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The mis-expression of posterior *Hox-4* genes in *talpid* (*ta*^{3}) mutant wings correlates with the absence of anteroposterior polarity

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Summary

Developing chicken wings homozygous for the *talpid* (*ta*^{3}/*ta*^{3}) mutation are polydactylous and have defects in the establishment of their anteroposterior polarity. We analysed the expression domains of the posteriorly restricted homeobox *Hox-4* genes in such mutant wings. The *Hox-4* genes are now expressed right across the anteroposterior axis instead of being expressed just posteriorly. This correlates well with the absence of clear morphological differences between the *talpid*^{3} digits and reinforces the idea that vertebrate *Hox-4* genes are involved in setting up the limb anteroposterior asymmetry.

Key words: *talpid*^{3}, *Hox-4*, chick wing development, anteroposterior polarity.

Introduction

Among candidate genes involved in limb pattern formation are genes containing a homeobox. Such genes are indeed probably part of the mechanisms responsible for either the epithelial-mesenchyme interaction-dependent growth (e.g. *Hox-7.1*, *Hox-8.1*; Robert et al., 1989; Hill et al., 1989; Davidson et al., 1991; Coehlo et al., 1991) or for the patterning along the anteroposterior (AP) and proximodistal (PD) axes such as the gene members of the HOX-4 and HOX-1 complexes or *Hox-3.3* (Dollé and Duboule, 1989; Dollé et al., 1989; Oliver et al., 1989; Izpisúa-Belmonte et al., 1991a; Nohno et al., 1991; Yokouchi et al., 1991). During both mouse and chicken limb bud outgrowth, the gene members of the HOX-4 complex (Featherstone et al., 1988; Duboule et al., 1990) are sequentially activated in a 3′ to 5′ sequence so that genes located at more 3′ positions in the complex are activated first and have a wide domain of expression in the limb (e.g. *Hox-4.3*; Izpisúa-Belmonte et al., 1990) whereas 5′-located genes are expressed later with a progressive restriction to more posterodistal areas (Dollé et al., 1989; Izpisúa-Belmonte et al., 1991a). Thus, the gene located at the 5′ extremity of the complex (*Hox-4.8*) has a transcript domain restricted to a region that largely overlaps with the zone of polarizing activity (Saunders and Gasseling, 1968), a region that is thought to organize the anteroposterior polarity of the developing limb.

The pattern of *Hox-4* gene expression in manipulated chick wing buds is consistent with the idea that *Hox-4* gene products encode positional information (Wolpert, 1989). Grafts of the polarizing region or application of retinoic acid respecify anterior cells to form posterior structures and result in mirror-image duplications in which an additional set of digits develop. Detailed analysis of such wing buds show that cells that express all members of the HOX-4 complex form posterior digits, whereas cells that express only more 3′ members of the complex form anterior digits (Izpisúa-Belmonte et al., 1991a; Nohno et al., 1991). These data strongly suggest that the wing anteroposterior asymmetry is established by the sequential activation of the HOX-4 complex genes whose products are asymetrically distributed (see Dollé et al., 1989; Duboule, 1991).

In this context, the *talpid*^{3} (*ta*^{3}) polydactylous mutant of the fowl is of particular interest since homozygous animals show strong defects along the anteroposterior axis of their developing limbs as well as along the major rostrocaudal axis (such as fusions of vertebrae). In limbs, the mutation affects both ectoderm (Ede, 1980) and mesoderm cells (Goettinck and Abbott, 1964). In the mesoderm, cell migration and skeletal patterns are abnormal (Ede and Kelly, 1964; Ede, 1968, 1971), and a retardation in cartilage hypertrophy is observed with failure in periosteal ossification (Hinchliffe and Ede, 1967, 1968).

Interestingly, fusion of elements across the antero-

posterior axis is often observed (e.g. between carpals, between metacarpals or radius and ulna) and the developing limb thus appears to contain three broad bands of condensed mesenchymal cells instead of the very precise precartilage pattern (Fig. 1; for a review and refs., see Hinchliffe and Johnson, 1980). A striking feature of ta^3 mutants is the apparent inhibition of cell death (Hinchliffe and Ede, 1967; Hinchliffe and Thorogood, 1974) leading to the absence of both posterior and anterior necrotic zones and the opaque patch. The absence, in ta^3, of both the anterior and posterior necrotic zones (Hinchliffe and Ede, 1967; Cairns, 1977), combined with an extensive apical ectodermal ridge (the thickened epithelium that controls bud outgrowth), is correlated with an excess of mesoderm leading to a pronounced polydactyly (from 8 to 10 rudimentary digits) in both wings and legs. Digits, however, are not normal and cannot be identified as posterior or anterior (Fig. 1). At the cellular level, the mutation appears to affect mesodermal cells migration and adhesivness (Ede and Flint, 1975).

We analysed the expression pattern of some 'posterior' *Hox-4* genes in *talpid*^{3} mutant limbs and report here that their transcripts are now expressed right across the anteroposterior axis. These results further demonstrate the involvement of Hox genes in setting-up the limb pattern and suggest some hypotheses that account for the *talpid*^{3} phenotype in developing chicken limbs.

Materials and methods

The in situ hybridizations were carried out as previously (Dollé and Duboule, 1989) but without a prehybridization step. The antisense RNA probes were those reported in Izpisúa-Belmonte et al. (1991a).

Talpid^3 mutant material was obtained from matings of heterozygous birds (ta^3/+ × ta^3/+). Homozygous *talpid* embryos can easily be distinguished from stage 19. The *talpid*^{3} gene is recessive and heterozygotes (ta^3/+) and homozygous wild type (+/+) are indistinguishable, so the *talpid* stock normal controls may be of either genotype.

The illustrations in Fig. 1 are camera-lucida drawings made from whole mounts of limbs fixed in Bouin's and stained with methylene blue (Van Wijhe's method in Cowdry, 1952).

Results

We analysed the expression patterns of the chicken *Hox-4.4*, *Hox-4.6* and *Hox-4.8* genes in normal limbs and in limbs dissected out from embryos produced from matings of birds heterozygous for the mutation *talpid*^{3} and which had either a normal or mutant phenotype. The pattern, in which expression of these genes is confined to bud mesenchyme, has been described elsewhere in some detail (Dollé et al., 1989, 1991; Izpisúa-Belmonte et al., 1991a; Yokouchi et al., 1991) and is briefly illustrated in Fig. 2A-D. In early bud stages (about stage 21-22, Hamburger and Hamilton, 1951), the *Hox-4.4* transcripts are found in a large part

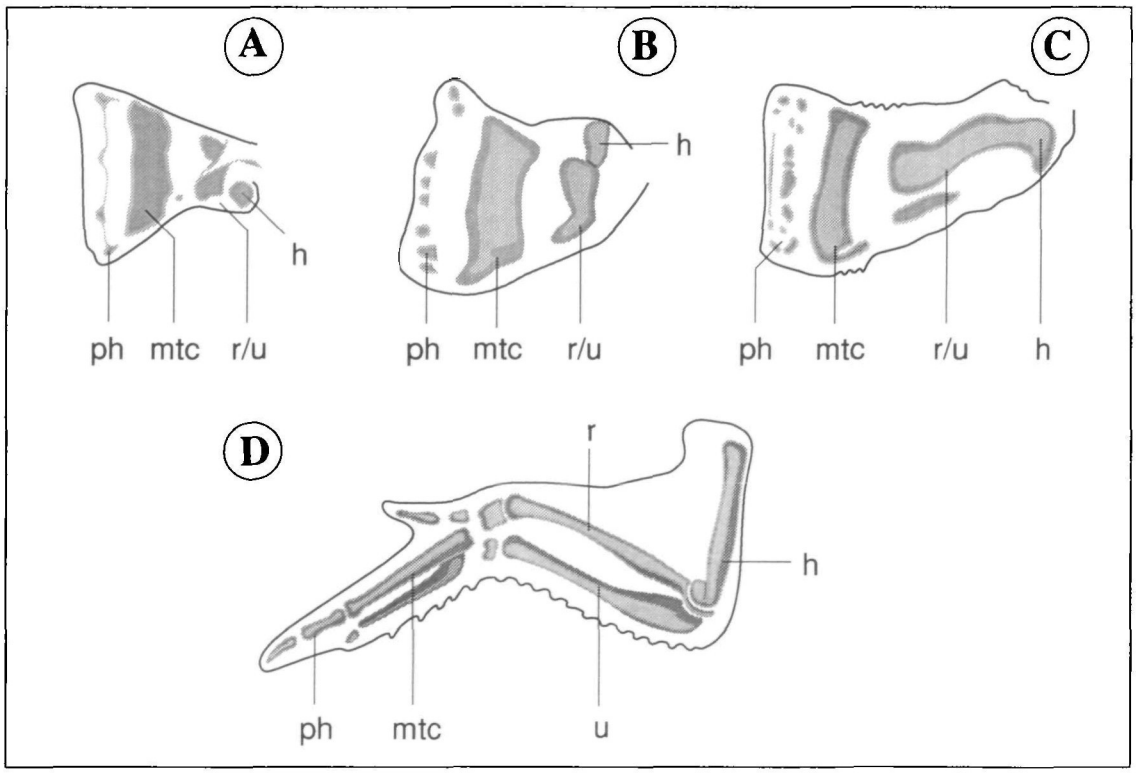

Fig. 1. Camera-lucida drawings of preskeletal patterns of chicken limbs homozygous for the *talpid*^{3} mutation. (A-C) drawings of homozygous *talpid*^{3} left forelimbs at three different stages; (A) 8.5 day; (B) 10 day and (C) 11 day. The preskeletal patterns are shown in grey. As a control, a normal left forelimb at day 10 is shown below (D). ph, phalanges; mtc, metacarpals; r, radius; u, ulna; h, humerus.

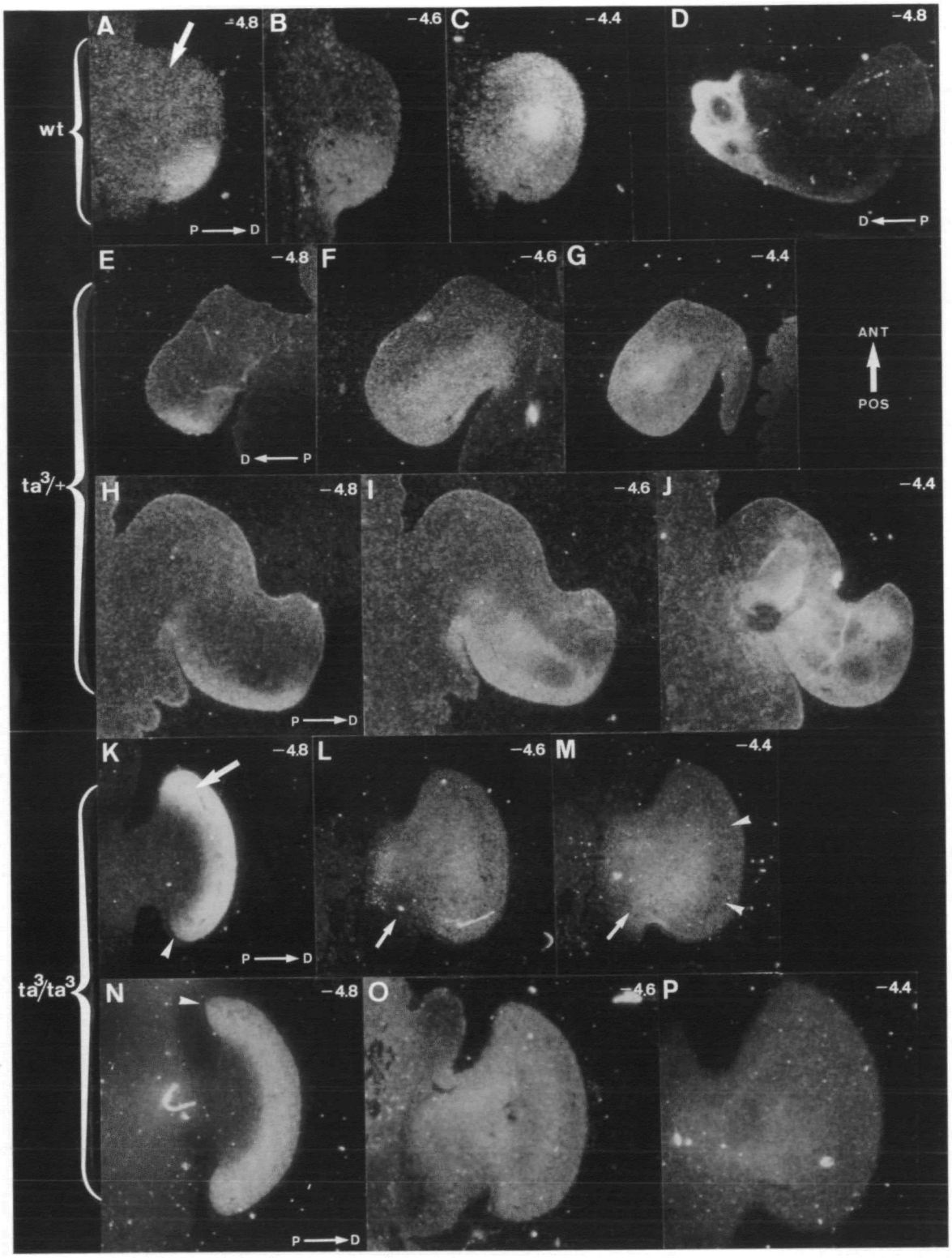

Fig. 2. Expression patterns of the *Hox-4.8*, *Hox-4.6* and *Hox-4.4* genes during the development of *talpid*^{3} chicken limbs. Panels A to C are control hybridizations on normal wing buds to illustrate the normal expression patterns of *Hox-4.4*, *Hox-4.6* and *Hox-4.8* in a stage 21 embryo. The Hox-4.8 transcript domains is also shown for an older (stage 30) embryo, which further emphasizes its posterodistal restriction (D). (E-G) Expression domains of the same three genes in heterozygous or homozygous ($ta^3/+$; $+/+$) wings at about stages 22. The control limbs shown in panels E to J could also be homozygous $+/+$ since $+/+$ and $ta^3/+$ phenotypes are undistinguishable; see the text. The domains are normal as is the case in older (stage 28) embryos (panels H-J). (K-P) *Hox-4* expression domains in homozygous ta^3/ta^3 wings, at two developmental stages (K to M and N to P) corresponding approximately to those shown under E-G and H-J, respectively. The wings are fan-shaped and the transcript domains have lost their posterior specificities. All the panels are orientated with anterior (ANT) to the top and posterior (POS) to the bottom. The proximodistal axis is indicated, for the different series, at the bottom right of panels A,D,E,H,K and N. The genotype of the various samples is indicated on the left margin and the probes used at the top right of each panel.

of the wing bud (Fig. 2C) except for a very small proximoanteriorly located part (not shown). In contrast, the *Hox-4.6* gene is expressed only in the posterior half of the bud, from the most proximal part to the distal tip (Fig. 2B) whereas *Hox-4.8* transcripts are found only in the posterior part of the bud tip, in a more distal and posterior area than that containing the *Hox-4.6* mRNAs (Fig. 2A). Thus, at these developmental stages, the *Hox-4.8* domain is contained within the *Hox-4.6* domain which itself is included in the *Hox-4.4* domain (Izpisúa-Belmonte et al., 1991a). Later in development, the transcripts domains become restricted to precartilaginous condensations and then to the perichondria of the future bones (Dollé and Duboule, 1989; Dollé et al., 1989; Yokouchi et al., 1991) but still conserve their coordinate patterns as illustrated by the posterodistal restriction in the expression domain of *Hox-4.8* at about stage 30, when *Hox-4.8* is expressed in the prospective areas for digit 4 and 3 as well as in a thin cell layer, posteriorly (Fig. 2D). Wing buds from normal embryos from the *talpid*^{3} matings (*ta*^{3}/+ or +/+) were analysed at about stages 24 (Fig. 2E-G) and 30 (Fig. 2H-J). In these limbs, the expression patterns of the three genes were indistinguishable from the wild-type patterns.

In contrast, in homozygous *ta*^{3}/*ta*^{3} wings, the transcript domains of the most posteriorly expressed Hox-4 genes were strikingly abnormal (Fig. 2K-P). We analysed homozygous embryos at two different stages which approximately correspond to the control stages shown in Fig. 2A-C and H-J. The results obtained were similar for younger or older embryos (see below) and will thus be presented and discussed together. *Ta*^{3}/*ta*^{3} wings have lost their anteroposterior asymmetry (Fig. 2K-P), compare e.g. panel K with panel A, and are fan-shaped (Fig. 2K-P). As in the normal limb bud, *Hox-4* genes are not expressed in the ectoderm, including the apical ectodermal ridge (Fig. 2, arrowheads in panels K and N). *Hox-4.4* is transcribed in cells throughout *ta*^{3}/*ta*^{3} wing bud (Fig. 2M), with an area of weaker intensity in the progress zone (arrowheads in M), at the distal tip of the wing (see also Dollé et al., 1989 for a similar observation in the mouse). At a later stage, the *Hox-4.4* signal is weakening but can still be detected in most of the limb (Fig. 2P). When more posterior genes, such as *Hox-4.6* or *Hox-4.8* are considered, the abnormalities in the transcript domains become obvious. The *Hox-4.6* gene is now expressed with no posterior restriction and the transcripts are thus widely distributed in the wing where they are expressed strongly in both pre-axial and post-axial mesoderm cells (Fig. 2M, compare with control panel B). Consequently, in *ta*^{3}/*ta*^{3} mutants wings, the *Hox-4.4* and *-4.6* genes have completely overlapping domains across the anteroposterior axis (Fig. 2L,M). However, proximodistal differences in the domains (see Dollé et al., 1989) are still visible in the most proximal part of the wing where the *Hox-4.4* expressing cells seem to extend slightly more proximal than those expressing both genes (Fig. 2, arrows in panels L,M). The same features also characterize expression of *Hox-4.8* in *talpid*^{3} wing buds. Firstly, the expression domain is not restricted to the posterior-most part of the limb but is equally distributed along the anteroposterior axis (Fig. 2). Secondly, the specific distal restriction of the *Hox-4.8* domain (Izpisúa-Belmonte et al., 1991a; Dollé et al., 1991) is conserved. Thus there is striking expression of *Hox-4.8* all across the broadened bud in the distal, subectodermal, region (Fig. 2). This area of high *Hox-4.8* expression correlates with the region where *Hox-4.4* transcripts seem to be less abundant (Fig. 2, compare panels M and K), which suggests possible interractions between the transcriptional regulation of the posterior *Hox-4* genes. Finally, there is no visible difference between the amounts of Hox transcripts in homozygous mutants versus normal animals.

Discussion

The absence of AP polarity in the developing limbs of *ta*^{3}/*ta*^{3} mutant chickens, as judged by morphological criteria and by preskeletal patterns, is correlated with the abnormal extension into anterior areas of the expression domains of those *Hox-4* genes that are normally expressed only in posterior mesoderm. Consequently, the Hox domains entirely overlap all across the anteroposterior axis, which leads to the absence of discrete domains in which cells express different combinations of *Hox-4* genes. In the context of models that have been proposed for the functions of *Hox* genes during limb pattern formation (Dollé et al., 1989; Izpisúa-Belmonte et al., 1991a,b; Duboule, 1991; Yokouchi et al., 1991), this new distribution of Hox expression domains could largely account for the observed phenotype. Indeed, the absence of discrete Hox domains could be responsible for the non-individualization of the various elements across the anteroposterior axis (Dollé et al., 1989; Yokouchi et al., 1991). In contrast, the persistence of discrete Hox domains along the proximodistal axis (e.g. the distal restriction of *Hox-4.8* transcripts) correlates with the existence of a proximodistal pattern (a succession of different elements) grossly similar to the normal pattern.

As far as the digit pattern is concerned, two different aspects should be considered; digit identity and the number of digits (Ede, 1971). Digit identity can be considered to be specified, largely, by the expression of the *Hox* genes (Izpisúa-Belmonte et al., 1991a; Yokouchi et al., 1991). According to this view, the absence of discrete Hox domains in the most distal areas of the growing wing should "homogenize" the positional information acquired by those cells located in the presumptive digit zones and the strong expression of *Hox-4.8* should result in the development of a series of similar "posterior" digits. It is striking that the *ta*^{3}/*ta*^{3} digits do all look the same, although their identity cannot be recognized clearly. In contrast, digit number will not be directly related to *Hox-4* expression but instead to the broadening of the bud (Wilby and Ede, 1975; Wolpert and Stein, 1984). The anteroposterior

extension of the distal mesoderm (correlated but not necessarily caused by the extension of the apical ridge) in *talpid*^{3} wing buds produce an increase in the number of digits.

The *talpid*^{3} limb phenotype can thus be seen as a combination of the effects of a broadening of the bud and to a defect in the mechanism that establishes the anteroposterior polarity and the wing asymetry. The analysis of the Hox-4 gene expression domains in such wings suggests that their expressions are correctly coordinated but spatial specificity has been lost. We proposed earlier (Dollé et al., 1989) that the expression of the *Hox-4* genes could be controlled by the polarizing region (Saunders and Gasseling, 1968) and retinoic acid, a candidate morphogen possibly produced by the polarizing region, can activate 5′-located genes in vivo (Izpisúa-Belmonte et al., 1991a; Nohno et al., 1991). According to this view, the *talpid*^{3} limb phenotype could be due to a 'diffusion' of the polarizing activity in all the wing bud or to a shift of this activity to a more proximocentral part. Alternatively, *Hox-4* expression in *talpid*^{3} may not be due to a change in the distribution of the activating signal but instead could reflect a change in the responsiveness of the cells. In this context, it is interesting that the activity of the polarizing region is defective in the *talpid*^{3} mutant whereas the mutant cells seem unable to respond to polarizing region grafts from normal embryos (Ede and Shamslahidjani, 1983).

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