Overexpression of *Hoxc13* in differentiating keratinocytes results in downregulation of a novel hair keratin gene cluster and alopecia

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Summary

Studying the roles of Hox genes in normal and pathological development of skin and hair requires identification of downstream target genes in genetically defined animal models. We show that transgenic mice overexpressing *Hoxc13* in differentiating keratinocytes of hair follicles develop alopecia, accompanied by a progressive pathological skin condition that resembles ichthyosis. Large-scale analysis of differential gene expression in postnatal skin of these mice identified 16 previously unknown and 13 known genes as presumptive *Hoxc13* targets. The majority of these targets are downregulated and belong to a subgroup of genes that encode hair-specific keratin-associated proteins (KAPs). Genomic mapping using a mouse hamster radiation hybrid panel showed these genes to reside in a novel KAP gene cluster on mouse chromosome 16 in a region of conserved linkage with human chromosome 21q22.11. Furthermore, data obtained by *Hoxc13 lacZ* reporter gene analysis in mice that overexpress *Hoxc13* suggest negative autoregulatory feedback control of *Hoxc13* expression levels, thus providing an entry point for elucidating currently unknown mechanisms that are required for regulating quantitative levels of Hox gene expression. Combined, these results provide a framework for understanding molecular mechanisms of *Hoxc13* function in hair growth and development.

Key words: *Hoxc13*, Transgenic, Target genes, Alopecia, KAP gene cluster, Mouse

Introduction

Development of skin and hair evidently employs many of the regulatory mechanisms used elsewhere during organogenesis (Oro and Scott, 1998). Based on their distinct spatio-temporal expression patterns in both developing and adult skin, many members of the phylogenetically conserved Hox gene family have been implicated in the patterning, growth and cyclical regenerative processes of skin and hair, as well as in certain skin pathologies including cancer (e.g. Scott and Goldsmith, 1993; Stelnicki et al., 1998; Chang et al., 1998). Although many of the embryonic patterning functions of Hox genes have been successfully defined by gene targeting (reviewed by Krumlauf, 1994; Capecchi, 1997), Hox mutant mice frequently suffer from perinatal lethality, thus precluding analysis of their presumptive roles in postnatal skin and hair follicles. Accordingly, the hairlessness observed in *Hoxc13* loss-of-function mutants is to date the only reported case of a Hox mutant with a skin phenotype (Godwin and Capecchi, 1998). Although these mice develop morphologically normal hair follicles, the brittleness of the rudimentary hair produced causes it to break off at the surface. In interpreting these results, it has been proposed that *Hoxc13* may either directly or indirectly regulate hair-specific keratin genes or, alternatively, play a role in controlling proliferation and differentiation of follicular keratinocytes involved in hair growth.

Hox genes encode transcription factors that have been postulated to act as master switches in development by controlling the activities of batteries of downstream ‘realiser’ genes, also referred to as cytodifferentiation or structural genes (Garcia-Bellido, 1975; Gehring, 1987). Consequently, defining the roles of Hox genes at molecular levels will require identification of these downstream targets in animal models that harbor defined genetic alterations. Information about Hox targets, however, is presently rather tenuous and most of the relatively small number of target genes have been identified in the fruit fly *Drosophila* (reviewed by Graba et al., 1997). In keeping with the diversity of cell types and developmental stages where Hox genes are expressed, these target genes exhibit great heterogeneity and may be grouped into three major functional classes, including regulatory genes that encode either transcription factors or signaling molecules, and the realiser genes, whose products are believed to be directly involved in terminal differentiation. To date, the isolation of
clusters of structurally and functionally similar Hox target genes that might correspond to the hypothesized realisator gene batteries has been elusive.

Studies with both Hox gain- and loss-of-function mutants suggest that the patterning functions of Hox genes are sensitive to their quantitative levels of expression (Pollock et al., 1992; Capecchi, 1997; Greer et al., 2000). This implies existence of control mechanisms that keep the concentration of specific Hox gene products within the range of critical threshold levels. It has been proposed that negative autoregulatory feedback might be a fundamental mechanism for providing this type of stability in expression of transcriptional regulators (Savageau, 1974; Thieffry et al., 1998). While recent data obtained with synthetic gene networks engineered in *Escherichia coli* support this concept (Becskei and Serrano, 2000), its in vivo validity in eucaryotic systems has not yet been demonstrated.

We have addressed essential aspects of these issues by using a transgenic mouse model overexpressing Hoxc13- termed GC13 mice. Our data show that overexpression of Hoxc13 in differentiating keratinocytes of hair follicles results in alopecia. We provide evidence for the existence of a negative autoregulatory feedback loop controlling Hoxc13 expression levels in hair follicles by examining expression of a Hoxc13- lacZ reporter gene construct in both normal and GC13 mice. By using suppression subtractive hybridization (Diatchenko et al., 1996) of cDNAs derived from normal versus GC13 postnatal skin, we have isolated differentially expressed genes as downstream targets of Hoxc13. Consistent with the hair defect, we found that among these genes the largest group consisted of novel, as well as previously known, keratin-associated protein (KAP)-encoding genes, whose expression was repressed by up to more than 20-fold in GC13 skin. Using a mouse-hamster radiation hybrid gene panel, we mapped most of these genes to a narrow distal region of mouse chromosome 16 syntenic to human chromosome 21, thus revealing the existence of a novel KAP gene cluster.

**MATERIALS AND METHODS**

**Sequence analysis**

The *Hoxc13*-coding sequence was derived from overlapping cDNAs obtained by 5' and 3' RACE (rapid amplification of cDNA ends) and confirmed by analysis of corresponding genomic sequences. Total RNA isolated from the posterior halves of E12 embryos was used as template for cDNA synthesis. The presumptive start codon is the most RNA isolated from the posterior halves of E12 embryos was used as template for cDNA synthesis. The presumptive start codon is the most

**Generation of transgenes and transgenic mice**

The GC13 overexpression construct was derived from cosmid cos2W3H (Peterson et al., 1994) as a 19 kb NotI-XhoI fragment and subcloned. Reporter gene construct LZ-GC13 was generated by replacing a singular Ascl fragment of GC13 that contained 430 bp of the 5'-region of *Hoxc13* exon 1 and approximately 50 bp of adjacent intronic sequences (Fig. 1A) with PCR-amplified *E. coli lacZ*, thus creating an in-frame-fusion between the *Hoxc13*- and *lacZ*-coding sequences. The *lacZ* gene was amplified from plasmid pCH110 (Amersham Pharmacia) and Ascl restriction sites were incorporated by PCR using, respectively, the following forward and reverse primers: 5'-GGCGCGGGAAGTCGTTTTTACAACGTCGTGACTGG-3'; 5'-GGCGCGGCCCTTACCGGAATACCGGGCAGAC-3'. GC13 transgenic mice were generated by pronuclear injection of FVB zygotes and mutant 61B1 and 61U4 transgenic lines were established by breeding with FVB mice. In both lines the mutant trait was transmitted to subsequent generations without recognizable changes. Transgenesis was determined by PCR analysis of genomic DNA isolated from tail biopsies or placenta. Forward and reverse primers 5'-GGAGAGGCTTCCGGTACGACATC and 5'-CCATTTGCCCTCC-TTTTCGGTGCC derived from sequences near the 3' and 5' ends of *GC13*, respectively, amplified a diagnostic 400 bp fragment that assumed tandem incorporation of multiple transgene copies. Injection of embryos with LZ-GC13 resulted in a total of six transgenic mice expressing *lacZ* in the same pattern (see Fig. 3) as examined by X-gal staining, essentially as described (Papenbrock et al., 1998). Three of these mice were sacrificed and examined at different embryonic stages, while the remaining were used as founders for establishing three independent LZ-GC13 strains used for further analyses. Preliminary blood and bone marrow analysis of GC13 mutant mice did not reveal hematopoietic defects as a potential cause for the frequent perinatal lethality (data not shown).

**In situ hybridization**

Whole-mount in situ hybridization in E13-E15 embryos was performed as described (Hogan et al., 1994) by using digoxigenin-labeled (Roche Diagnostics) antisense RNA probes synthesized either from plasmid pc13ISH (Peterson et al., 1994), or from plasmid pc13rev containing a partial cDNA of 697 bp that included 532 bp of 3' coding sequence and 165 bp of 3' untranslatable flanking sequence cloned into vector pcR111-TOPO (Invitrogen). Between the two probes, no difference in expression pattern and signal intensity was detectable. In situ hybridization to 10 µm frozen skin sections using 35 S-labeled sense and antisense (control) RNA probes synthesized from pc13rev was performed at 50°C as described (Awgulewitsch and Utset, 1991). Samples were exposed to Kodak NTB2 autoradiographic photoemulsion for 4 days.

**BrdU assays and immunofluorescence microscopy**

Pieces of skin (approx. 0.3×1.0 cm) dissected from anterior dorsal trunk of 2-day-old postnatal mice were incubated in RPMI medium supplemented with 100 µM BrdU for 4 hours, fixed in 3% paraformaldehyde for 30 minutes, and embedded in Tissue-Tek™ OCT compound (Sakura Finetechnology, Tokyo). Cryosections (10 µm) were stained with rabbit anti-β-galactosidase (β-gal) polyclonal antibodies (Eppendorf – 5 Prime), followed by rhodamine-conjugated donkey anti-rabbit IgG (Chemicon International). For BrdU/β-gal double-staining, sections were additionally incubated with FastImmune Anti-BrdU FITC-conjugated antibodies with DNase (Becton Dickinson Immunocytometry Systems) for 45 minutes at room temperature and fixed in 1% paraformaldehyde for 5 minutes before mounting in *ProLong*™ Antifade Reagent (Molecular Probes). Image analysis was performed using a Leitz™ photomicroscope equipped with a Photometrics™ Quantix CCD camera.

**Differential screening**

Screening for differentially expressed genes in skin of GC13 mice was performed essentially as described previously (Tkatchenko et al., 2000). Subtracted/normalized cDNA libraries were generated using the PCR-Select cDNA Subtraction Kit (Clontech). Secondary PCR products were cloned into pCRII-TOPO vector using TOPO TA Cloning Kit (Invitrogen) and resulting libraries were organized in 384-well plates. Bacterial clones from these plates were manually printed onto Hybond-XL nylon membranes (Amersham Pharmacia Biotech) using a 384 pin tool (V&P Scientific) to prepare two identical sets of bacterial colony filters. Hybridization of duplicate filters containing 384 randomly picked clones of either library with subtracted cDNA probes detected 130 differential signals corresponding to 71 under-
Phylogenetic tree analysis and genomic mapping

Phylogenetic tree analysis of amino acid sequences deduced from KAP-related cDNA sequences was performed by using the Genebee Multiple Alignment program (Belozersky Institute, Moscow State University; http://www.genebee.msu.su/services/malign reduced.html). For GenBank Accession Numbers of previously known and novel KAP gene sequences used for this analysis, see Fig. 6A.

Known and novel KAP genes were mapped by using a mouse/hamster radiation hybrid (RH) panel (Research Genetics). PCR data sets obtained with this panel were analysed at the Jackson Laboratory RH database (http://www.jax.org/resources/documents/cmda.html) for determining genomic map positions. PCR cycling conditions were as follows:

HGTpe1.1 (62°C), 5′/5′-GCTCAGGGAGGATACCTTGCAG-3′; R/5′-GCTCTTGCTCAGATATGAC-3′

Pmg1 (62°C), 5′/5′-GATGTGATCCACCCCTATTCTC-3′; R/5′-GTTTGGCAATACAGGAACACT-3′

Hael1 (70°C), 5′/5′-GCCAGTTACACACAGCCTCAG-3′; R/5′-GGCCG-3′

Tagagcaaggattg
g
gagtctgaaaattggggt-3′

tggaggaatacttgctg-3′

Fig. 1. Transgene and Hoxc13 protein sequence. (A) Top: Schematic of genomic clone cos2W3H (Peterson et al., 1994) containing the Hoxc-13 transcription unit within the proximal 19 kb NolI (N)XhoI (X) restriction fragment; map positions of Hoxc-13, Hoxc12 and Hoxc11 homeoboxes are indicated. Bottom: Close-up of 19 kb genomic fragment used as transgene. Boxed regions indicate sequences included in overlapping Hoxc-13-specific cDNAs with presumptive coding regions shown in grey and black (homeobox), and noncoding 5′ and 3′ flanking sequences shown as white boxes; p(A), presumptive AATAAA polyadenylation signal. The LC-GC13 reporter gene was cloned by replacing the 430 bp AscI (A) fragment from the first exon of Hoxc13 with E. coli lacZ as indicated; this created a Hoxc13lacZ open reading frame fusion. (B) Comparison of deduced amino acid sequence of Hoxc13 with those of its paralogous genes Hoxa13, Hoxb13 and Hoxd13 (Mortlock et al., 1996; Zelts er et al., 1996; Hérault et al., 1996). Signs shown at top of the alignment indicate the following degrees of sequence conservation: no sign, average weight of column pair exchanges is less than weight matrix mean value; black dot, average weight of column pair exchanges is less than mean value plus one s.d.; *; average weight of column pair exchanges is more than mean value plus one s.d.; ++, average weight of column pair exchanges is more than mean value plus two s.d.; *+, average weight of column pair exchanges is more than mean value plus two s.d. The homeodomain is underlined. The Hoxc-13-coding sequence has been deposited in the GenBank database, Accession Number AF193796.

RESULTS

GC13 transgenic mice exhibit severe hair and skin defects

To further assess the role of Hoxc13 in hair growth, we generated transgenic mice that overexpressed this gene,

and 59 overexpressed sequences. Sequence analysis of these cDNAs identified a total of 74 different known and unknown sequences, thus revealing an average redundancy of 1.8. High density array filters were prepared with these potentially differential cDNAs to determine their relative levels of expression in normal and GC13 mutant skin by reverse Northern quantitative hybridization as described (Tkatchenko et al., 2000).
employing as a transgene a 19 kb genomic fragment (GC13) containing Hoxc13 (Fig. 1A). Based on genomic and cDNA sequencing, we determined the deduced protein sequence of 328 amino acid residues containing the Hoxc13 homeodomain (Peterson et al., 1994) near its C terminus (Fig. 1B). Sequence comparison revealed, outside the homeodomain, partial similarity at the N terminus to the other three paralogous group 13 Hox proteins (Mortlock et al., 1996; Zeltser et al., 1996; Hérault et al., 1996).

We established two GC13 mutant transgenic lines (61B1 and 61U4) of essentially identical phenotype (Fig. 2). Mutant mice could be identified at birth by a short tail and taut skin (Fig. 2A, part a), as well as kinky whiskers (Fig. 2A, part c), and frequently an overall smaller stature in comparison to non-transgenic littermates (Fig. 2A, part a). Juvenile mutants exhibited retarded growth of coat hair (Fig. 2A, part a), while adult animals developed progressive alopecia and flakiness of the skin at varying degrees (Fig. 2A, part b). Regions most severely affected were ventral and anterior-dorsal trunk and head. Skeletal analysis revealed that the shorter tail was due to a reduction in the number of tail vertebrae by about ten (Fig. 2A, parts e,f). This loss affected exclusively caudal vertebrae without lateral processes and did not involve homeotic transformations of any of the five most anterior caudal vertebrae C1 through C5, which continued to have lateral processes (Fig. 2A, parts e,f).

The overtly recognizable and, with age, rapidly advancing pathological changes in mutant skin were mirrored by progressive histological abnormalities (Fig. 2B). Most obvious changes were epidermal thickening and enlarged hair follicles; the former was apparent already in 5-day-old postnatal skin (Fig. 2B), which is when hair follicles are in the growth phase (anagen) of the first hair cycle (Paus et al., 1999). Follicle enlargement appeared to result primarily from thickened outer root sheaths (ORSs), as observed in anagen follicles of the second hair cycle in 30-day-old skin. In skin of adult mutants older than 2-3 months, many follicles had degenerated into cyst-like structures filled with large amounts of (presumably) keratinaceous material (Fig. 2B). Both the squamous and suprabasal cellular layers, which appeared overall irregular and disorganized, contributed to the dramatic epidermal thickening, underlying the scaly appearance of the skin characteristic of ichthyosis. Severely affected animals typically exhibited ulceration of the skin (data not shown). Scanning electron microscopy of mutant whisker shafts

Fig. 2. Phenotypic changes in transgenic mice overexpressing Hoxc13. (A) Externally discernible changes of skin and skeleton. (a) One week old GC13 (61B1) mutant pup (top) in comparison with non-transgenic littermate (below); note the smaller size, shorter tail and retardation of hair growth exhibited by the mutant. (b) dorsal view of 4.5-month-old GC13 (61U4) mutant female mouse exhibiting severe scaling and crusting of skin, as well as alopecia. Ventral/facial view of a 10-day-old GC13 (61B1) mutant pup (d) in comparison with non-transgenic littermate (c) shows retarded growth of ventral hair coat and kinkiness of mutant whiskers. Alcian Blue and Alizarin Red staining of posterior skeletons of 2-day-old (61B1) mutant (f) and non-transgenic littermate (e) reveals reduction in the number of tail vertebrae in the mutant; ventral view of sacral and caudal vertebrae C1 through C5 shows no changes in the number of vertebrae with lateral processes (arrowheads) between mutant and control. (B) Hematoxylin and Eosin stained sagittal sections (5 μm) of dorsal neck skin derived from normal and GC13 mutant (61B1) littermates sacrificed at 5 days, 30 days and 1 year of age; note the thickened cellular epidermis (E) in mutant skin of each stage and the enormously thickened squamous layer (S) in older mutants; enlargement of mutant anagen hair follicles (F) seen at 30 days appears to result from thickened outer root sheaths; older GC13 mice have distended, cyst-like follicles. (C) Scanning electron micrographs of nasal whiskers from 3-month-old normal and GC13 (61B1) mutant mice. Scale bar in B: 200 μm for B.
revealed changes in cuticular septation that often exhibited lesions and appeared generally denser and less well defined (Fig. 2C). These architectural defects were mirrored by lack of or irregular septation in plucked mutant coat hair, which often showed fracturing near the root (data not shown).

**Negative autoregulatory feedback control of Hoxc13 expression**

Mutant embryos at stages E13 through E15 exhibited higher levels of Hoxc13 expression exclusively in those structures that we observed to be phenotypically affected, namely vibrissae and tail bud at E13 (data not shown), as well as pelage hair follicles beginning at E15 (Fig. 3A). These data suggest the presence of control elements required for Hoxc13 expression in developing hair follicles and tail bud within the GC13 transgene. This assumption was confirmed by analyzing the expression pattern of a lacZ-tagged version of the GC13 transgene termed LZ-GC13 (Fig. 1A), which was expressed in tailbud and vibrissae at E13, and in coat hair follicles at later (≥E15) stages (Fig. 3B, parts a,c). To monitor LZ-GC13 expression in GC13 mutant background, we generated LZ-GC13/GC13 double transgenic mice. Surprisingly, these mutant mice showed reduced levels of X-gal staining in neural tube and tail bud at E16.5 (Fig. 3B, parts c,d), i.e. the same sites where we detected increased levels of Hoxc13 expression by WMISH in GC13 mice (Fig. 3A). These results suggest the existence of a negative autoregulatory feedback loop, which is probably required for maintaining balanced physiological levels of Hoxc13 expression. To obtain evidence for the existence of such a control mechanism during postnatal hair growth and development, we examined Hoxc13 RNA and LZ-GC13 b-gal expression in sectioned skin of 5-day-old normal and GC13 mutant mice. While the in situ hybridization data clearly indicated substantially increased levels of Hoxc13 expression in the upper bulb and lower shaft regions of GC13 versus normal anagen hair follicles (Fig. 3C), the levels of LZ-GC13 reporter gene activity in the same regions presented the reverse situation, i.e. LZ-GC13 expression was drastically reduced in LZ-GC13/GC13 mutant compared with normal hair follicles in LZ-GC13 mice (Fig. 3D). Combined, these data suggest employment of a negative autoregulatory feedback mechanism that, either directly or indirectly, controls Hoxc13 expression levels during development of both axial skeleton and hair follicles.

**Hyperproliferation of keratinocytes in skin of GC13 mice**

To examine whether the enlargement of hair follicles and the thickening of the epidermis in mutant skin reflect changes in the proliferation rates of precursor cells, we performed bromodeoxyuridine (BrdU) assays with 2-day-old normal and mutant skin. The results showed an increase in the number of
The mutant hair phenotype is thus directly linked to 5 d GC13 mice. We chose this developmental stage because skin, we screened for differentially expressed genes in skin of underlie the pathological changes observed in GC13 mutant animals. As a first step toward defining the molecular mechanisms that downstream targets

Identification of hair-specific genes as Hoxc13 downstream targets

As a first step toward defining the molecular mechanisms that underlie the pathological changes observed in GC13 mutant skin, we screened for differentially expressed genes in skin of 5 d GC13 mice. We chose this developmental stage because the more advanced pathological manifestations seen at later stages could result in secondary and increasingly nonspecific changes in gene expression, whereas earlier skin contains only a small fraction of fully differentiated hair follicles (Paus et al., 1999). Using a strategy based on suppression subtractive hybridization (SSH) (Diatchenko et al., 1996; Tkatchenko et al., 2000), we isolated 74 potentially differential cDNA clones. Reverse northern quantitative hybridization of these cDNAs using complex cDNA probes (Fig. 5A), identified a total of four upregulated and 25 downregulated sequences (Fig. 5B). The overexpressed cDNAs corresponded to four known genes that included, most conspicuously, keratin complex 2 gene 1 (K1), whose overexpression in mutated form in transgenic mice reproduced features of epidermolytic hyperkeratosis (Bickenbach et al., 1996), a skin disorder with similarities to the one observed here. Of apparent relevance is also the overexpression of a member of the 14-3-3 protein gene family. The 14-3-3 proteins are known to play a role in keratinocyte differentiation and proliferation and interact with K8 and K18 keratins in a cell-cycle- and phosphorylation-dependent manner (Liao and Omary, 1996).

Remarkably consistent with a Hoxc13 role in hair follicle development and hair growth is our finding that the 25 downregulated cDNAs included five sequences that corresponded to previously known hair-specific KAP-encoding genes, as well as nine novel sequences that were found to have closest similarities to KAP genes of the high glycine tyrosine (HGTp) class, including the murine HGTpII.1 (Krtap6-1 – Mouse Genome Informatics) and HGTpI-alpha (Krtap8-2 – Mouse Genome Informatics; Aoki et al., 1997).
Fig. 5. Identification of genes differentially expressed in GC13 skin. (A) Reverse northern quantitative hybridization. 30 ng of each of 74 plasmid DNAs were spotted in triplicate on two filters hybridized in parallel with normal and GC13 skin complex cDNA probes. Washed filters were exposed to an imaging plate and scanned images (STORM 860 scanner, Molecular Dynamics) analyzed and quantified using Image Quant (Aoki et al., 1997). The previously reported expression patterns of the phylogenetic tree and genomic mapping analysis indicate that 

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</table>

(Figs 5B, 6A). We propose to designate the nine novel HGTp-type KAP genes Krtap16-1 to Krtap16-9 because our phylogenetic tree and genomic mapping analysis indicate that they belong to a novel cluster of structurally similar genes (see below). The previously reported expression patterns of the identified known KAP genes, HGTpII.1 and HGTpI-alpha (Aoki et al., 1997), Pmg1 (Krtap14 – Mouse Genome Informatics), Pmg2 (Krtap15 – Mouse Genome Informatics; Kuhn et al., 1999) and Hacll (Krtap11-1 – Mouse Genome Informatics; Huh et al., 1994), as well as the IF keratin gene Krt1-1 (Bertolino et al., 1988), extensively overlap with the GC13 transgene expression pattern in differentiating keratinocytes of the hair shaft (Figs 3, 4). This, combined with the high levels of downregulation of between 2.5- and 21.5-fold for this subgroup, suggests that these genes are downstream targets of Hoxc13.

Apart from the keratin and KAP genes, a conspicuous member of the group of repressed genes is agouti-related protein (Agrp), whose 13-fold downregulation might be of relevance to the overall smaller stature and reduced body weight, as well as the skin phenotype of GC13 mice. Like its close structural relative, agouti protein, Agrp is a paracrine signaling molecule that has been implicated in the control of body weight as a hypothalamic Mc4r melanocortin receptor antagonist, as evidenced by obesity in transgenic mice that ubiquitously overexpressed Agrp (Ollmann et al., 1997). Recent findings of expression in numerous tissues in chicken, including skin, could suggest a role for Agrp in regulating peripheral melanocortin systems as well (Takeuchi et al., 2000). However, further information about Agrp expression pattern and function in skin will be required for understanding the significance of its downregulation in GC13 skin.

**Genomic mapping of a novel KAP gene cluster controlled by Hoxc13**

Insight into the structural and phylogenetic relationships among the identified KAP-related cDNAs was gained by generating a phylogenetic tree using a multiple sequence alignment program (see Materials and Methods). The data revealed that the nine novel KAP cDNAs were structurally more related to each other and to HGTpII.1 and HGTpI-alpha than to the remaining known KAP genes identified (Fig. 6A), thus suggesting that they correspond to members of a new distinct subgroup which we propose to designate Krtap16-n, according to human genome organization (HUGO) keratin gene nomenclature (http://www.gene.ucl.ac.uk/). Furthermore, our sequence comparison of the nine novel KAP-related cDNA sequences, Krtap16-1 to Krtap16-9, against public databases revealed highest similarity to a previously unidentified cluster of KAP-related sequences located in the human chromosome...
Fig. 6. Phylogenetic tree analysis and genomic mapping of KAP genes controlled by Hoxc13. (A) Phylogenetic tree analysis of previously known and novel KAP genes found to be downregulated in GC13 mice. Clustering of the novel Krtap16 genes together with the previously known HGTP II.1 and HGTP I-alpha genes suggests closer phylogenetic relationships among members of this group than to the other genes (Pmg1, Pmg2 and Hael1) included in the analysis. GenBank Accession Numbers for the sequences used: AF03691 (Pmg1), NM013713 (Pmg2), U30686 (Hael1), D86420 (HGTP II.1), D86422 (HGTP I-alpha), AF345291 (Krtap16-1), AF345292 (Krtap16-2), AF345293 (Krtap16-3), AF345294 (Krtap16-4), AF345295 (Krtap16-5), AF345296 (Krtap16-6), AF345297 (Krtap16-7), AF345298 (Krtap16-8), AF345299 (Krtap16-9). (B) Schematic representation of genomic mapping results: KAP genes (shown in regular type) analyzed here map between the two distal markers Grik1 and Sod1 (shown in bold type) on mouse chromosome 16 (MMU16), a region of conserved linkage with human chromosome HSA21q22.11 region. Novel KAP genes Krtap16-1 to Krtap16-8 cluster together with HGTP II.1 and Hael-1, distal to Tiam1 and occupy a conserved position in the HSA21q22.11 region as indicated; the more distantly related Pmg1 and Pmg2 genes map proximal to Tiam1.

21q22.11 region (Hattori et al., 2000). As this region is of conserved linkage with the distal region of mouse chromosome 16, we predicted clustering of the Krtap16 genes in that part of the mouse genome. This assumption was confirmed by mapping Krtap16-1 to Krtap16-8 by the two distal markers Tiam1 and Sod1 on mouse chromosome 16 using a mouse/hamster radiation hybrid panel (Fig. 6B). Of the known KAP genes identified as presumptive Hoxc13 targets, we mapped the HGTP II.1 and HGTP I-alpha, which are structurally similar to the Krtap16 genes, to the same region, as well as the more distantly related Hael-1. However, the structurally distinct Pmg1 and Pmg2 genes, which are known to be closely linked to each other (Kuhn et al., 1999), were mapped proximal to the Tiam1 marker (Fig. 6B).

DISCUSSION

Our data show that increased levels of Hoxc13 expression in hair follicles during pre- and postnatal development cause severe hair and skin defects including alopecia and a progressive hyperproliferative disorder resembling ichthyosis. The hair phenotype is similar to the hair growth defects observed recently in Hoxc13-targeted mice (Godwin and Capecchi, 1998), thus suggesting that the hair defects exhibited by our two independent GC13 transgenic strains are Hoxc13 specific. Accordingly, one can conclude that the level of Hoxc13 expression is a crucial parameter for proper hair growth and development. These results support the concept of antipodal regulation of Hox genes stating that overexpression and underexpression may result in similar phenotypic changes (Pollock et al., 1992). Apparently, this concept is of relevance also for other developmental regulatory genes encoding transcription factors as demonstrated by the influence of human PAX6 gene dose on eye development in transgenic mice (Schedl et al., 1996). In that case, overexpression on wild-type background caused distinct eye abnormalities similar to the murine Pax6 mutant phenotype small eye.

Negative feedback regulation of Hoxc13 expression

The apparent great sensitivity of certain cell populations to changes in the expression levels of crucial transcriptional regulators suggests the existence of control mechanisms capable of providing stable physiological expression levels. Useful models for such mechanisms in mammalian systems might be synthetic gene circuits engineered in E. coli, which facilitate examining the behavior of individual system components quantitatively and, thus, allow to make predictions about system stability (Gardner and Collins, 2000). Following this approach by using circuits that consisted of a regulator and repressor modules has demonstrated that negative feedback can increase system stability by dramatically reducing variability in gene expression (Becskei and Serrano, 2000), an essential requirement for homeostasis. In view of these findings, our own data that indicate involvement of a negative feedback mechanism in the control of Hoxc13 expression levels provide significant new insight into the regulation of a Hox gene, in this case a gene that plays a pivotal role in hair growth and development. As both over- and underexpression of individual members of a group of genes encoding structural components of hair, i.e. the hair keratin genes, has been shown to result in severe hair growth abnormalities including brittleness and hair loss (e.g. Powell and Rogers, 1990; Schlake et al., 2000), one may conclude that their normal expression levels need to be well balanced and tightly controlled. Presumably, this control will not tolerate wide fluctuations in the expression levels of
upstream transcriptional regulators, a group of genes that, as we have shown here, includes Hoxc13.

The sequential activation of Hox genes in the embryo occurs in distinct, though overlapping, anterior-posterior (A/P) expression domains whose order is co-linear with the map positions of the corresponding Hox genes in their transcriptionally unipolar clusters (Krumlauf, 1994). Accordingly, genes located at the 3' end of a Hox cluster will become activated first and occupy the most anterior domain, while subsequent expression of genes located further 5' will become increasingly posteriorly restricted.

Within their initial A/P expression domains, each Hox gene is typically active in cells of different morphogenetic lineages, and thus may contribute to the formation of various organ systems (Krumlauf, 1994). During subsequent stages of embryogenesis, these initial expression domains undergo dynamic changes and many Hox genes continue to be active in a wide variety of tissues during postnatal development and adulthood. Orchestrating these complex patterns of gene activity requires elaborated regulatory circuits.

Although little is known about mechanisms controlling quantitative levels of expression, considerable insight has been gained into qualitative and temporal control mechanisms. Accordingly, translocation experiments within the Hoxd cluster that involve placement of anteriorly expressed genes into the genomic environment of ‘posterior’ genes, suggest that, within cluster context, the time point of activation is not autonomously regulated by the control elements specific for the translocated gene, but largely determined by its relative proximity to the 5' end of its cluster (van der Hoeven et al., 1996). Furthermore, deletion of genomic segments upstream of the Hoxd cluster revealed that the successive 3'→5' activation is regulated by control elements located upstream of the cluster, which apparently mediate a silencing mechanism (Kondo and Duboule, 1999). Release of this silencing mechanism is believed to render downstream target genes progressively accessible for transcription (Kmita et al., 2000), involving the function of multiple and dispersed local control elements that regulate a multitude of subsequent temporal and regional specificities in expression (Krumlauf, 1994). Some elements have been shown to function as components of positive auto- and crossregulatory circuits which are believed to be essential for maintaining Hox gene activity (e.g. Gould et al., 1997), whereas repressor elements might be required for the refinement of expression boundaries (e.g. Bieberich et al., 1990; Nonchev et al., 1997). Although this has not yet been demonstrated, we speculate that some of these inhibitory elements might play a role in stabilizing quantitative levels of expression as system components in negative feedback circuits, and Hoxc13 might serve as a paradigm for elucidating these quantitative regulatory mechanisms.

Identification of Hoxc13 downstream target genes

A prerequisite for understanding the complex patterning functions of Hox genes is to identify their downstream target genes. Although the number of Hox targets in Drosophila alone has been estimated to range in the thousands (Biggin and McGinnis, 1997), to date only relatively few presumptive downstream genes have been isolated in both flies and vertebrates. About two to three dozen Drosophila Hox targets have been isolated by using various approaches (reviewed by Graba et al., 1997), including genetic studies (Bienz, 1994), enhancer trapping (Wagner-Bernholz, 1991), immunoprecipitation of in vivo formed Hox protein/DNA complexes (e.g. Gould et al., 1990; Graba et al., 1992), cDNA subtraction (Feinstein et al., 1997) and the selection of genomic fragments that confer reporter gene activity on Hox gene expression in yeast cells (Mastick et al., 1995). Only a handful of mammalian Hox downstream targets, including the mouse homolog of the Drosophila tumor suppressor gene lethal(2) giant larvae (Tomotsune et al., 1993), Ncam (Jones et al., 1993), the human Lamin B2 DNA replication origin (de Stanchina et al., 2000), the cyclin-dependent kinase inhibitor p21 (Bromleigh and Freedman, 2000) and the tumor suppressor gene p53 (Raman et al., 2000) have been identified by immunoprecipitation and a combination of in vivo and in vitro protein/DNA-binding methods. For the majority of these fly and mammalian downstream genes, it can currently not be ascertained whether they are being directly or indirectly targeted by Hox proteins.

We have used suppression subtractive hybridization (SSH) of cDNAs (Diatchenko et al., 1996) combined with reverse northern quantitative hybridization to identify genes that are differentially expressed in 5-day-old postnatal skin of GC13 mutant mice. Our approach is conceptually similar to a more conventional cDNA subtraction successfully pursued by Feinstein and co-workers that resulted in the isolation of Ubx target genes in Drosophila (Feinstein et al., 1995). In that case, cDNAs differentially expressed in fly embryos ubiquitously expressing Ubx upon heat shock induction were enriched by subtraction and used as a complex probe for screening a genomic library, thus isolating five previously known genes activated by Ubx.

Apart from using a different animal model system, our screen differs in several important ways from those studies. First, in contrast to other subtractive hybridization methods, which usually favour enrichment of highly expressed mRNAs, a key feature of SSH is equalization of enrichment levels among highly, moderately and rarely expressed messages; this greatly increases the overall efficiency of the screening process. Second, instead of ubiquitously expressing Hoxc13, overexpression was restricted to the normal activity domains of this gene; this strategy should minimize the risk of isolating presumptive targets that might be irrelevant for the normal function of this gene. Third, our screen aimed at identifying unregulated, as well as downregulated targets. As our results indicate, the latter group is highly relevant, as it comprises by far most of the downstream genes identified. This raises the question of to what extent this particular outcome could have been predicted.

To answer this question, it is useful to consider two competing models for the role of DNA binding of Hox proteins in their functional activity and specificity, as proposed by Biggin and McGinnis (Biggin and McGinnis, 1997). The first predicts that sequence-specific binding of Hox proteins requires cooperative interactions with co-factors such as Pbx and Exd. This so called ‘co-selective binding model’ is supported by data that show distinct DNA-binding specificities of different Hox protein/Pbx complexes (Pöppel et al., 1995; Mann and Chan, 1996). In contrast, the second model suggests that most Hox proteins bind independently (without prior heteroduplex formation with co-factors) to a large number of binding sites that contain a simple core recognition sequence.
where they principally act as repressors. According to this ‘widespread binding model’, DNA-bound Hox protein will be transformed from transcriptional repressor to activator only upon secondary interaction with specific co-factor(s). Under the premise of increased ratios of Hoxc13 protein/co-factor(s) in our GC13 mice, this model would predict a shift towards a presumptive transcriptional repressor function of Hoxc13 and thus a downregulation of target genes, a speculation that is consistent with our data. As this model is based on direct Hox protein-DNA interactions, one might further conclude that most of the downregulated genes identified here are direct transcriptional targets for Hoxc13. However, invoking the ‘co-selective binding model’ for predicting the effects of excessive Hoxc13 protein under the assumption of limited co-factor supply might favor a more neutral outcome with respect to the activation or repression of presumptive targets, because Hox/Pbx complexes have been shown to be capable of acting either as transcriptional activators or repressors depending on association with additional co-factors (Saleh et al., 2000). Furthermore, one might predict indirect effects due to generally reduced availability of presumptive co-factors shared among Hoxc13 and other Hox proteins, i.e. excessive Hoxc13 might act as a co-factor sink.

**Clustering of realisator genes**

KAP genes form by far the largest group of presumptive Hoxc13 targets identified in this screen, i.e. 14 out of a total of 29 genes, including nine that have been classified as novel KAPs. All of these KAPs are downregulated by between approx. 2.5-fold (Pmg1) to more than 20-fold (Krtap 16-9) when compared with normal levels (Fig. 5A,B). KAP gene products presumably are involved in alignment and lateral crosslinking of keratin intermediate filaments (IFs), thus playing an important role in epidermal and follicular keratinocyte differentiation (Powell and Rogers, 1997; Fuchs, 1995). Structural similarities allow us to distinguish several different groups of KAPs whose distinct expression patterns reflect the functional diversity among keratinocytes. While certain structurally unique epidermal KAPs, such as fillagrin and loricin, are encoded by spliced transcripts (Markova et al., 1993; Rothnagel et al., 1994), all follicular KAPs identified to date appear to be encoded by small intron-less genes (Powell and Rogers, 1997). Based on their amino acid composition, the latter group has been classified into high sulfur proteins (containing up to 40% cysteine), and the high-glycine/tyrosine (HGT) proteins (Powell et al., 1991; Powell and Rogers, 1997). Both classes include several subfamilies summarized designated as Krtaps (keratin-associated proteins) followed by a number specifying the family and its subfamilies summarily designated as Krtaps (keratin-associated proteins) followed by a number specifying the family and its subfamilies summarized

Krtap8 gene on bovine chromosome 1 (HSA21q22.1; Harlizius et al., 1997).

The conserved genomic organization and clustering of KAP genes may reflect a constraining force of relevance for coordinate expression. This view is consistent with the sequential activation of keratin and KAP genes in distinct subpopulations of keratinocytes during follicle differentiation (Powell and Rogers, 1997). Accordingly, the first class of genes expressed is the group of IF keratin genes, followed by the HG Tp class and later by high sulfur protein genes. Furthermore, expression data obtained with several similar HGT-p-type KAPs indicate temporal progression of activation of members of the same subgroup (Aoki et al., 1997), i.e. genes that (according to our genomic mapping results) cluster closely together with the novel Krtap16 genes. This apparent sequential activation of KAP genes is reminiscent of the Hox gene activation patterns, and once the HGT-p-type Krtap16 gene cluster has been characterized in more detail, it will be of great interest to see whether spatio-temporal expression patterns of its members occurs in a co-linear fashion with respect to their map position – a feature currently unique to the Hox clusters (Krumlauf, 1994).

It has been proposed that Hox genes coordinate the activities of thousands of realisator genes in a concerted fashion, because independent control of the realisators would result in chaotic development (Gehring, 1987). One would predict that during evolution this logistical demand has been coped with by clustering of functionally similar realisator genes. Our identification of such a realisator gene cluster controlled by Hoxc13 supports this concept and provides an experimental system for studying the mechanisms of its regulation.

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