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Point mutations in a distant sonic hedgehog *cis*-regulator generate a variable regulatory output responsible for preaxial polydactyly

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Precise spatial and temporal control of developmental genes is crucial during embryogenesis. Regulatory mutations that cause the misexpression of key developmental genes may underlie a number of developmental abnormalities. The congenital abnormality preaxial polydactyly, extra digits, is an example of this novel class of mutations and is caused by ectopic expression of the signalling molecule Sonic Hedgehog (SHH) in the developing limb bud. Mutations in the long-distant, limb-specific *cis*-regulator for *SHH*, called the ZRS, are responsible for the ectopic expression which underlies the abnormality. Here, we show that populations of domestic cats which manifest extra digits, including the celebrated polydactylous Hemingway's cats, also contain mutations within the ZRS. The polydactylous cats add significantly to the number of mutations previously reported in mouse and human and to date, all are single nucleotide substitutions. A mouse transgenic assay shows that these single nucleotide substitutions operate as gain-of-function mutations that activate *Shh* expression at an ectopic embryonic site; and that the sequence context of the mutation is responsible for a variable regulatory output. The plasticity of the regulatory response correlates with both the phenotypic variability and with species differences. The polydactyly mutations define a new genetic mechanism that results in human congenital abnormalities and identifies a pathogenetic mechanism that may underlie other congenital diseases.

INTRODUCTION

In the vertebrate genome, a series of non-coding sequence elements are present which are distinguished by substantial conservation across vast evolutionary distances (often given the acronym CNE for conserved non-coding elements) (1,2). These elements are significant in length ranging from 100 bp to greater than a kilobase and have proved in a number of cases to be *cis*-regulators. CNEs are associated with genes that undergo highly orchestrated regulation especially those involved in key developmental processes (3–5). The developmental signalling molecule sonic hedgehog (SHH) has a complex expression pattern regulated by a number of these highly conserved, modular elements (6). One of these, the ZRS, is the *cis*-regulator that controls the expression of *Shh* in the developing limb bud specifically in a domain called the zone of polarizing activity (ZPA) (7–9). The ZPA is located along the posterior

margin of the limb bud and through the production of SHH regulates digit identity and number (10). The ZRS is also an extreme example of a long-range regulator. In the human genome, the ZRS resides at a distance of 1 Mb from the *SHH* gene and is located within the intron of another gene that itself has no role in limb development (7,8,11). The ZRS is highly conserved over a length of ~800 bp and has an ancient role in development of appendages having recently been identified in the chondrichthyan fishes (12).

Human preaxial polydactyly (PPD) on chromosome 7q36 [also presents as PPD type II, triphalangial thumb (TPT) and triphalangial thumb-polysyndactyly (TPTPS)] includes a broad range of digit abnormalities on the preaxial or anterior side of the hands and feet (13–15). Mouse models for PPD (such as the *Ssq* and *Hx* mutants) were instrumental in defining the developmental basis for this abnormality and showed that preaxial polydactyly results from ectopic expression of *Shh* at

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a site along the margin opposite the ZPA; i.e. at the anterior side of the limb bud (16,17). The data are compelling that PPD is a regulatory disorder (8) resulting from mutations within the ZRS (7,18). The ZRS mutations culminate in the misexpression of *Shh* generating an ectopic ZPA and consequently, the production of supernumerary digits.

Here, we investigate the ZRS mutations to better understand the disease process that gives rise to extra skeletal elements. Analysis in polydactylous cats, including the celebrated Hemingway's cats, adds significantly to the number of mutations associated with this abnormality. Mutations that redefine the activity of a *cis*-regulator by modifying the normal spatial expression pattern are, presently, unique to the ZRS. Using a mouse transgenic assay, we show that the mutations generate a highly variable expression pattern dependent on the position and the sequence context of the mutation within the ZRS. In a number of cases, the features of the expression pattern generated by the mutation relates to the observed phenotype. Specific point mutations relate to distinct morphological changes in the limb which proffers the notion that novel morphological forms need not require complex molecular modification. *Cis*-regulatory changes in a single step can transform a fundamental structural feature in mammals.

RESULTS

Mutations in the ZRS cause polydactyly in domestic cats

Regulatory mutations, particularly in *Drosophila*, have been invaluable in mapping functional *cis*-regulators and in determining the genetic circuitry important for developmental regulation (19). To uncover further clues for understanding gene regulation by the ZRS, a survey to extend the number of mutations in this element was undertaken. Polydactylous cats (Fig. 1A), also called 'mitten' cats, are a well-known curiosity of the pet world. Valued as good luck charms by sea captains on sailing ships in the past centuries, polydactylous cats are frequently found along the northeastern coastal regions of North America. Genetic surveys from the 1970s estimate a high frequency of the polydactyly allele in coastal cities such as Halifax, Nova Scotia and Boston (20). The most celebrated of the affected animals are the Hemingway's Cats descended from a single polydactylous cat given to the American author Ernest Hemingway by a ship's captain in the 1930s (21). Highlighted in a BBC/PBS documentary (22), around 40 descendants populate the grounds of the Ernest Hemingway Home in Key West, Florida (www.hemingwayhome.com) of which about half have extra toes. In addition, although perhaps more rare, polydactylous cats are also known to occur in Great Britain. Therefore we initiated a study to examine extra-toed cats in both North America and Britain to pose the question—are polydactylous cats subject to the same genetic mechanism that generates extra digits in human and mouse?

The wild-type cat ZRS sequence was initially determined from the genome of five unrelated cats and the analysis showed no differences in the 800 bp CNE (Supplementary Material, Fig. S1). Subsequently, genomic DNA from cats in the Hemingway colony was obtained (provided by Dr Kristen Jensen, DVM) and the sequence from three wild-type and four mutant cats was determined. A partial pedigree of the

Hemingway's cats analysed is shown in Figure 1B. All affected cats carried the same A to G nucleotide substitution at base 479 in the cat CNE which was designated the *Hw* mutation (Fig. 1 B and D and Supplementary Material, Fig. S1); three of the cats were heterozygous and one (cat ED in the pedigree, Fig. 1B) was homozygous. The homozygous cat, however, did not show a more severe phenotype and in accord, was polydactylous only on the forelimbs. The study was extended to other cats from different regions of North America (Fig. 1C). Genomic DNA was obtained from two unrelated North American polydactylous cats and shown to have the same mutation as the Hemingway's cats (Fig. 1C) suggesting that this is a common allele in polydactylous cats in North America.

In contrast the *Hw* mutation was not found in cats obtained in Britain. Genomic DNA was obtained from eight British polydactylous cats from six individual owners in different areas of England (Fig. 1C). Two new point mutations were identified in these cats, the *UK1* and the *UK2* mutations at position 257 and 481, respectively (Fig. 1C and D) showing that preaxial polydactyly in domestic cats has arisen on, at least, three independent occasions. It emerged that the positions of the *UK2* and the *Hw* mutations are located one nucleotide apart, residing within a conserved nucleotide triplet in a region that otherwise, shows considerable sequence divergence in mammals (red box in Fig. 1D). These two mutations implicate this conserved triplet as having an important role in *Shh* expression despite the species differences.

The addition of the cat polydactylous mutations to the previous list of those for mouse and human yields a total of 13 associated with preaxial polydactyly (7,18,23–25). All the mutations are single nucleotide changes and all, but one, are found in the most highly conserved 800 bp CNE of the ZRS (Fig. 1D). The point mutations pinpoint conserved, functional subdomains and thus highlight structural features that will be instrumental in establishing the mechanism of ZRS activity. We predict that the scattering of the point mutations across the CNE shows that most, if not all, of the highly conserved domain is required for accurate spatial expression of the *Shh* gene in the limb. Moreover, since no single subdomain is responsible for ectopic expression, a mechanism that affects a general feature of the enhancer appears to be crucial for misexpression of *Shh*.

The PPD associated point mutations generate a plastic regulatory response

Transgenic assays showed that the *Hx* and the M100081 mouse mutations generate ectopic expression in the developing limb buds (23,26). To examine the capacity of the human point mutation to generate ectopic activity, we took advantage of the mouse transgenic assay. Initially, two of the human ZRS point mutations were assayed that represented different extremes of the polydactylous phenotypic spectrum. The mutation associated with the Cuban family (7,15) causes an unusual, severe phenotype. The defects range from triphalangial thumb to hexadactyly and the feet are similarly affected. Radial dysplasia is also found and one individual had bilateral absence of the tibia. The Belgian2 mutation, in contrast, is associated with a family manifesting a milder

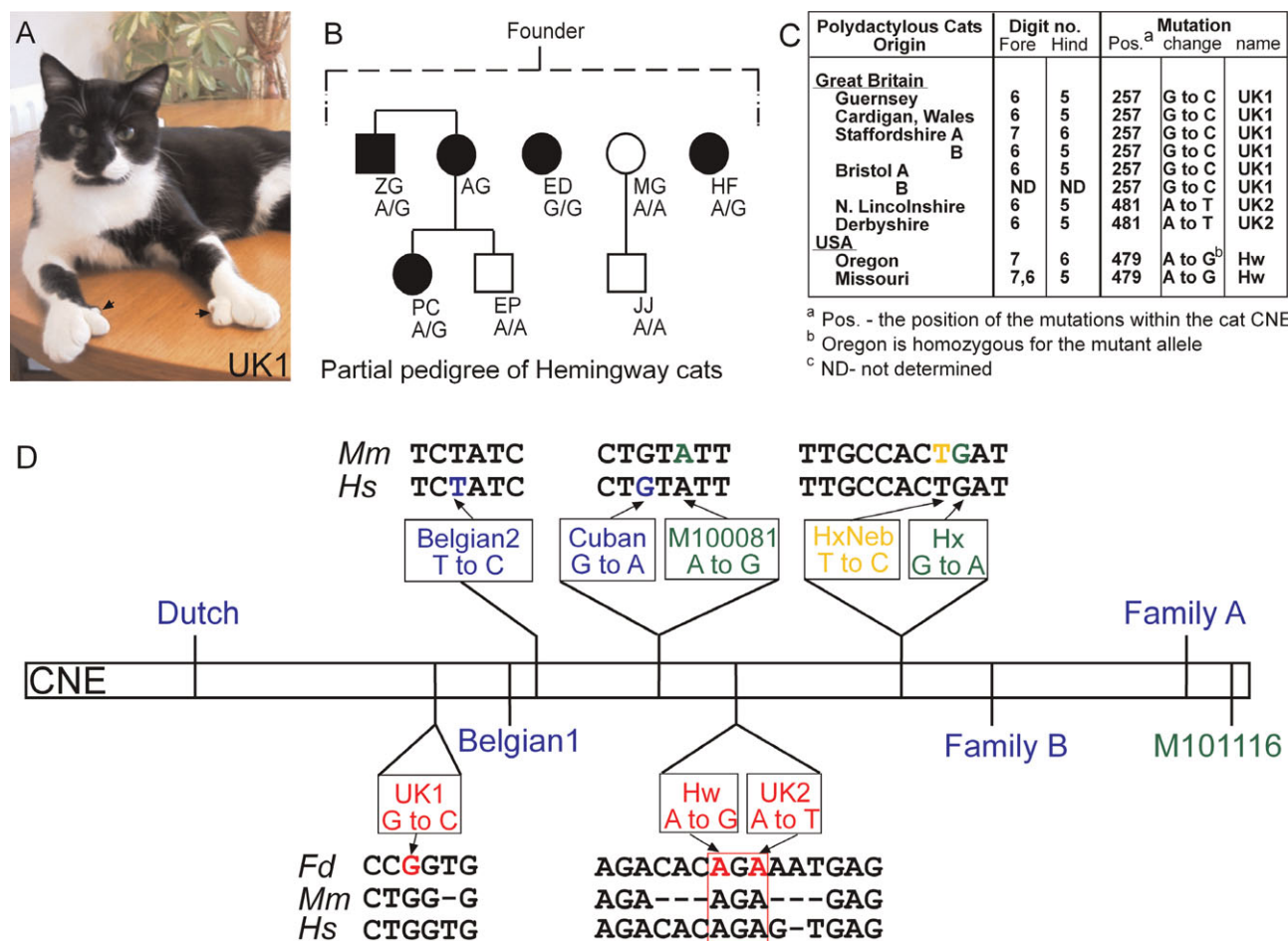


Figure 1. Genetic analysis of polydactylous cats and a summary of the mutations. (A) Picture of a polydactylous cat (carrying the UK1 mutation) showing the extra digits on the fore paws (arrows). (B) A partial pedigree of the Hemingway cats that were sampled. All affected cats are presumably derived from a single founder. The affected cats are depicted with solid squares (males) and circles (females). The initials for each individual cat are shown and the genotype of each cat is shown below the initials. The wild-type allele has an A at position 481 of the cat sequence and the mutant allele a G. (C) List of the polydactylous cats from Great Britain and the USA showing the origin of each, the limb phenotype and mutation that was determined. (D) Summary of the 12 mutations that reside in the ~800 bp conserved non-coding element (CNE, represented by rectangle). The relative position of the cat mutations are shown below. The position of the mutation is designated by red nucleotide and the surrounding sequence and corresponding sequence in mouse and human are shown (Fd, *Felis domesticus*; Mm, *Mus musculus*; and Hs, *Homo sapiens*). The conserved nucleotide triplet is designated by the red box. Other mutations used in the analysis are shown above the CNE. The name and the position of the mutations are shown (mutations identified in Hs are indicated in blue while Mm mutations are in green). The relative position of the human Cuban mutation and the mouse M100081 are shown and are one nucleotide apart. The 11 bp Hx subdomain is shown and the position of the Hx and HxNeb mutations are indicated.

phenotype more commonly observed in PPD families. Predominantly triphalangeal thumb, sometimes accompanied by a small nubbin, is found on the hands of the affected family members (7) but no long bone involvement has been reported (personal communication, P de Beer).

The mouse ZRS was modified to carry the different human point mutations and the transgenic embryos generated were analysed at ~E11.5; the mid-stage at which ectopic expression of *Shh* is detected in polydactylous mutants (16,17). Reporter constructs containing wild-type and mutant forms of ZRS drive expression of the *LacZ* reporter gene. The wild-type ZRS directs expression in a pattern that reflects endogenous *Shh* in the limb which is restricted to the posterior margin (7) (Fig. 2A). The mutation associated with the Cuban family (7,15) resulted in seven transgenic embryos (Table 1) expressing the reporter gene along the posterior margin.

However, three of these embryos differed from wild-type in that *LacZ* expression was broader and continued along the distal margin (arrows in Fig. 2B). In addition, these embryos showed an additional site of expression at an ectopic position along the anterior margin of the limb bud (arrowheads in Fig. 2B). Expression at the ectopic site extended along the anterior edge towards the distal end of the limb bud. In the hind limb the ectopic and the posterior expression merges and extends around the outer margin of the autopod. A second construct using the Cuban mutation contained within the human ZRS was also assayed (Fig. 2C). The expression pattern in the hindlimb was similar to that observed for the mouse ZRS (3 out of 10 expressing embryos; Table 1) in that the ectopic and posterior expression merged distally; however, in neither the fore nor the hind limb did we observe expression extending as far into the interior of the

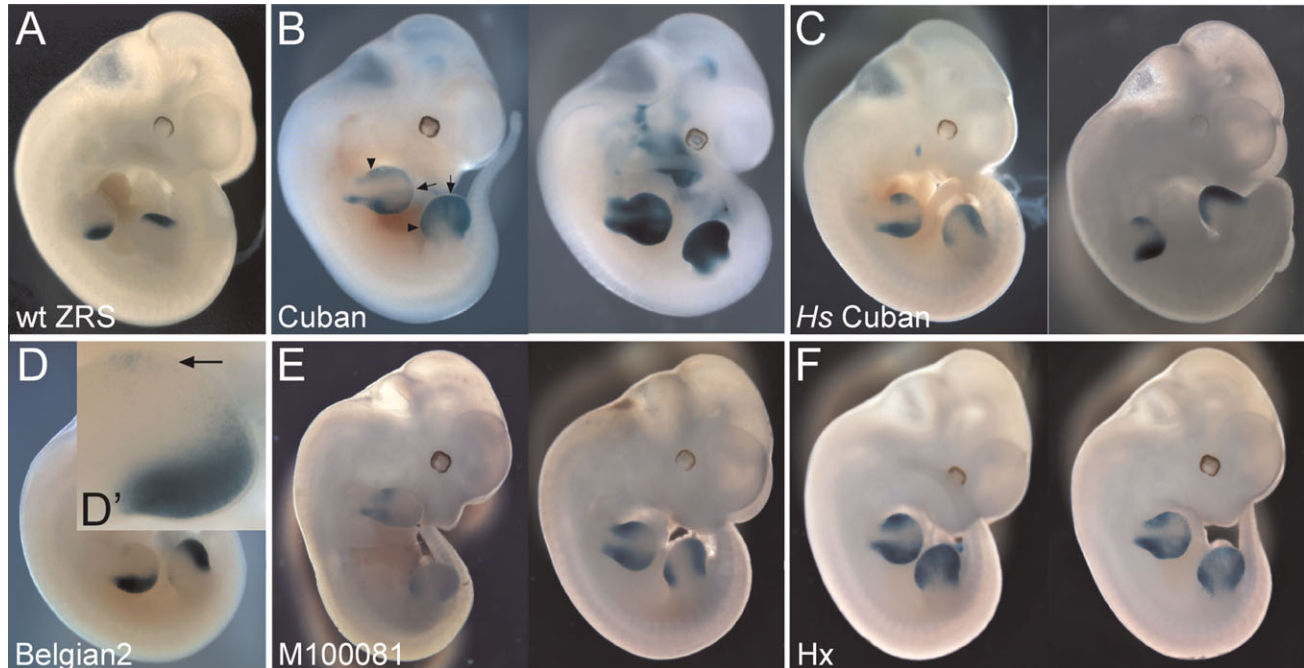


Figure 2. Transgenic analysis of the human and mouse mutations showing the variable expression output. All embryos are at approximate stage E11.5. (A) The typical pattern of LacZ in transgenic embryos carrying the mouse wild-type (wt) ZRS. The stain resides at the posterior margins of both the fore and hind limbs. (B) Transgenic embryos carrying the human Cuban mutation on the mouse ZRS. Two embryos are shown to indicate that the pattern is consistent in embryos even when the overall level of expression differs. One embryo is designated to show the position of the ectopic expression along the anterior margin indicated by an arrowhead and the extension of the posterior to the distal margin by an arrow. The Cuban mutation on the human ZRS (Hs) is shown in (C). The expression does not extend as far into the interior of the limb as seen in (B). The Belgian2 mutation in (D) reveals a low level of anterior expression. The arrow in the D' inset points to the cells expressing LacZ. (E) Expression of the mouse M100081 mutation one nucleotide removed from the Cuban mutation. Note that the expression pattern is similar to Cuban showing the extension to the distal edge. (F) Expression pattern of the Hx mutation with appreciable ectopic expression but the distal expression is not apparent.

Table 1. Summary of transgenic embryos carrying preaxial polydactyly mutations

Mutation ^a	Number of Tg ^b	Number of expressing ^c	Number of ectopic ^d
Cuban	8	7	4
Hs Cuban	19	10	3
Belgian 2	17	13	5
Hx	6	4	4
M100081	3	2	2
Fd wt	13	9	0
Fd Hw	11	8	4 ^e
Fd UK2	22	8	2
Hw	8	6	3
UK2	10	6	2
Hx Neb	7	5	1 ^f

^aColumn contains name of the mutation used to make transgenics. All are in the mouse ZRS except where stated; i.e. *Hs* is human and *Fd* is cat ZRS.

^bNumber of embryos that carried the transgene.

^cNumber of transgenic embryos that express in the limb.

^dNumber of expressing transgenics that stain along the ectopic domains of the limb.

^eOnly fore limbs express ectopically.

^fExpresses abnormally and only in forelimbs.

limb bud. (Fig. 2C). The Belgian2 mutation incorporated into the mouse ZRS showed appreciably weaker expression in the anterior domain (Fig. 2D). Five of the transgenic embryos showed a small number of scattered cells located along the

anterior margin expressing the reporter gene. The degree of expression of the Cuban and the Belgian2 mutations in transgenic embryos is therefore in accordance with the severity of the limb abnormality.

The two mouse mutations M100081 and *Hx* also display phenotypes which involve the long bones (23,27). The M100081 mutation, which is two nucleotides downstream of the Cuban mutation, frequently shows a shortened tibia; while the *Hx* mutation displays severe twisting of both the fore and hind limbs due to shortening of the radius and the tibia. The modified ZRS containing the mouse M100081 (18) mutation generated a posterior pattern (Fig. 2E) akin to the Cuban mutation with the additional site of strong expression along the anterior margin of the limb bud. The *Hx* mutation on the other hand does not show the extreme posterior expression (Fig. 2F). Ectopic expression mediated by the ZRS containing the *Hx* mutation reflected the endogenous misexpression of *Shh* detected in the polydactylous limb (17,18), i.e. expression extends along the anterior edge from the distal end of the limb bud to a region near the proximal edge of the AER.

These data confirm that first, the mechanism for generating misexpression in human is the same as mouse and therefore, the human phenotype results from *Shh* ectopic expression during limb formation. Secondly, a correlation is suggested by the transgenic assay that a relationship exists between the specific mutation and the phenotype. It is not clear how the expression expanded distally and at the posterior margin

detected, for example, in the transgenic assay using the Cuban mutation would affect the phenotype of the limb; however, studies in the chick limb bud system do aid in understanding the ectopic anterior expression. Analysis in the chick suggests that differences in levels of SHH in the ectopic domain may be relevant to the range of PPD phenotypes. Based on transplantation experiments in the developing chick limb bud, there is a direct correlation between the number of cells transplanted from the ZPA to the anterior margin and the extent of digit duplications (28). Also in these experiments the long bones are often affected; however, a direct correlation with dosage has not been made. Subsequent experiments directly testing exogenous application of *Shh* (either as a bead soaked in SHH or as cells expressing SHH) to the anterior margin of developing limbs shows that number and identity of extra digits is primarily dependent on SHH dosage (29). The severity of the polydactylous phenotype is, therefore, likely to directly relate to the degree of ectopic *Shh* expression generated by the point mutation.

The cat *Hw* mutation is forelimb specific

The correlation between the severity of phenotype and the expression pattern raised an intriguing question concerning the mutations in the domestic cats. The *Hw* and the *UK2* mutations reside at proximal locations within a conserved nucleotide triplet (red box in Fig. 1D). The Hemingway's cats are predominantly affected only on the fore limbs (~80% of the affected cats show polydactyly on the forepaws only; personal communication, K. Jensen). In contrast, cats that carried the *UK2* mutation were affected on all four paws. To examine the possibility that point mutations within this short, conserved nucleotide stretch drive ectopic expression but possess different activities, constructs were made for transgenic analysis using the cat ZRS, and either the *Hw* or *UK2* mutations.

The transgenic embryos carrying wild-type cat ZRS showed a posteriorly restricted pattern as expected; however, the pattern differed from that of the mouse ZRS in that the expression extended further forward along the distal margin (compare the position of the arrow in Fig. 3A with arrow in Fig. 3D). This distal expression was stage-dependent and was most noticeable in the fore limbs; whereas, this pattern was never detected in embryos carrying the mouse ZRS. The nucleotide difference between the mouse and cat CNE domain is ~16% which provides the basis for generating the species-specific patterns. Constructs carrying the cat ZRS and the *Hw* mutation generated expression at the ectopic, anterior margin (Fig. 3B); however, the ectopic expression occurred only in the forelimbs in accordance with the predominant Hemingway phenotype. In contrast, the *UK2* mutation drove ectopic expression in all four limbs in the transgenic assay (Fig. 3C). Thus the cat *UK2* and *Hw* mutations lie two nucleotides apart but generate different ectopic expression patterns that correlate with the limb phenotype.

To examine the significance of the non-conservation surrounding the nucleotide triplet (Fig. 1D), the *Hw* mutation was introduced into the corresponding position in the mouse ZRS. The resulting transgenic embryos showed ectopic activity but, in contrast, expression was detected in both fore

and hind limbs (Fig. 3D). The patterns of ectopic expression at the anterior margin driven by the *UK2* mutations differed from the pattern observed with the mouse *Hx* and M100081 mutations. The ectopic expression was low and occurred in restricted foci or patches of cells at the anterior site. No differences were detected between the *UK2* mutation incorporated into the cat or the mouse ZRS (Fig. 3E). Moreover, taking only the forelimbs into account, the *Hw* mutation showed an ectopic pattern akin to that of the *UK2* mutation with lower and more restricted expression at the anterior margin and again, the species background did not play a significant role.

The mouse transgenic response to the species-specific sequence differences indicates the variation that is possible even within a highly conserved regulatory element. Although it is unclear how the expression pattern relates to species phenotypic differences, it does show the regulatory plasticity that is possible due to sequence differences. These data indicate the potential to generate species expression differences from minor sequence changes within regulatory elements.

The *Hw* and *UK2* mutations pinpoint the conserved nucleotide triplet as essential for appropriate expression. If indeed these conserved nucleotides represent a functional element, a 3 bp stretch is unusually short for a protein-binding domain. This site is presumably affected by other surrounding nucleotides which are not conserved between mouse and cat (Fig. 1D), and the 3 bp compose a site that is variable in evolution. Interestingly, fore-limb specificity is not a property of the nucleotide triplet since the *UK2* mutation, just two nucleotides away, results in expression in all four limbs when contained in either the cat or the mouse enhancer. Hence, both the position and sequence context of the mutation influences the ectopic expression pattern of the mutation.

Neighbouring mutations have different activities

Variability of expression within a highly conserved domain was unexpected. Conservation of small sub-domains within the ZRS might be expected to delineate functional regulatory units and indeed, the similarity between the expression patterns of the Cuban and the M100081 underscore this assumption. To further investigate the expression potential of a highly conserved domain, we focused on the sub-domain of 11 bp that surrounds the mouse *Hx* mutation (Fig. 1D). This sub-domain is completely conserved across vertebrates from mammals to fish, an evolutionary period of over ~400Myr, suggesting that each nucleotide is relevant to ZRS activity. Since the mouse *Hx* mutation drives strong expression at the anterior margin, we asked whether the conserved subdomain represented a functional unit. The neighbouring nucleotide at the 5' side of the *Hx* mutation (*HxNeb*, yellow nucleotide in Fig. 1D) was changed from a T to a C and used in the transgenic assay. Unexpectedly, this change was very weak at producing anterior activity; only one in five expressing embryos (Table 1) showed ectopic activity and the expression pattern (data not shown) was unusual at both the anterior and posterior margins. Not all mutations within conserved nucleotide positions produce ectopic activity at a high rate; thus, the *Hx* and *HxNeb* mutations agrees with the *Hw/UK2* data that mutations even when closely apposed can affect the expression output in dramatically different ways.

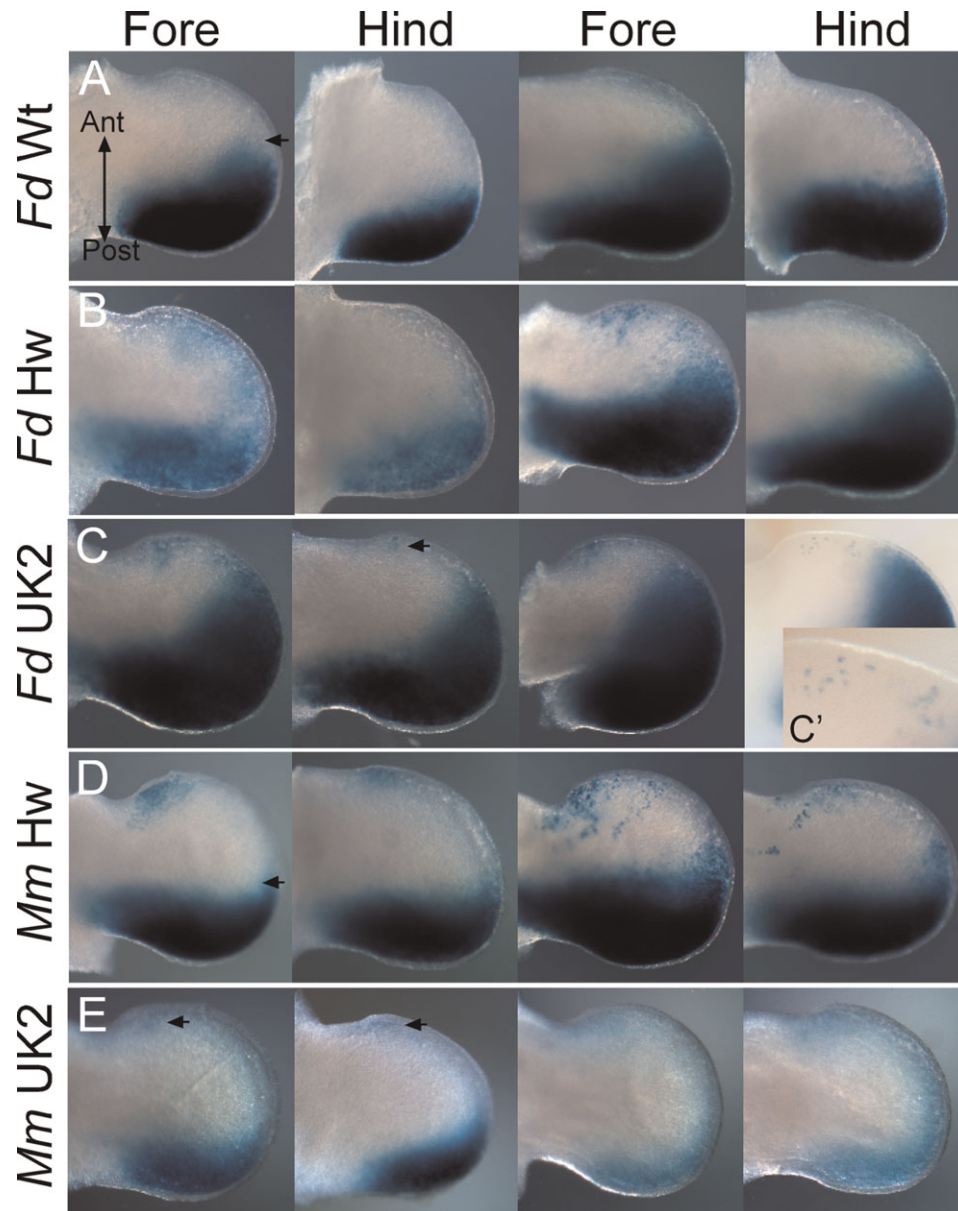


Figure 3. Limb bud expression in transgenic embryos expressing the cat mutations. The fore and hind limb buds from two embryos for each transgenic construct are shown. (A) LacZ expression from the cat wild-type ZRS. In all limb buds shown, the view is from the dorsal side and the anterior and posterior axes are indicated. Posterior expression occasionally extended along the distal margin (indicated by arrow). (B and C) Expression of the Hemingway (*Hw*) and the *UK2* cat mutations, respectively, which reside in a conserved nucleotide triple separated by one basepair. (B) Ectopic expression at the anterior margin is detected only in the fore limbs. Expression is lower in the anterior than the posterior and often appears in patches of cells. In (C) ectopic, anterior expression is also detected in the hindlimbs. The inset C' shows labelled cells scattered along the anterior margin at a higher magnification. (D and E) Expression of the *Hw* and the *UK2* mutations, respectively, contained in the mouse ZRS. Expression in (D) occurs in the ectopic domain in both the hind and the fore limbs. *UK2* mutation (E) shows low anterior expression in both fore and hind limbs.

DISCUSSION

Analysis of polydactylous cats identified three new mutations associated with preaxial polydactyly. As found for human and mouse (8), the cat mutations reside within the ZRS suggesting that the nature of preaxial polydactyly in cats is equivalent to other mammals. Since these mutations produce a limb-specific phenotype in human with no other discernible physiological defects, we submit that this type of polydactyly has no further detrimental affect on the cat's health.

The ZRS mutations that cause preaxial polydactyly operate via a novel disease mechanism. The notion that point mutations can alter the expression pattern rather than inactivate the gene is a crucial step in understanding the disease process. Taking into consideration other genetic analyses of the *Shh* regulatory region, more light is shed on the mechanism of ZRS activity. Clearly, combination of the data from both the transgenic analysis and the ZRS targeted deletion (9) demonstrates that the regulator is both necessary and sufficient to generate the *Shh* limb-specific spatial expression

pattern. In addition, mice that are hemizygous for the ZRS deletion exhibit no detectable limb abnormalities showing that loss of ZRS activity is not responsible for preaxial polydactyly. These observations confirm that the ZRS point mutations are not inactivating but rather act via gain of function mechanisms and thus, the point mutations appear to actively re-direct *Shh* expression to the ectopic site.

Thus far, 12 mutations that lie in the highly conserved 800 bp region of the ZRS have been reported and all are single nucleotide substitutions (7,18,23,30). The mutations are scattered throughout the CNE suggesting that the whole of the element is important in regulating *Shh* in the ZPA of the limb. Each mutation is expected to affect individual protein binding sites. Comparing sequence around the mutations showed that very little similarity exists between the different sites implying therefore, that a number of different protein/DNA interactions are affected. One exception is the comparison of the Hx and the Cuban mutations which both result from G to A substitutions in a four nucleotide sequence ACTG; however, there appears to be no further shared sequence motif that predicts a candidate DNA binding protein (31). The unlikely scenario is that each mutation causes ectopic expression by its own unique mechanism and therefore, we predict it is more likely that the point mutations, perhaps by a number of different routes, affect a general property of the *cis*-regulator.

Another facet of the disease mechanism is that the expression output mediated by the different ZRS point mutations varies. The relevance of these observations is apparent when comparing the expression output of a mutation in the transgenic assay with the general phenotype of the affected family. The Belgian2 (7) and the Cuban (13) mutations, which represent the extreme phenotypic variation, revealed qualitatively different ectopic expression levels in the transgenic assay. This is not restricted to the ectopic expression since the Cuban, and the mouse Hx and M100081, mutations also affects the pattern at the posterior margin. This correlation between expression pattern and phenotype was further corroborated by the expression of the reporter construct containing the cat Hw mutation which is predominantly responsible for forelimb polydactyly. In addition, the Hw nucleotide change revealed species-specific differences and suggests that the sequence context of the mutation can influence the expression output. Thus the degree of mis-expression is dependent on the nature of the mutation.

Although the limb spatial regulatory activity is contained with the ZRS, other genetic abnormalities suggest that additional regulators may modulate limb expression. For example, a recessive mutation is responsible for acheiropodia, a condition in humans that results in severe distal truncations of both the arms and legs (32). A small intrachromosomal deletion that is near, but does not include, the ZRS has been reported as responsible. Initially, it was postulated that the phenotype was caused by interruption of *LMBR1* expression, however, a more plausible explanation is one that includes disruption of long range *Shh* regulation. Thus a modulator of ZRS activity may reside upstream of the ZRS. An interesting mouse phenotype called short digits (*Dsh*) results from a chromosomal inversion around the *Shh* gene and causes misexpression during limb-bud development but at later stages than in PPD (33). *Shh* is ectopically expressed in chondrifying skeletal elements reflecting the expression pattern of the related protein IHH. Loss of an ancient suppressor of *Ihh* expression

activity mediated by the inversion has been postulated to be responsible for the ectopic expression (33,34). At present the complexity of regulators responsible for limb expression is unclear since only the ZRS has been identified. Also, since the transgenes drive ectopic expression outside the endogenous locus, the point mutations within the ZRS are all that is required to generate ectopic expression. However, if other modulators of limb expression exist, it will be of interest to determine the genetic interactions that may occur.

Understanding the range of potential effects of single nucleotide substitutions on *cis*-regulatory elements is of interest for other reasons. Differences in gene regulation due to nucleotide differences called single nucleotide polymorphisms are of increasing interest and are hypothesized to play a major role in defining individual variations within a population (35). In addition, the basis for differences between species (36,37) may be changes at the level of gene regulation. In accord, our analysis showed that point mutations in a key developmental *cis*-regulator can result in additional skeletal elements supporting the notion that small regulatory changes can have profound effects on the morphology of the animal.

MATERIALS AND METHODS

Transgenic analysis

All transgenic constructs were based on a β -globin minimal promoter and bacterial *lacZ* gene (vector 1230, a gift from Robb Krumlauf) (38). Point mutations were made using a QuickChange IIXL site directed mutagenesis kit (Stratagene). In all cases, the entire modified ZRS inserts were sequenced before use. DNA for microinjection was prepared by electroelution [Elutrap (S&S)]. Microinjection and β -gal staining were performed using standard techniques (7).

Cat mutational analysis

Cat buccal swab DNA samples were prepared from brushes (in list of protocols from <http://medicine.ucsd.edu/hypertension/>), the ZRS amplified by PCR, treated with Shrimp alkaline phosphatase and Exonuclease (USB) and sequenced using Big Dye V3 chemistry (ABI). Sequence analysis was conducted using Sequencher software (Gene Codes Corp).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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