysed by crossing them with the *R26R* reporter line (Soriano, 1999), which produces  $\beta$ -gal staining upon exposure to the CRE recombinase. As expected, the three lines gave somewhat different staining patterns. However, these patterns were not as robust and widespread as the corresponding *Hoxd11/lacZ* patterns. The reasons for this discrepancy, as well as the potential interest of these lines are discussed.

## Results and Discussion

### Construction of the CRE Expressing Alleles

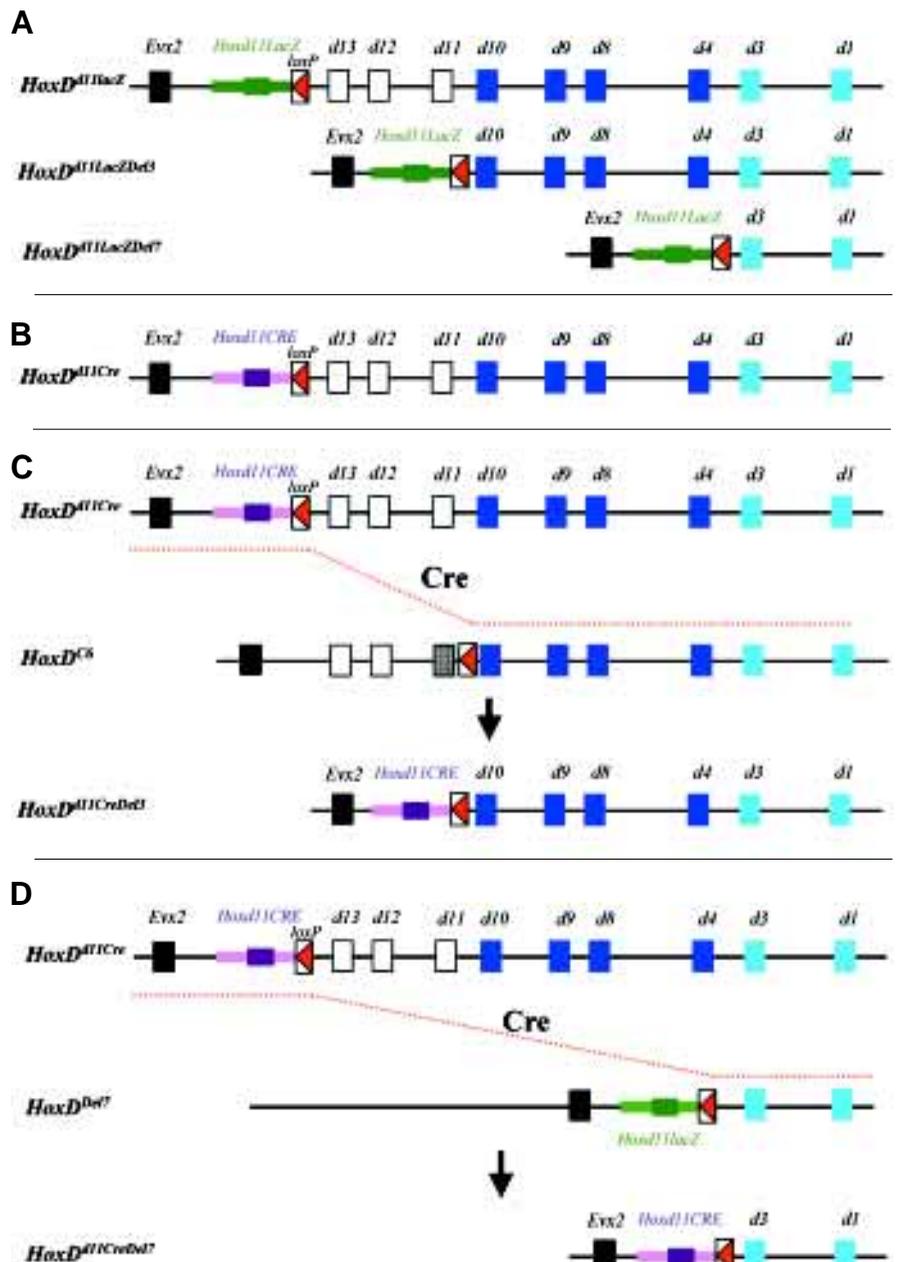
To produce the *Hoxd11/Cre* fusion gene, the CRE coding sequences were inserted in frame with *Hoxd11*, within a *SpeI* site introduced at the 5' extremity of the *Hoxd11* coding sequence. SV40 polyadenylation sequences were added in 3', replacing the part of exon I that extends from the start codon of the HOXD11 protein, until the *NotI* site. Consequently, this partial gene replacement lead to the synthesis of the CRE recombinase without any residues derived from *Hoxd11*. This was made on a rather large *Hoxd11* transgene (Gérard et al., 1993), such that transcription was under the control of *Hoxd11* regulatory sequences. In order to integrate this construct within the *HoxD* complex, we flanked the transgene with homologous arms allowing for recombination between *Hoxd13* and *Evx2*, at the 5' extremity of the cluster (Fig. 1). The recombination site was the same as the one used for the *Hoxd11/lacZ* transgene in a previous series of gene transpositions (van der Hoeven et al., 1996). This *Hoxd11/Cre* transgene was similar to the knock in of *lacZ* within *Hoxd11* (Gérard et al., 1993), except that in this latter case, the *lacZ* sequence was introduced into the *NotI* site of *Hoxd11*, generating a protein with the first 146 codons of *Hoxd11* fused to the  $\beta$ -galactosidase sequence. This *HoxD<sup>d11LacZ</sup>* configuration was subsequently used to produce further transposition upstream *Hoxd13* (Fig. 2A; *HoxD<sup>d11LacZ</sup>*, see, Zakany and Duboule, 1996; 1999).

The targeting vector was electroporated into D3 ES cells and clones with homologous recombination events were selected. After injection into mouse blastocysts, this allele was transferred

into the germ line and a mouse strain was derived (*HoxD<sup>d11Cre</sup>*). These mice carried a wild type copy of the *HoxD* complex, with, in addition, a transgene in which the *Cre* recombinase gene was under the control of the *Hoxd11* promoter. This transgene was located between *Hoxd13* and *Evx2* (Fig. 2B). The *HoxD<sup>d11Cre</sup>* mice (Fig. 2B; referred to as 'CreDel0' from now onwards) were the starting material from which we derived the two other lines of mice described below, by using targeted meiotic recombination (TAMERE; Héroult et al., 1998).

The second line of *Cre* expressing mice was generated by producing a male carrying the *CreDel0* allele, along with another allele of *HoxD* containing a *loxP* site between *Hoxd11* and *Hoxd10* (*HoxD<sup>C6</sup>*; Gérard et al., 1996; Fig. 2C). We also introduced a transgene expressing the CRE enzyme under the control of the *Sycp1* promoter, in order to express this enzyme at the time of chromosome pairing, during meiotic prophase (Vidal et al., 1998). In this way, around five percent of meiotic cells had a targeted crossing over between the two *loxP* sites, inducing the deletion of the three genes located in-between (*Hoxd13*, *Hoxd12* and *Hoxd11*; Fig. 2C, red dotted line). The corresponding *HoxD<sup>d11CreDel3</sup>* (referred to as 'CreDel3') mice were recovered from litters derived from this male. This configuration was similar to the previously reported *HoxD<sup>d11lacZDel3</sup>* allele (Fig. 2A; Zakany and Duboule, 1996), with the difference that *Cre* coding sequences were substituted to *lacZ* sequences. Unlike the *d11lacZ* transgene, the *d11Cre* transgene was designed not to be expressed as a fusion product.

For the third line of 'deleter mice', the same TAMERE method was used, but with yet another *HoxD* allele, which already lacked seven genes from the complex. This allele was produced by a *loxP/Cre* dependent deletion in *cis*, starting from the *HoxD<sup>d11lacZ</sup>* chromosome described in Fig. 2A (top; Zakany and Duboule, 1999). This *HoxD* allele was added to the *HoxD<sup>d11Cre</sup>* chromosome (Fig. 2B), along with the *Sycp1Cre* transgene and progeny of the males from this genotype were looked for the presence of the recombination event, referred to as *HoxD<sup>d11CreDel7</sup>* (Fig. 2D; 'CreDel7' from now onwards). Altogether, these three lines of *Cre* expressing mice were related to the three *lacZ* lines depicted under Fig. 1A, though with the modifications mentioned above. In all three cases, the same *Hoxd11/Cre* transgene was present, always at the same respective distance from the 5'-located gene *Evx2*, but at various



**Fig. 2. Schematics of the chromosomes used in this study. (A)** The three chromosomes show the previously produced lines with a *Hoxd11/lacZ* fusion upstream *Hoxd13*. In these lines, a single *loxP* site was left (arrowhead), without the selection cassette. Details can be found in van der Hoeven *et al.*, 1996 (*d11/lacZ*); Zakany and Duboule, 1996 (*d11/lacZDel3*) and Zakany and Duboule 1999 (*d11/lacZDel7*). **(B)** Schematic of the chromosome produced by the recombination event shown in Fig. 1. The *Hoxd11/Cre* fusion gene is positioned between the *Evx2* and *Hoxd13* genes, in the same transcriptional orientation as *Hoxd13*. A *loxP* site is left, along with the PGKneo selection cassette. **(C)** Production of the *CreDel3* allele using meiotic recombination between the *d11/Cre* chromosome (same as in B), on the one hand, and the *HoxDC<sup>6</sup>* allele, a configuration that contains a *loxP* site between *Hoxd11* and *Hoxd10* (Gérard *et al.*, 1996). Upon inter-chromosomal recombination (dashed line), the *Hoxd11/Cre* transgene is brought immediately upstream of *Hoxd10*, along with a concomitant deletion of the *Hoxd11*, *Hoxd12* and *Hoxd13* genes. This *HoxDd11CreDel3* allele is referred to as 'CreDel3' in the text. As for the chromosome shown in B, the selection cassette is still present. **(D)** Production of the *CreDel7* allele using meiotic recombination between the *d11/Cre* chromosome (same as in B), on the one hand, and the *HoxD<sup>Del7</sup>* allele, a configuration that contains a *loxP* site between *Hoxd4* and *Hoxd3* (Zakany and Duboule, 1999). Upon inter-chromosomal recombination (dashed line), the *Hoxd11/Cre* transgene is brought immediately upstream of *Hoxd3* along with a concomitant deletion of the *Hoxd4* to *Hoxd13* region. This *HoxDd11CreDel7* allele is referred to as 'CreDel7' in the text. As for B and C, the selection cassette is present in this chromosome.

positions within the *HoxD* complex, along with deletions of either three or seven genes.

#### Expression of the *Hoxd11/Cre* Recombinants

Our previous work on the above described *lacZ* alleles (Van der Hoeven *et al.*, 1996; Zakany and Duboule, 1996; 1999) revealed that the expression profile of the *Hoxd11/lacZ* transgene was progressively more extended along with the transgene being relocated anteriorly, i.e. in the 3' part of the complex. Thus, while the *HoxD<sup>d11lacZ</sup>* mice had  $\beta$ -gal staining quite similar to the endogenous *Hoxd13* pattern (Fig. 3, Del0), the *HoxD<sup>d11lacZDel3</sup>* mice displayed a much wider expression profile, resembling that of the now neighbouring gene *Hoxd11* (Fig. 3; Del3). This observation was reinforced by the analysis of the *HoxD<sup>d11lacZDel7</sup>* mice, which

showed a well distributed expression profile, reminiscent of that shown by endogenous *Hoxd3*, though with features of more posterior genes such as the expression in developing digits (Fig. 3; Del7). Therefore, we anticipated that similar *HoxD* alleles carrying *Cre* coding sequences would generate somewhat related patterns of transgene expression.

We investigated this issue by using the *ROSA26* reporter line (R26R; Soriano, 1999), a line of mice that expresses the *lacZ* gene only in the presence of a functional CRE recombinase enzyme. When present, the enzyme induces the deletion of a DNA fragment which otherwise interferes with transcription. Therefore, we produced mice trans-heterozygous for each one of the *CreDel* configurations as well as for the R26R allele, with the aim of mapping the functional domains of CRE activity in our deleter mice.

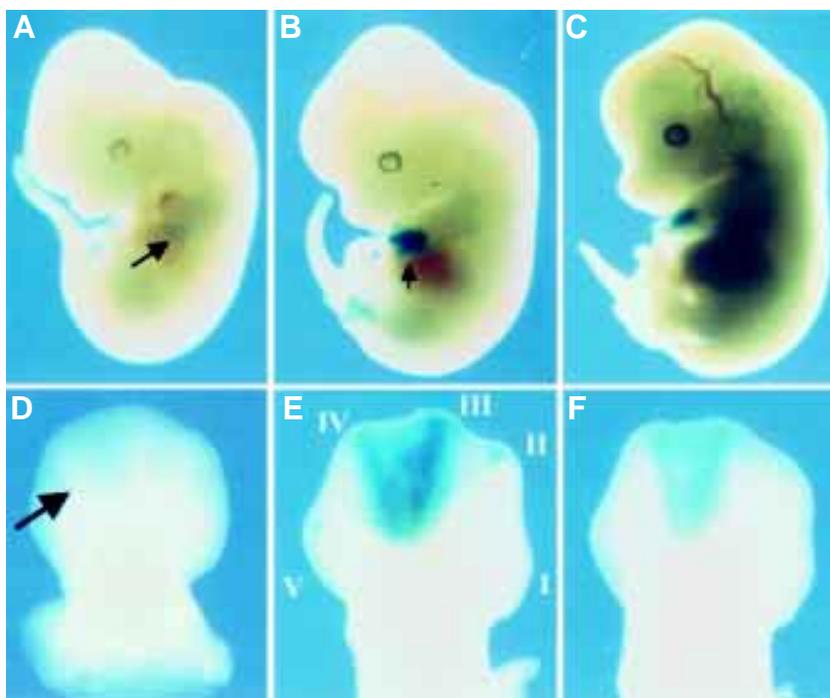
**HoxD<sup>d11Cre</sup>**

The earliest developmental stage at which  $\beta$ -gal staining was detected was in E11.5 embryos. A weak activity was scored in the posterior part of the growing forelimb buds, mostly between digits III and IV (Fig. 4 A,D; arrows). One day later, at 12.5, this expression domain was more visible and staining started to appear in digits II and V, whereas it was reinforced in digits III and IV (Fig. 4 B,E). Expression did not significantly change at day 13.5 (Fig. 4 C,F). Staining also started to appear in the developing hindlimb, with the same topological restriction than for the forelimb (data not shown).

Altogether, this staining, while reminiscent of the *lacZ* staining observed in *HoxD<sup>d11lacZ</sup>* mice (Fig. 3, Del0), was clearly less intense and not as widely distributed. Expression was detected in both developing limbs, but not in the posterior trunk (compare Fig. 3 with 4A). While this result suggested that the approach would indeed generate mice with a *Hox*-like CRE functional domain, it indicated that this particular line of deleter mice would likely not be effective enough to achieve the original purpose of this experiment. Potential reasons for this observation are discussed below.

**HoxD<sup>d11CreDel3</sup>**

The analysis of CreDel3 mice revealed a stronger and wider distribution of the staining. In E10.5 embryos, a weak signal was detected in the most distal part of the developing limbs (not shown). This staining soon increased in intensity, and E11.5 old foetuses displayed a visible but restricted staining in the presumptive digits III and IV (Fig. 5 A,D), similar to the pattern observed previously with the CreDel0 mice (compare with Fig. 4). In contrast, however, the staining became much stronger in subsequent developmental



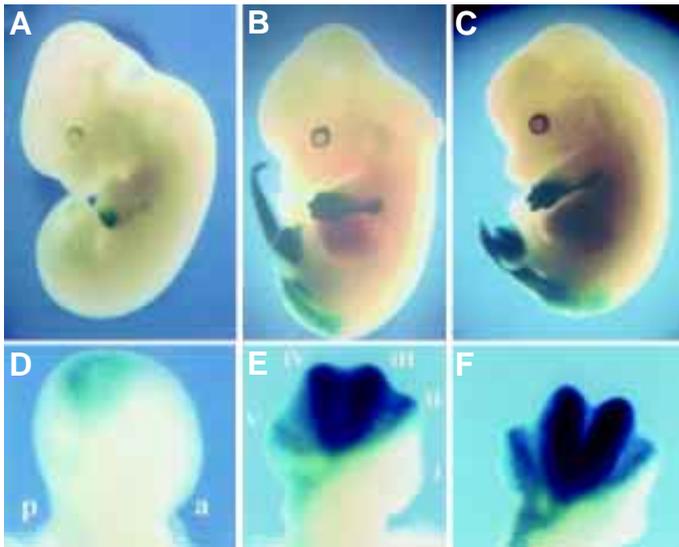
**Fig. 4. Xgal staining of embryos of different ages, carrying the *CreDel0* allele. (A-C)** Full embryos at 11.5 (A), 12.5 (B) and 13.5 (C) days post coitum. Staining was highly restricted to the developing limbs. **(D-F)** Isolated forelimbs (D,E) and hindlimb (F) at day 11.5 (D) and 12.5 (E,F). Staining is primarily observed in digits III and IV, though some weaker signal was seen in other digits.



**Fig. 3. Xgal staining of 11.5 embryos carrying the *Hoxd11lacZ* (Del0), *Hoxd11lacZDel3* (Del3) and *Hoxd11lacZDel7* (Del7) alleles.** In these three embryos, the same reporter transgene is present, but at three different positions within the *HoxD* complex. The stained domains are progressively more extended towards the anterior part, along with a more 3' position of the transgene. Thus, Del0 staining resembled *Hoxd13*, whereas Del7 is reminiscent of *Hoxd3* in the trunk (see van der Hoeven et al., 1996; Zakany and Duboule, 1996; 1999 for details).

stages and E12.5 old embryos showed a rather robust coloration in both fore- and hindlimbs (Fig. 5 B,E and data not shown). In addition, staining was scored in the posterior region of the trunk in both neuroectoderm and mesoderm derivatives, from about the level of somite 28 downwards, with increasing intensity in the tail (Fig. 5B). One day later, the staining was still quite robust, both in developing limbs and trunk (Fig. 5 C,F). Coloration seemed to be slightly reinforced in E13.5 embryos.

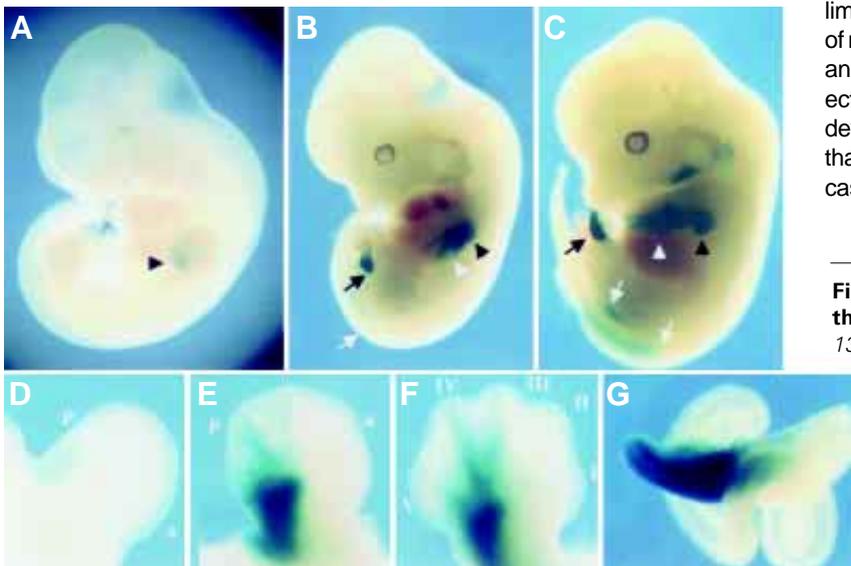
Staining was particularly strong in developing forelimb digits III and IV, with some residual signal in digits II and V (Fig. 5F). In hindlimbs, staining was comparable though less intense, giving rise to this partial 'two-digits' patterns, already well visible for the CreDel0 line (not shown). In the trunk, in particular the central nervous system, staining was clearly not homogenous, showing a mosaic appearance with positive cells neighbouring negative ones, unlike the situation found in *HoxD<sup>d11lacZDel3</sup>* embryos (Fig. 3; Del3 and Zakany and Duboule, 1996). This 'salt and pepper' staining was also scored in parts of the developing limbs, where staining was weak, such as in prospective digits II and V. This could be due to a low level of CRE enzyme, which, consequently would not work efficiently on all cells expressing the transgene. Altogether, the CreDel3 mice seemed to be better candidates to be used as deleter mice than their relatives CreDel0 mice. These mice nevertheless did not completely fulfill our expectations based upon the analysis of the related *HoxD<sup>d11lacZDel3</sup>* strain (Fig. 3). The domains where the CRE enzyme was fully functional, as illustrated by the blue staining, nonetheless indicated a possibility to use these mice for some digit-specific, CRE-dependent modifications.



**Fig. 5. Xgal staining of embryos of different ages, carrying the *CreDel3* allele. (A-C)** Full embryos at 11.5 (A), 12.5 (B) and 13.5 (C) days post coitum. Early on, staining was restricted to the developing limbs. Subsequently however, it appeared in the tail and posterior part of the trunk. **(D-F)** Isolated forelimb at day 11.5 (D), 12.5 (E) and 13.5 (F). Staining was more widely distributed than in the *CreDel0* line, encompassing from digit II to digit V. In early stages, the staining was nevertheless mostly concerned with digit III and IV (D), as for the *CreDel0* mice.

#### *HoxD*<sup>d11CreDel7</sup>

The analysis of *CreDel7* mice revealed a pattern quite different from what was expected, based on the analysis of the *HoxD*<sup>d11lacZDel7</sup> mice (Fig. 3, Del7; Zakany and Duboule, 1999). Indeed staining was observed only in a restricted number of structures where *d11LacZ* expression had been originally detected. For example, in 10.5 old embryos, a weak staining was scored in a proximal region of the developing forelimbs (Fig. 6 A,D). The intensity of this unexpected staining was reinforced at subsequent stages (Fig. 6 E,F), whereas the strong staining seen in distal limbs of *HoxD*<sup>d11lacZDel7</sup> mice (Fig. 3; Del7) was absent, or barely detectable



**Fig. 6. Xgal staining of embryos of different ages, carrying the *CreDel7* allele. (A-C)** Full embryos at 11.5 (A), 12.5 (B) and 13.5 (C) days post coitum. Early on, staining was restricted to the developing forelimbs. Subsequently, weak staining appeared in the posterior part of the trunk. **(D-F)** Isolated forelimbs at day 11.5 (D), 12.5 (E) and 13.5 (F). Staining became quite robust, but in a completely ectopic domain, spanning a proximal-posterior domain. **(G)** A strong staining was observed in a part of the intestinal hernia (arrow in C), in a way reminiscent of the *Del7* embryos (Zakany and Duboule, 1999).

at any stages examined (Fig. 6). Surprisingly as well, hindlimbs were totally negative (not shown), even though *HoxD*<sup>d11lacZDel7</sup> mice did show a robust staining in the distal parts of all appendages (Fig. 3; Del7; Zakany and Duboule, 1999). A weak signal was detected in the posterior trunk, at the expected position for the *Hoxd11* transgene, but the anterior expression seen for the *HoxD*<sup>d11lacZDel7</sup> mice was absent. In fact, only few of the expression features previously detected in *HoxD*<sup>d11lacZDel7</sup> mice were left after replacement of the *d11lacZ* transgene by the *d11Cre* transgene. For example, some staining was scored in the mandible (Fig. 6C), likely reflecting positive Crest cell, whereas a robust coloration appeared in the caecum (Fig. 6 C,G), a piece of the intestinal hernia which normally expresses all *Hoxd* genes but *Hoxd12* and *Hoxd13* (Zakany and Duboule, 1999).

Therefore, in these *CreDel7* mice, the differences observed with respect to the *d11lacZ* version were not only quantitative, but also clearly qualitative. The CRE-derived  $\beta$ -gal pattern was quite different, appearing either as a subset of the expected *HoxD*<sup>d11lacZDel7</sup> pattern (Fig. 3), or as an ectopic expression profile, such as in proximal limbs (Fig. 3), or as a faithful recapitulation of expected expression domains was nevertheless observed in a few instances (e.g. in the intestinal hernia).

#### Increasing the Doses of CRE and Expression of the Cre RNA

One possibility to account for the differences between the observed and the expected patterns of CRE-dependent *lacZ* staining is a low level of excision, which may be due to a reduced level of enzyme produced by the recombinant *d11Cre* transgene. We investigated this possibility by looking at mice homozygous for the targeted *Hoxd11/Cre* transgene, i.e. mice which should produce twice as much as recombinase. In all three lines, double amount of the CRE enzyme slightly increased the intensity of the staining. However, this slight increase did not reveal any novel sites of expression (not shown).

We further investigated whether the fact that these CRE functional profiles were weak and restricted was due to a poor efficiency in the translation of the enzyme, or to a weak activity of the protein. We looked at the presence of *Cre* RNAs by whole mount *in situ* hybridisation in 12.5 days old embryos. The distribution of *Cre* RNAs faithfully reproduced that of the  $\beta$ -gal staining, indicating that wherever the CRE enzyme was present, it probably allowed for a fair excision at the *R26R* locus, hence the efficiency of the enzyme was not the limiting factor in the process. This was clear for both lines of mice, which showed a restriction of *Cre* RNA in digits III and IV (Fig. 7). In the case of the *CreDel7* mice, the same ectopic expression was detected in a proximal part of the developing limb with a *Cre* antisense RNA probe (Fig. 7C), than that scored with the  $\beta$ -gal staining (Fig. 6). In this latter case, expression of the RNA was not scored distally,



**Fig. 7.** Whole mount *in situ* hybridisation with a *Cre* antisense RNA probe, carried out on 11.5 embryonic limbs from the *CreDel0* (A), *CreDel3* (B) and *CreDel7* (C) lines. The distribution of the RNA corresponds to the domains where the *CRE* enzyme was found active (compare with previous figures).

demonstrating that the difficulty to obtain sustained and faithful expression of our *Cre* transgenes occurred at the level of transcription, rather than at the level of translation. The sites where  $\beta$ -gal staining were observed indeed corresponded accurately with the sites where *Cre* RNAs were detected.

Previous work had shown that the position of the same transgene within the *HoxD* complex would dictate important aspects of the resulting expression profiles (van der Hoeven *et al.*, 1996; Zakany *et al.*, 1996; Héroult *et al.*, 1999; Kmita *et al.*, 2000). We took advantage of this observation to generate this series of *Hoxd11/Cre* transgene relocations, which in principle, should have provided us with a set of nested expression patterns allowing for *CRE*-specific recombinations in progressively more extended domains, e.g. in the developing trunk and limbs. While some recombinase activity was detected in the expected domains, thus validating the approach to some respect, the amount of available enzyme was not high enough to trigger recombination at a satisfactory level, i.e. with an efficiency allowing for at least 90 percent of the cells to express the *lacZ*. Three factors may be responsible for this partial failure to recapitulate the expected patterns, all three related to some differences between the construction of these alleles and the former *Hoxd11lacZ* alleles.

Firstly, the site where the HOXD11 and  $\beta$ -galactosidase proteins had previously been fused allowed for a substantial N-terminal piece of the HOXD11 protein to be present, whereas this latter protein was completely replaced in the *Cre* construct. It is possible that an enlarged fusion protein may have been stabilized, or may have favoured the activity of the  $\beta$ -galactosidase catalytic domain. While this could explain a higher activity of the *lacZ* fusion *versus* the *Cre* proteins, it can hardly account for the restricted distribution of the *Cre* RNA observed in these animals (Fig. 7). Secondly, the cloning strategy involved some modifications in the DNA sequences immediately upstream the conventional *Hoxd11* ATG, and it appears that another translation initiation codon could be used, which is located 13 residues upstream the ATG originally described (Gérard *et al.*, 1993). Therefore, it is likely that the initiation starting at this upstream position lead to a short and non-functional peptide, whereas only initiation events occurring at the second ATG could produce a low level of *CRE*. Here again, while this problem may have resulted in a decreased amount of the protein, it can hardly account for the abnormal RNA distribution.

An explanation to these unexpected results can perhaps be found in the persistence, in these alleles, of the cassette used for selection process in ES cells, since the PGK *neomycin* selection cassette used in these configurations contained a single *loxP* site on one of its extremity (Fig. 1B). Because the *LoxP* site was located in the 5' part of the cassette, and since the cassette was in the reverse orientation with respect to the transcription of *Hoxd11*, excision was not possible in the experimental schemes proposed

in this study. Consequently, all three configurations described in this paper still carried the selection cassette in the 3' part of the coding region. A series of studies have reported the necessity to removed such selection cassettes from the targeted loci, to prevent a foreign promoter (PGK in this case) to induce alterations in the transcription of nearby located genes (Rijli *et al.*, 1994; Beckers and Duboule, 1998; Héroult *et al.*, 1999). The presence of this cassette in our lines of mice may explain the observed differences in the transcription of the chimaeric gene, both qualitatively and quantitatively.

In a more general context, these three lines of transgenic mice do not appear to be fully appropriate for studying *Hox* gene function, for example through multiple conditional inactivations. In such instances indeed, it is necessary to produce high amounts of the *CRE* protein in the appropriate *Hox* domains, a condition which doesn't appear to be fulfilled in these lines. However, it is likely that these deleter strains can be used for subtle targeted events, for instance the analysis of a particular developmental process in a single digit, or in the intestinal hernia, two sites where a relatively high load of the *CRE* enzyme was scored.

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