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Physical and genetic interactions between *Alx4* and *Cart1*

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SUMMARY

Alx4 and Cart1 are closely related members of the family of transcription factors that contain the paired-type homeodomain. In contrast to other types of homeodomains, the paired-type homeodomain has been shown to mediate high-affinity sequence-specific DNA binding to palindromic elements as either homodimers or as heterodimers with other family members. Alx4 and Cart1 are co-expressed at several sites during development, including the craniofacial mesenchyme, the mesenchymal derivatives of neural crest cells in the first branchial arch and the limb bud mesenchyme. Because of the molecular similarity and overlapping expression pattern, we have analyzed the functional and genetic relationships between Alx4 and Cart1. The two proteins have similar DNA-binding activity in vitro and can form DNA-binding heterodimers; furthermore, they activate transcription of reporter genes that contain high-affinity DNA-binding sites in cell culture in a similar manner. Therefore, at least by these criteria, the two proteins are functionally redundant. Analysis of double mutant animals reveals several genetic interactions. First, mutation of Cart1 exacerbates Alx4-dependent polydactyly in a manner that is dependent on gene dosage. Second, there are complex genetic interactions in the craniofacial region that reveal a role for both genes in the fusion of the nasal cartilages and proper patterning of the mandible, as well as other craniofacial structures. Third, double mutant mice show a split sternum that is not detected in mice with any other genotype. Interpreted in the context of the biochemical characterization, the genetic analysis suggests that Alx4 and Cart1 are indeed functionally redundant, and reveal both unique and redundant functions for these genes in development.

Key words: *Cart1*, Alx4, Paired-type homeodomain, Polydactyly, Mouse

INTRODUCTION

The homeodomain is an evolutionarily conserved motif that mediates DNA binding in a large number of developmentally important transcription factors. Biochemical analysis shows that most homeodomain proteins can bind the sequence 5′ TAAT 3′ as monomers (Gehring et al., 1994). This raises an interesting question: how can the similar DNA-binding activities of different homeodomain proteins account for their ability to precisely regulate development? One possible resolution to this paradox has been the realization that different protein-protein interactions are utilized to alter the specificity of DNA binding, such that most homeodomain proteins form dimeric complexes that recognize extended DNA sequences with increased affinity; the increased complexity of the dimeric recognition sequence is sufficient to impart biologic specificity. One example of the way in which protein interactions influence DNA binding is represented by the ability of some proteins encoded by the homeotic selector genes of *Drosophila*, such as *Ultrabithorax*, to bind specific DNA sequences only as part of a complex with the *extradenticle* protein, which contains a divergent homeodomain (Chan et al., 1994; van Dijk et al., 1994). This cooperative dimeric DNA binding to sequences sufficiently complex to impart biologic specificity is believed to underlie at least some of the biologic functions of homeodomain-containing proteins.

Another form of dimeric DNA binding mediated by homeodomain proteins involves the ability of a subfamily of homeodomain proteins, those that contain the paired-type homeodomain, to cooperatively form DNA-binding homodimers. Wilson et al. (1993) have demonstrated that the paired-type homeodomain, and not other homeodomains, interacts with palindromic repeats of the consensus monomer binding sequence, 5′ TAAT 3′, separated by a variable number of nucleotides; the structural features that mediate this form of binding are contained entirely within the homeodomain. The specificity of DNA binding with regard to the preferred spacing between the two half-sites is largely determined by the amino acid identity of residue 50, with those proteins that harbor a glutamine or lysine at this position displaying a preference for a three nucleotide spacer (P3 site, 5′ TAAT NNN ATTA 3′), and proteins that harbor a serine residue at this position showing a preference for a two nucleotide spacer (P2 site, 5′ TAAT NN ATTA 3′) (Wilson et al., 1993). In addition to defining the optimal spacing between the half sites, the identity of residue 50 also contributes to the DNA-binding specficity by generating preferred residues within the spacer region. Thus, proteins that

contain the paired-type homeodomain with a glutamine at residue 50 are predicted to bind the P3 sequence with high affinity, thereby forming a family of transcription factors with redundant DNA-binding activity (Wilson et al., 1993, 1995). The similar DNA-binding activity of these proteins might be expected to generate a family of transcription factors with redundant functions, and the ability of these proteins to form heterodimeric DNA-binding complexes may further contribute to the redundancy at sites of co-expression of these proteins.

Alx4 and Cart1 are two recently identified members of the vertebrate family of proteins that contain the paired-type homeodomain without a paired domain (Zhao et al., 1993; Qu et al., 1997a). The two proteins are highly related within the homeodomain (55 out of 60 residues identical, 60 out of 60 similar), have a glutamine at position 50 and, based on the data described above, are predicted to cooperatively bind the P3 sequence. Outside the homeodomain, the proteins are highly divergent, with little sequence conservation except for a small block of approximately 20 amino acids at the C terminus, which is shared by many other proteins that contain the paired-type homeodomain and has been referred to as the paired-tail (Mathers et al., 1997).

During development, *Alx4* and *Cart1* are expressed almost exclusively in mesenchymal cells, with many areas of overlapping expression (Zhao et al., 1994; Qu et al., 1997b). Both genes are prominently expressed in craniofacial mesenchyme, especially in mesenchymal cells of the frontonasal mass. In addition, overlapping expression is detected in the mesenchymal cells of the first branchial arch. *Alx4* shows high level expression in the mesenchyme of the anterior limb bud, while *Cart1* is expressed at low levels in mesenchymal cells throughout the developing limb bud. Finally, both genes are expressed in mesenchymal cells in the lateral mesoderm that will eventually contribute to the cartilages and bones of the sternum and ribs (Zhao et al., 1994; Qu et al., 1997b). Despite the molecular similarlity and the overlapping expression of the two genes, mice homozygous for targeted disruptions of the respective genes show no phenotypic overlap. *Alx4* knockout mice show three major phenotypes: (1) completely penetrant preaxial polydactyly, which is associated with the formation of an ectopic anterior zone of polarizing activity (ZPA) in the anterior limb bud during development, (2) a ventral body wall defect that results in herniation of the abdominal contents at the time of birth in greater than 98% of homozygous animals, and (3) a temporal delay in the formation of the parietal bone of the skull (Qu et al., 1997b). Mice homozyogous for a *Cart1* null mutation have a neural tube closure defect over the midbrain that results in exencephaly and acrania (Zhao et al., 1996). Remarkably, this defect is substantially rescued by the administration of folic acid to the mother during gestation (Zhao et al., 1996). Importantly, on the 129/Sv genetic background, heterozygotes for either gene are apparently normal (Zhao et al., 1996; Qu et al., 1997b).

Alx4 and Cart1 regulate two processes, limb patterning and neural tube closure, that are genetically complex. In the case of both genes, the analysis of mutant mice provides evidence for genetic complexity. On the 129/Sv background, $Alx4^{+/-}$ mice have no polydactyly and homozygotes have a single extra digit; in contrast, heterozygous F_1 progeny of a 129/Sv × C57BL/6 mating show hindlimb polydactyly (Qu et al., 1997b, 1998). Further evidence for a role for strain-specific modifier genes comes from the analysis of *Strong's luxoid* (*lst*) polydactylous mice, which harbor inactivating mutations in the *Alx4* gene (Qu et al., 1998). Homozygotes on the *lst* strain background have severe polydactyly, with up to nine digits per limb, and heterozygotes have hindlimb polydactyly, with a single extra preaxial digit (Qu et al., 1998). Similarly, the neural tube closure defect of $Cart1^{-/-}$ mice is subject to strain variability in penetrance; exencephaly is observed in 100% of homozygotes on the 129/Sv background, but in only 70% of 129/Sv × C57BL/6 hybrid animals that are homozygous for a *Cart1* mutation (Zhao et al., 1996). The strain-dependent variations in phenotype of both *Alx4* and *Cart1* mutant mice support the idea that these genes participate in processes that are both developmentally and genetically complex.

Because Alx4 and Cart1 are structurally related within the homeodomain, and, on the basis of exisiting data, are likely to possess similar DNA-binding activity as both homodimeric and heterodimeric DNA-binding complexes, we considered it likely that the two genes might perform redundant functions at sites of co-expression during development. To address this hypothesis, we have characterized the DNA-binding activities of the two proteins in vitro, their transcriptional regulatory properties in cell culture, and their genetic interactions in vivo. We find that Alx4 and Cart1 do in fact show similar DNA-binding and transcriptional regulatory properties, demonstrating that, at least for some activities, they are functionally redundant. In vivo, the two genes show strong genetic interactions, including exacerbation of the phenotypes of the single gene mutants, gene dose-dependent interactions and the generation of novel skeletal abnormalities at sites of co-expression. The results demonstrate that the two genes have both unique and overlapping functions, and reveal new functions for these genes during development.

MATERIALS AND METHODS

Gel-shift assays

Peptides containing the homeodomains of Alx4 (residues 185-265) and Cart1 (residues 128-195) were expressed as $(His)_6$-tagged peptides in *E. coli* BL21 and purified to homogeneity by chromatography on Ni^{+2}-NTA agarose as previously described (Qu et al., 1998). Gel-shift reactions contained 15 mM Tris pH 7.5, 75 mM NaCl, 1.5 mM EDTA, 0.3% NP-40, 0.8 μg dIdC, 4 mM spermidine, 4 mM spermine, 1.5 mM DTT and 7.5% glycerol. After incubation on ice for 10 minutes, ^{32}P-labelled probe was added and the mixture was incubated at room temperature for 15 minutes before separation on 5% polyacrylamide gels that contained 0.5× TBE. In competition experiments, a 50-fold molar excess of the competing oligonucleotide was added during the preincubation phase. In preliminary experiments, DNA binding was shown to be dependent on protein concentration. The sequence of the gel-shift probes is shown (top strand):

P1/2: 5′ CCTGAGAA**TAAT**CTGAGGACTGTACA 3′
P2: 5′ CCTGAGAA**TAAT**CG**ATTA**CTGTACA 3′
P3: 5′ CCTGAGAA**TAAT**CCG**ATTA**CTGTACA 3′
P4: 5′ CCTGAGAA**TAAT**CCGG**ATTA**CTGTACA 3′
P5: 5′ CCTGAGAA**TAAT**CCTGG**ATTA**CTGTACA 3′
P3mut: 5′ CCTGAGAATGGTCCGAGGACTGTACA 3′

Reporter gene assays

Three copies of the indicated sequences were cloned upstream of a basal promoter (from the adenovirus *E1b* gene) and chloramphenicol acetyl transferase (CAT) gene in the plasmid pE1b-CAT to generate

the indicated reporters (P1/2-CAT, P2-CAT, etc.). The Alx4 expression plasmid pCMX-Alx4 has previously been described (Qu et al., 1997a); the coding sequences of the Cart1 cDNA were cloned into pCMX (Umesono et al., 1991) to generate the expression plasmid pCMX-Cart1. Transient transfectons utilized 0.5 μg of the indicated reporter construct, 0.5 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia) and 0.1 μg of the indicated expression plasmids. DNA samples were transfected into 293 (human embryonic kidney) cells using Lipofectamine (Gibco); in preliminary experiments, it was shown that 293 cells do not express either Alx4 or Cart1, do not harbor P3 DNA-binding activity and do not efficiently express any of the indicated reporter constructs. 40 hours after transfection, lysates were made, and CAT and β-galactosidase activities were determined by previously described methods.

Mice

Mice harboring targeted mutations of the *Alx4* and *Cart1* genes have previously been described (Zhao et al., 1996; Qu et al., 1997). As both Alx4-dependent polydactyly and Cart1-dependent exencephaly are sensitive to changes in the strain background, all analysis was carried out in 129/Sv mice. Embryos and mice were genotyped by polymerase chain reaction (PCR) using the following primers:

Alx4: wild-type: 5′ CCTGACTTGTGGTGTCACTGC 3′ (forward primer) and 5′ CAGCATTCTAGGTCTCCCACAG 3′ (back primer) yields a 350 bp product;

mutant: 5′ TCTATAGATCTCTCGTGGGATCA 3′ (forward primer) and 5′ CAGCATTCTAGGTCTCCCACAG 3′ (back primer) yields a 400 bp product.

Cart1: wild-type: 5′ AGGAAGTTGGCAGACGAATAAAG 3′ (forward primer) and 5′ AGCCATTTCGCTGCCACGTAC 3′ (back primer) yields a 500 bp product;

mutant: 5′ ACTTCCTGACTAGGGGAGGAG 3′ (forward primer) and 5′ AGCCATTTCGCTGCCACGTAC 3′ (back primer) yields a 450 bp product.

PCR conditions were 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute for a total 35 cycles.

Skeletal preparations

Newborn mice were killed, eviscerated, placed in a 70°C water bath for 1 minute and skinned. Skeletons were fixed in 100% ethanol for 3 days, followed by staining with Alcian blue (15 mg of Alcian blue in 80 ml ethanol/20 ml glacial acetic acid) for 8-12 hours. Skeletons were rinsed in 100% ethanol overnight and cleared in 2% KOH for 12 hours. Staining for bone was carried out using Alizarin red (50 mg/l in 2% KOH) for 3 hours. Skeletons were then cleared in 2% KOH and stored in glycerol. Skeleton preparations were analyzed for at least five animals of each genotype.

Whole-mount in situ hybridization

Whole-mount in situ hybridization experiments were performed as previously described (Qu et al., 1997b). Probes were digoxigenin labelled and visualization was by alkaline phosphatase reaction. The probes used were: a 1.2 kb *Eco*RI fragment containing the 3′UTR of the Alx4 cDNA, a 400 bp fragment of the Cart1 cDNA, a 1.2 kb fragment of mouse Hox D13 cDNA, a 600 bp fragment of mouse patched and a 640 bp fragment of mouse Shh cDNA (Dolle et al., 1989; Echelard et al., 1993; Zhao et al., 1994; Goodrich, et al., 1996; Qu et al., 1997b). At least two animals of each genotype and developmental stage were analyzed by whole-mount in situ hybridization. In the case of Shh and HoxD13 expression at E11.5 in the limb bud, at least four embryos (16 limb buds) were analyzed for each genotype.

RESULTS

DNA binding Alx4 and Cart1

To address the ability of Alx4 and Cart1 to bind DNA, the homeodomains of each protein were expressed with a $(His)_6$ tag in *E. coli* and purified by chromatography on Ni^{+2}-NTA agarose. To facilitate the detection of heterodimeric complexes, the proteins contained different amounts of sequence outside the homeodomain, such that by SDS-PAGE analysis, the Alx4 peptide was 15.5 kDa and the Cart1 peptide was 12 kDa (Fig. 1A).

Based on previous data (Wilson et al., 1993), the purified proteins were used in gel-shift reactions on a panel of probes containing differing arrangements of the 5′ TAAT 3′ half site. Both proteins bound a probe that contained a single half-site as monomers (P1/2) (Fig. 1B). Consistent with previously published data (Wilson et al., 1993), both monomeric and dimeric binding was detected to probes that contained palindromic arrangements of the two half sites separated by a variable number of nucleotides (P2, P3, P4 and P5). With each probe, the amount of DNA binding as both monomeric and dimeric protein-DNA complexes was similar for the two proteins (Fig. 1B). Importantly, only the P3 site (5′ TAAT CTG ATTA 3′) was bound with strong cooperativity by both proteins, resulting in a large increase in the amount of dimeric protein-DNA complex (Fig. 1B); modest cooperativity was detected for binding to the P2 and P4 sites (a detailed analysis of the cooperative nature of the binding will be described elsewhere). No binding was detected to probes that did not contain a the 5′ TAAT 3′ half site (data not shown). Thus, Alx4 and Cart1 show similar DNA-binding activity across a panel of palindromic sequences, with cooperative dimeric binding greatest to P3 elements.

As a separate way of testing the DNA-binding activity of these homeodomains, the ability of a 50-fold excess of unlabelled oligonucleotide to compete binding to a labelled P3 probe was tested (Fig. 1C). None of the other arrangements of the 5′ TAAT 3′ half-site competed for binding when present in 50-fold molar excess, nor did a mutant probe (P3mut) in which both half-sites were specifically mutated. We conclude that, in vitro, both proteins show a similar preference for the P3 sequence over other tested sequences. The results are consistent with previously published data on the specificity of paired-type homeodomain-DNA interactions (Wilson et al., 1993).

To test the ability of the proteins to form DNA-binding heterodimers on the P3 DNA sequence, gel-shift assays were carried out using a mixture of the two proteins. Complexes corresponding to the two individual homodimeric complexes were detected, as was a new complex with intermediate mobility, indicative of an Alx4-Cart1 heterodimer (Fig. 1D). Importantly, the results do not rule out the possibility that Alx4 or Cart1 bind other DNA sequences as part of a complex with other proteins. The results do demonstrate, however, that with regard to binding to the P3 sequence, Alx4 and Cart1 have essentially identical activity and, furthermore, the two proteins interact physically to form heterodimeric DNA-binding complexes. Thus, the Alx4 and Cart1 homeodomains possess similar DNA-binding activity on the DNA sequences tested, a result consistent with the high degree of sequence similarity.

Transcriptional activation by Alx4 and Cart1

The overlapping DNA-binding activities of Alx4 and Cart1 prompted us to compare their abilities to regulate transcription in cell culture. We generated a panel of synthetic reporter

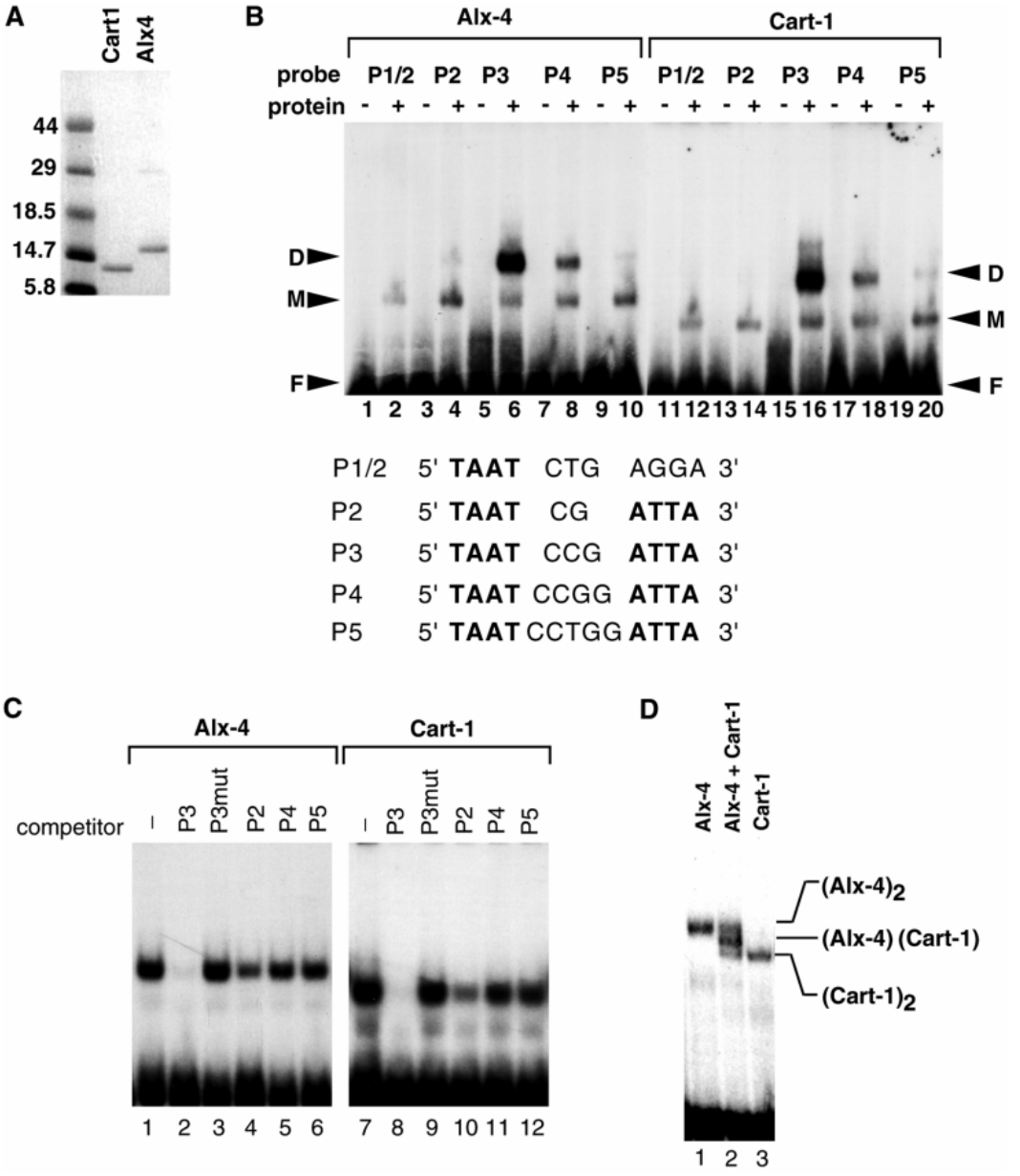

Fig. 1. Alx4 and Cart1 have similar DNA-binding activity to palindromic elements. (A) The Alx4 and Cart1 homeodomains were expressed in *E. coli* and purified to homogeneity. The purified peptides were separated by SDS-PAGE and the gel was stained with Coomassie blue. The position of the molecular mass markers is shown. (B) The purified Alx4 and Cart1 proteins were used in gel-shift reactions using the indicated gel-shift probes. The migration of the monomeric protein-DNA complex (defined by binding to the P1/2 probe, which contains a single copy of the sequence 5′ TAAT 3′) is designated by an M; the migration of dimeric protein-DNA complex is designated by a D; and F designates the free probe. Note the preference for dimeric protein-DNA complex formation on the P3 probe. (C) Alx4 and Cart1 homeodomains were used in gel-shift reaction using the P3 probe and the different oligos were used in 50-fold molar excess as unlabelled competititor DNA. Note that only the P3 oligo competes for binding to either protein. (D) Gel-shift reactions were carried out using a P3 sequence as probe and Alx4, Cart1, or a mixture of Alx4 and Cart1. The complex with intermediate mobility, indicative of a heterodimeric protein-DNA complex, is indicated.

constructs harboring the indicated sequences upstream of the chloramphenicol acetyl-transferase gene (P2-CAT, P3-CAT, etc.). All of the reporters were expressed at very low levels after transient transfection of human 293 cells (Fig. 2); this was consistent with the absence of gel-shift activity using 293 cell nuclear extracts on the indicated probes (Qu et al., 1998, and data not shown). However, cotransfection of either an Alx4 or Cart1 expression plasmid resulted in high level induction of CAT activity from the P3-CAT reporter (Fig. 2). Induction was dependent on the presence of P3 sites in the reporter; the weaker binding to P2 and P4 sites detected in vitro was not sufficient to support transcriptional activation. The magnitude of the induction by Alx4 and Cart1 was similar over a large range of input plasmid DNA, suggesting that the two proteins are approximately equipotent in activating the P3 reporter construct (Fig. 2 and data not shown). Thus, both proteins appear to regulate transcription in a similar manner.

Co-expression of Alx4 and Cart1 with the P3 reporter resulted in additive, but not synergistic, activation, supporting the idea that Alx4 and Cart1 regulate transcription in a similar manner (Fig. 2). Because dimerization by Alx4 and Cart1 is a consequence of cooperative DNA binding, and neither protein exists as a dimer in solution, direct detection of heterodimer formation by co-immunoprecipitation was not possible (data not shown). We conclude that, with regard to in vitro DNA binding and transcriptional regulation in cell culture, Alx4 and Cart1 have similar activities; furthermore, the two proteins interact physically in vitro, and probably in vivo. The functional overlap and physical interaction between the two proteins provides a framework for the interpretation of the genetic interactions presented below.

Genetic interactions between Alx4 and Cart1

The similarities between Alx4 and Cart1 in biochemical

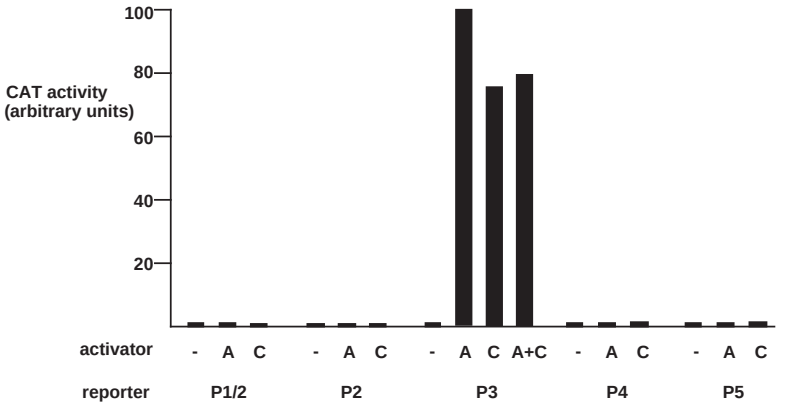

Fig. 2. Transcriptional activation by *Alx4* and *Cart1*. Reporter constructs harboring three copies of the indicated sequence upstream of a basal promoter from the adenovirus *E1b* gene were transfected into human 293 embryonic kidney cells along with 100 ng of the indicated expression construct (or 50 ng each of *Alx4* and *Cart1* expression plasmid, A+C). 2 days after transfection, cell lysates were prepared and CAT activity was determined. Note both Alx4 and Cart1 efficiently activate a reporter containing P3 elements.

properties suggested that the genes and proteins may function in a redundant manner during development. This motivated us to examine the genetic interactions between Alx4 and Cart1 by generating double mutant animals. Because the phenotypes of both *Alx4*$^{-/-}$ and *Cart1*$^{-/-}$ animals vary according to the strain background, both mutations were maintained on the 129/Sv background.

Alx4 and *Cart1* heterozygotes were crossed to generate double heterozygotes, which were apparently normal. Double heterozygotes were then intercrossed to generate progeny of all possible genotypes. Embryos were dissected and examined at E19.5, shortly before birth. Animals homozygous for either *Alx4* or *Cart1* were identified on the basis of the phenotypes specific for each gene. All *Alx4*$^{-/-}$ animals had abdominal wall defects that were severe and resulted in herniation of the abdominal contents, regardless of concommitant *Cart1* mutation; likewise, all *Cart1*$^{-/-}$ animals had exencephaly, regardless of the *Alx4* genotype.

Cart1 mutation modifies Alx4-dependent polydactyly

A major determinant of anterior-posterior polarity within the developing limb bud is the Zone of Polarizing Activity (ZPA), a group of mesenchymal cells in the posterior aspect of the limb bud (Saunders and Gassling, 1968; for review, see Johnson and Tabin, 1997). The polarizing activity of the ZPA is mediated in large part by Sonic hedgehog (Shh) (Riddle et al., 1993), which exerts its patterning activity, at least in part, by regulating the expression of genes of the HoxD cluster, including *HoxD13*. In many strains of polydactylous mice, including *Alx4* mutant animals, an ectopic ZPA and ectopic domains of *Shh* and *HoxD13* expression are formed along the anterior aspect of limb bud during development (Chan et al., 1995; Masuya et al., 1995, 1997; Qu et al., 1997b). The ectopic *Shh* expression, together with the fact that Alx4 is normally expressed in the anterior mesenchyme of the limb bud during develoment, suggests that Alx4 normally functions, directly or indirectly, to repress *Shh* expression and ZPA formation in anterior mesenchymal cells of the limb bud. The ectopic ZPA that forms in *Alx4* mutant animals is much smaller than the normal ZPA, and arises later in develoment (E11.0 for the ectopic ZPA vs. E9.25-9.5 for the normal ZPA) (Qu et al., 1997).

The *Cart1* gene is expressed at low levels in mesenchymal cells of the limb bud without any evidence of an anterior-posterior gradient, and the limbs and axial skeleton of *Cart1*$^{-/-}$ animals are normal (Zhao et al., 1994, 1996). Nonetheless, *Cart1* mutation exacerbates the polydactyly of *Alx4*$^{-/-}$ animals. *Alx4*$^{-/-}$/*Cart1*$^{-/-}$ mice have an increased number of digits (typically seven on the hindlimb, and six or seven on the forelimb); in addition, at least one tibia is reduced in size in double mutant animals (Fig. 3). Both the increased number of digits and the tibial reduction (hemimelia) show variable expressivity: although every double mutant animal showed one hindlimb with seven digits and tibial hemimelia, often the contralateral limb had six digits and normal zeugopods; the expressivity was similarly variable in the forelimb. In contrast, we have never detected hemimelia in *Alx4*$^{-/-}$ animals. Although hemimelia is associated with other genetic forms of polydactyly, such as mutation of the zinc finger transcription factor Gli3, the mechanism of the relationship between these two is not clear (Schimmang et al., 1992; Hui and Joyner, 1993). The polydactyly was also exacerbated in *Alx4*$^{-/-}$/*Cart1*$^{+/-}$ mice, and hemimelia was detected, although it was not as severe as in double homozygotes (Fig. 3). We conclude that *Cart1* mutation modifies the polydactyly of *Alx4*$^{-/-}$ mice; furthermore, exacerbation of the polydactyly is dose dependent, with *Alx4*$^{-/-}$/*Cart1*$^{+/-}$ animals showing a phenotype that is intermediate in severity compared to *Alx4*$^{-/-}$ animals and double mutant homozygotes. The results are consistent with the hypothesis that Cart1 and Alx4 function in a redundant manner to repress *Shh* expression and ZPA formation in the anterior mesenchyme of the limb bud.

Directly or indirectly, Alx4 functions to repress Shh expression in the anterior mesoderm of the limb bud. As noted above, the ectopic domains of *Shh* and *HoxD13* expression arise later and are physically smaller than the normal posterior domains. ZPA grafting experiments in the chick have documented a clear relationship between the amount of ZPA tissue grafted and the extent of the resulting limb duplication; as few as 30 ZPA cells grafted to the anterior limb bud yield a single extra digit, while larger grafts are required for complete duplications (Tickle, 1981). Given the correlation between the size of ZPA grafts and limb duplications in the chick, we considered the possibility that the enhanced polydactyly in double mutant mice might be associated with an ectopic ZPA that arises earlier in development or is physically larger. To test this idea, we performed whole-mount in situ hybridization experiments on wild-type, *Alx4*$^{-/-}$ and double mutant embryos using probes for both Shh and HoxD13. While there was, in general, a trend towards a larger ectopic ZPA in the double

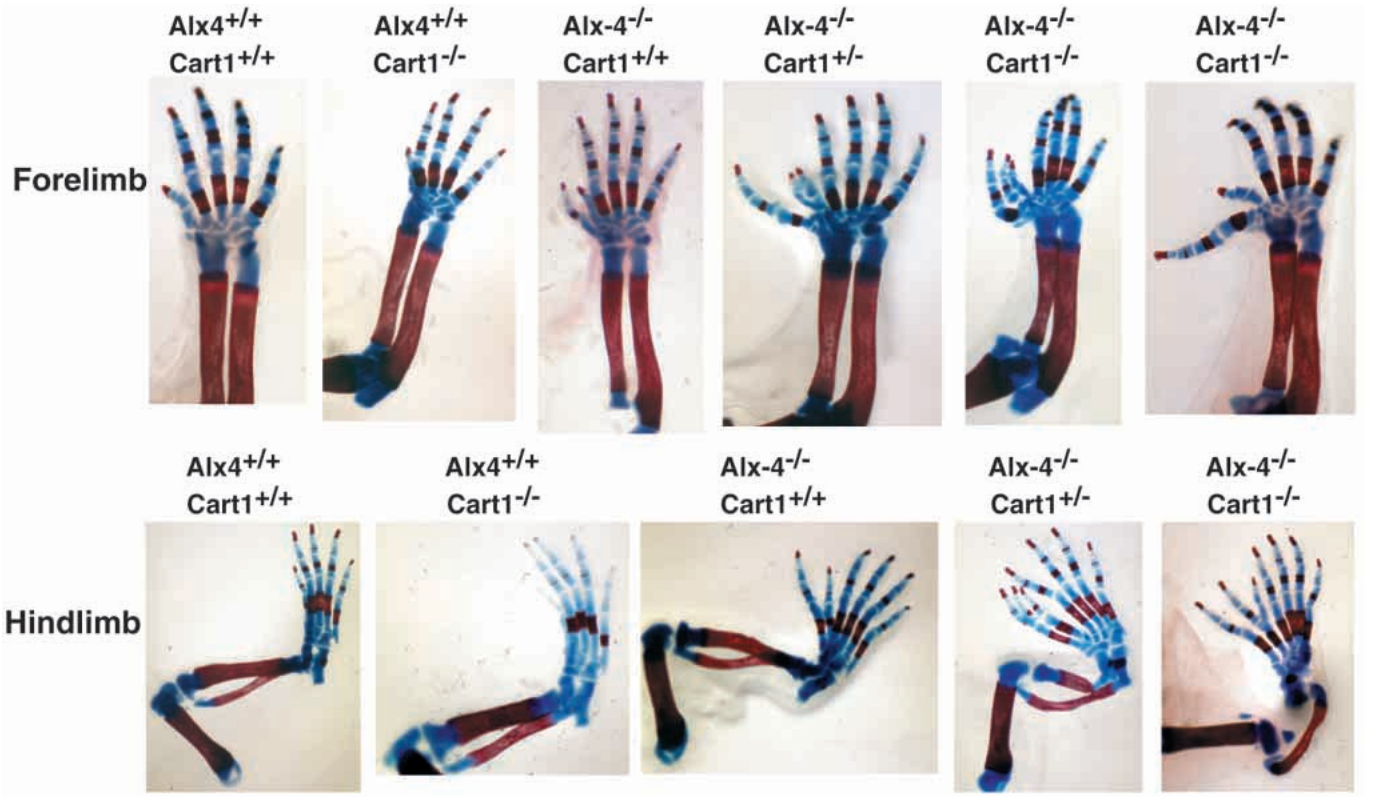

Fig. 3. Cart1 mutation enhances Alx4-dependent polydactyly. Mice heterozygous for mutations in *Alx4* and *Cart1* were intercrossed to generate embryos of all possible genotypes. Shown are skeletion preparations stained with Alizarin red (bone) and Alcian blue (cartilage) of the forelimbs and hindlimbs of E19.5 embryos of the indicated genotypes. In the forelimb, note the increase in length of the ectopic first digit in double mutant animals; in the hindlimb, there is reduction of the tibia in *Alx4*$^{-/-}$/*Cart1*$^{+/-}$ and *Alx4*$^{-/-}$/*Cart1*$^{-/-}$ animals. Note also the increase in hindlimb digit number in double mutant animals.

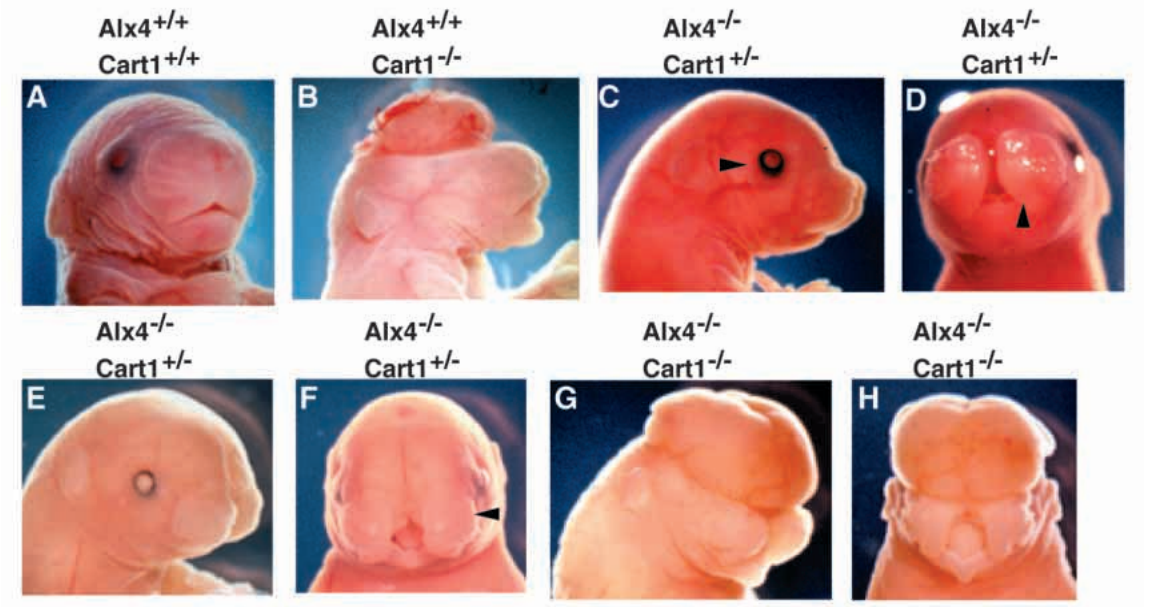

Fig. 4. Craniofacial anomalies in *Alx4*/*Cart1* double mutant mice. Shown are the faces of E19.5 embryos of different genotypes. *Alx4* mutant animals have normal appearing faces, while *Cart1* mutant embryos have exencephaly that leads to distortion of structures in the face (B); however, there is no facial or palatal clefting. The facial and nasal cleft in *Alx4*$^{-/-}$/*Cart1*$^{+/-}$ animals is of variable severity, with some animals showing a severe midline defect (E,F), and others showing a smaller defect (C,D). In double mutant animals, the facial clefting is even more severe and is associated with Cart1-dependent exencephaly (G,H).

mutant mice, the pattern was not invariant (data not shown). Given the fact that the polydactyly shows variable expressivity, with some double mutant limbs harboring seven or eight digits, and some six, this result is perhaps not surprising. This result is also consistent with previous analysis of *lst* mice, in which the size of the ectopic ZPA found during development does not precisely correlate with the severity of the polydactyly phenotype (Chan et al., 1995).

Craniofacial abnormalities in double mutant animals

Both *Alx4* and *Cart1* are prominently expressed in craniofacial mesenchyme, cells derived from the cranial neural crest that will form most of the cartilage and bone structures of the face; expression of both genes is especially high in the frontonasal mass (Zhao et al., 1994; Qu et al., 1997b). In addition, both genes are expressed in mesenchymal cells of the first branchial arch, cranial neural crest derivatives that are fated to form the maxilla, mandible and a number of small bones of the base of the skull.

The major phenotype of *Cart1*$^{-/-}$ mice is exencephaly due to a failure to close the neural tube over the midbrain (Zhao et al., 1996); this phenotype was not modified by concommitant *Alx4* mutation. The exencephaly detected in *Cart1*$^{-/-}$ animals results in a number of secondary defects in the formation of craniofacial bones and cartilages, including broadening and shortening of the nasal bones and cartilages, and absence of the bony plates that form the skull (Zhao et al., 1996). In *Alx4*$^{-/-}$ mice, the only abnormality in the craniofacial region is a decrease in the size of the parietal bone at the time of birth (Qu et al., 1997). If Alx4 and Cart1 function in a redundant manner during development, then combinatorial defects in structures derived from craniofacial and first branchial arch mesenchyme might be detected. Because *Cart1*$^{-/-}$ mice have a number of facial defects that are likely to be secondary to the exencephaly, we focused our attention on *Alx4*$^{-/-}$/*Cart1*$^{+/-}$ animals.

Alx4$^{-/-}$/*Cart1*$^{+/-}$ animals showed a number of craniofacial defects not present in either *Alx4*$^{-/-}$ or *Cart1*$^{+/-}$ animals. The most striking of these was the presence of a midline fusion defect that resulted in cleft face and cleft palate (Fig. 4). This defect was completely penetrant in animals of this genotype, although the severity was variable. Consistent with the idea that this defect was a consequence of redundancy, the cleft face was also detected in animals of other genotypes with at least three mutant alleles. Thus, although *Cart1*$^{-/-}$ animals have facial abnormalities, these do not include cleft face, but this defect was present in *Alx4*$^{+/-}$/*Cart1*$^{-/-}$ and *Alx4*$^{-/-}$/*Cart1*$^{-/-}$ animals (Fig. 4 and data not shown). Skeletal preparations of the craniofacial region showed that the cleft faces were associated with a failure of the cartilages that will form the nasal septum to fuse in the midline (Fig. 5). Thus, animals that harbor three mutant alleles in any combination display cleft face associated with failure to fuse the septal cartilages. The fact that this phenotype was detected in Alx4$^{-/-}$/Cart1$^{+/-}$ animals demonstrates that this abnormality is not secondary to the neural tube closure defects that are detected only in Cart1$^{-/-}$ mice.

In addition to the midline fusion defect, several other craniofacial abnormalities were present. There was a decrease in the size of the mandible in the anterior-posterior dimension. In particular, the portion of the mandible anterior to the alveolar process was truncated (Fig. 6), an interesting

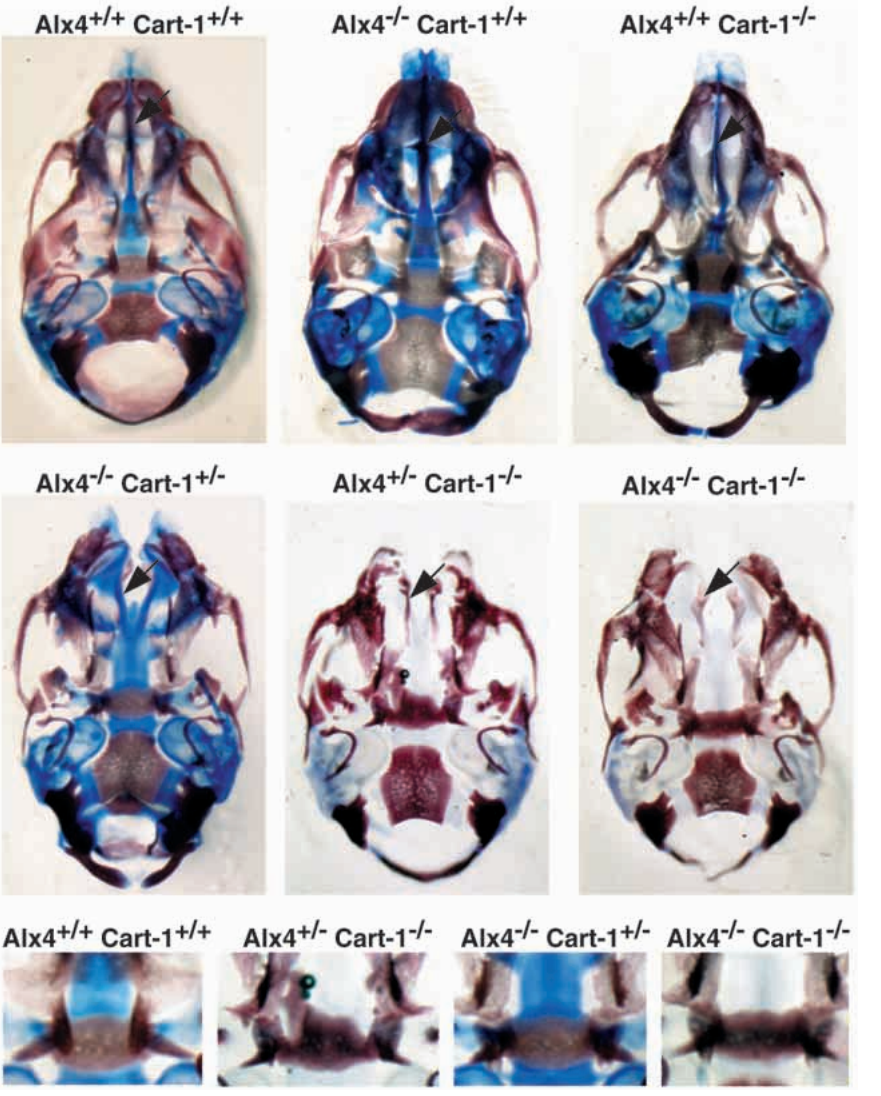

Fig. 5. Failure to fuse the cartilages of the nasal septum in *Alx4/Cart1* mutant mice. Shown are dorsal views of Alizarin red (bone)/Alcian blue (cartialge) stained skulls; the plates of the skull and the mandible have been dissected off to allow the bones of the base of the skull to visualized. The top panels show skeletal preparations of E19.5 embryos of the indicated genotypes. The arrows indicate the septal cartilages, which fail to fuse in *Alx4*$^{-/-}$/*Cart1*$^{+/-}$, *Alx4*$^{+/-}$/*Cart1*$^{-/-}$ and *Alx4*$^{-/-}$/*Cart1*$^{-/-}$ embryos, resulting in cleft face. In addition, the basispenoid (bottom panels) bone is misshapen in double mutant animals. The decreased cartilage staining of the double mutants is due to variability in staining, not a decrease in the amount of cartilage formed.

finding in view of the fact that Alx4 expression in the first branchial arch is restricted to mesenchymal cells along the anterior aspect. This abnormality was most prominent in double homozygotes, but was also detected in Alx4$^{-/-}$/Cart1$^{+/-}$ and Alx4$^{+/-}$/Cart1$^{-/-}$ mice (data not shown). These genotypes were also associated with malformation of the basisphenoid bone at the base of the skull (Fig. 5). In addition to the facial abnormalities, there was a decrease in the size of the frontal, parietal, occipital and temporal plates of the skull in *Alx4*$^{-/-}$/*Cart1*$^{+/-}$ mice compared to *Alx4*$^{-/-}$ animals (data not shown). Finally, *Alx4*$^{-/-}$/*Cart1*$^{+/-}$ animals were born with open eyes; this abnormality was not observed in animals with other mutant genotypes, as the eyes are not present in Cart1$^{-/-}$ mice. The entire constellation of abnormalities gave rise to newborn mice with abnormal facial appearance that was of variable severity, ranging from animals with a modestly shortened mandible and small facial cleft to animals with markedly misshapen heads and large facial clefts (Fig. 4). Thus, Alx4 and Cart1 function is required for the fusion of the nasal cartilages in the midline, as well as for full outgrowth of the mandible. Although the mechanisms responsible for the intricate patterning of the skeletal elements of the face are not as well understood as limb patterning, the presence of dose-dependent genetic interactions in the midline fusion of facial structures is consistent with a model of functional redundancy.

Sternal abnormalities

Mice mutant for both *Alx4* and *Cart1* displayed a novel sternal phenotype not present in either single mutant. In all double homozygotes, the sternum was split and the rib insertions were abnormal; in some animals, there was a decrease in the number of ribs that were attached to the sternum (Fig. 7). In contrast to the other phenotypes, this defect was detected only in double homozygotes. The developmental basis of this phenotype is not clear, but both genes are expressed in mesenchymal cells in the lateral mesoderm, the cells that are fated to form the sternum (Zhao et al., 1994; Qu et al., 1997b).

Sonic hedgehog signaling in *Alx4*/*Cart1* mutant mice

The polydactyly observed in *Alx4*$^{-/-}$ mice is a consequence of ectopic expression of Shh in mesodermal cells along the anterior aspect of the limb bud (Qu et al., 1997). Many of the phenotypes detected in *Alx4*/*Cart1* double mutants are midline fusion defects that are observed in mice that harbor mutations that result in ectopic activation of the Shh signaling pathway. These include the following: (1) preaxial polydactyly is detected in Gli3 mutant mice and patched mutant mice, as well as in trangenic mice that ectopically express *Shh* in the anterior limb bud, (2) neural tube closure defects similar to the Cart1 neural tube defect are observed in transgenic mice that ectopically express *Shh* in the dorsal neural tube, as well as in *Gli3* mutant mice, (3) sternal defects are observed in K14-Shh transgenic mice as well as in *Gli2/Gli3* mutant mice, and (4) cleft palate and cleft face is observed in K14-Shh transgenic mice as well as in *Gli2/Gli3* mutant mice (Schimmang et al., 1992; Echelard et al., 1993; Hui and Joyner, 1993; Goodrich et al., 1997; Mo et al., 1997; Oro et al., 1997). Together with the previous demonstration that *Alx4* mutant mice have ectopic *Shh* expression in the limb bud, these similarities suggested that the abnormalities detected in *Alx4/Cart1* mutant mice might be a consequence of aberrant Shh signaling at several sites during development.

To directly test this idea, we examined the expression of both *Shh* and *patched*, a putative transcriptional target of Shh signaling (Goodrich et al., 1996), in *Alx4/Cart1* double mutant mice. Using in situ hybridization on wild-type, *Alx4*$^{-/-}$, *Cart1*$^{-/-}$ and double mutant embryos at E9.5, E10.5, E11.5, E12.5 and E13.5, we did not find any differences in expression of either *Shh* or *patched* between wild-type and double mutant animals, with the exception of the ectopic activation of *Shh* and *patched* in the limb buds of *Alx4* mutant mice, which has already been described (Chan et al., 1995; Goodrich et al., 1996; Qu et al., 1997b). Thus, at sites other than the limb bud, we find no direct support for the idea that the developmental abnormalities detected in *Alx4/Cart1* mutant animals are a consequence of deregulated Shh signaling. While the developmental basis of the midline defects in *Alx4/Cart1* double mutant mice remains to be defined, these are likely to occur by Shh-independent mechanisms. This is analogous to the situation with *Gli2* and *Gli3*, in which double mutant analysis reveals both Shh-dependent and -independent phenotypes (Mo et al., 1997).

DISCUSSION

The *Alx4* and *Cart1* genes encode two closely related proteins; they are most closely related within the homeodomain (55/60 residues identical, 60/60 similar) and less so over the remainder of the proteins. The homology within the homeodomain and the co-expression at several sites in the developing embryo suggested that these proteins might display both functional and developmental redundancies. Biochemical analysis shows that Alx4 and Cart1 have essentially identical DNA-binding activities, at least on the panel of target sequences surveyed, and that the two proteins can form DNA-binding heterodimers. The redundancy at the level of DNA binding is associated with functional similarity in terms of the ability to activate reporter genes in cell culture. Although the results do not exclude the possibility that the proteins may have unique biochemical functions that we have not identified, they demonstrate that Alx4 and Cart1 have significant functional overlap. The functional similarities are associated with strong genetic interactions. Specifically, Cart1 functions as a dose-dependent enhancer of Alx4-dependent polydactyly, there are complex phenotypes generated in the craniofacial region and a novel phenotype, a split sternum, is present in double homozygotes. All of the genetic interactions occur at sites of co-expression during development, suggesting functional redundancy underlies these abnormalities. The results reveal previously unidentified roles for *Cart1* in limb patterning, for *Alx4* in the patterning of craniofacial structures, and for both genes in the the formation of the sternum and the ribs.

Biochemical properties of Alx4 and Cart1

Previous analysis of the DNA-binding properties of the paired type homeodomain suggested that both Alx4 and Cart1 would bind the palindromic sequence 5′ TAATNNNATTA 3′ (P3 site) cooperatively as homodimers (Wilson et al., 1993, 1995). Our data confirm this prediction; in addition, we demonstrate that the two proteins are able to form DNA-binding heterodimers,

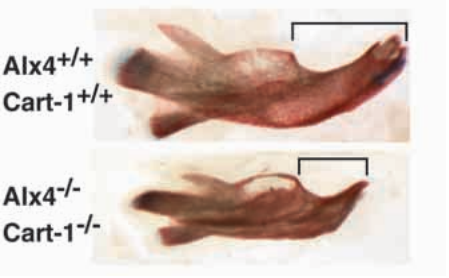

Fig. 6. Mandibular truncations in double mutant mice. Shown are mandibles from E19.5 wild-type and double mutant embryos. Note that the shortening of the mandible is predominantly restricted to the region anterior (distal) to the alveolar process (bracketed), which surrounds the molars.

at least in vitro. Both proteins function as sequence-specific transcription factors with similar efficiency. It is interesting to note that the biologic response is more stringently restricted to P3 sites than the in vitro DNA-binding activity. The results demonstrate that transcriptional activation is mediated exclusively through P3 sites and suggest that biologic function is more likely to depend on dimeric binding to palindromic sequences than on binding to the more ubiquitously distributed half sites, imparting biologic specificity to this class of proteins.

The molecular mechanisms that can cause novel synthetic phenotypes in double mutants are not limited to redundancy; other causes include blocks at consecutive points in a biochemical pathway or the involvement of the two genes in separate biochemical pathways that impinge on a developmental process. Does functional redundancy between *Alx4* and *Cart1* underlie the generation of the double mutant phenotypes? Three lines of evidence support such a model. First, Alx4 and Cart1 show remarkable similarity in both DNA binding and transcriptional regulation in the assays we have employed. Second, the novel phenotypes in double mutants are detected only in structures that are derived from regions that are sites of co-expression during development. Third, the polydactyly and craniofacial abnormalities show sensititivity to gene dose, such that animals with three out of four mutant alleles show more severe phenotypes than animals mutant for only one gene. These observations suggest that the genetic

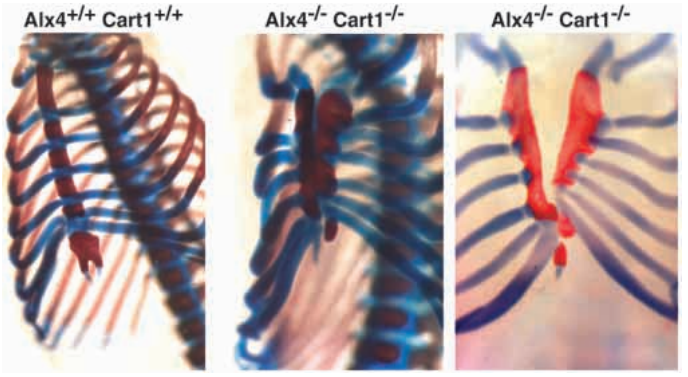

Fig. 7. Sternal abnormalities in double mutant mice. The sternum and ribs of wild-type and double mutant newborns, stained with Alizarin red and Alcian blue, is shown. Note the split sternum and abnormal rib fusions in the double mutants.

interactions are a consequence of functional redundancy. However, it remains formally possible that the genetic interactions that we detect are not a consequence of the similar biochemical functions, but instead are the result of indirect developmental interactions.

Cart1 as an enhancer of Alx4-dependent polydactyly

During development, *Alx4* is expressed in mesenchymal cells in the anterior half of the limb bud (Qu et al., 1997b). Targeted disruption of Alx4 results in preaxial polydactyly, which is associated with the formation of an ectopic anterior ZPA during limb bud development. As monitored by expression of Shh mRNA, the ectopic ZPA present in *Alx4* homozygous embryos is much smaller than the normal posterior ZPA, and arises later in development. This partial ZPA duplication is consistent with the partial limb duplication detected in *Alx4*$^{-/-}$ animals. One potential explanation for the very small size of the anterior ectopic ZPA in *Alx4*$^{-/-}$ embryos is that functionally redundant genes may contribute a similar function, i.e., repression of *Shh* expression in the anterior limb bud. The demonstration that *Cart1* mutation is a dose-dependent enhancer of Alx4 polydactyly is consistent with this idea, and uncovers a role for Cart1 in limb patterning. The evidence that Cart1 plays a role in AP patterning in the limb bud is intriguing in view of the fact that Cart1 is expressed diffusely throughout the limb bud mesoderm, without any gradient (Zhao et al., 1994; S. Q. and R. W., unpublished data). If Alx4 and Cart1 are biochemically redundant, why does *Cart1* expression in the posterior aspect of the limb bud not repress *Shh* expression in the ZPA? It is possible that the levels of *Cart1* expression in the posterior mesenchyme are insufficient to repress *Shh* expression, or that a co-factor needed for Alx4 and Cart1 activity is not present in the posterior mesenchyme.

A second point of interest relates to the fact that the polydactyly of *Alx4*$^{-/-}$ animals is subject to strong strain-specific effects. We have recently identified inactivating mutations in *Alx4* as the molecular basis of the polydactylous mouse mutant *Strong's luxoid* (*lst*) (Qu et al., 1998). It has been apparent for decades that the severity of the polydactyly phenotype in animals heterozygous or homozygous for the *lst* mutation is subject to strain-specific modifier genes (Forsthoefel, 1968). Thus, depending on the strain background, *Alx4*$^{+/-}$ animals may be normal or may have hindlimb polydactyly, and homozygotes have anywhere from 6 to 9 digits, with or without tibial hemimelia (Qu et al., 1998). Could strain-specific differences in *Cart1* expression in the limb bud mesenchyme account for these results? Against this possibility is the fact that *Alx4*$^{+/-}$/*Cart1*$^{-/-}$ mice (on the 129/Sv background) are not polydactylous, while the severe polydactyly in *Alx4*$^{-/-}$ animals on the *lst* strain background is associated with heterozygous hindlimb polydactyly. Thus, while differences in *Cart1* expression can modify the polydactyly of *Alx4*$^{-/-}$ animals, they do not induce polydactyly in Alx4 heterozygotes. Therefore, strain-specific differences in *Cart1* expression in the limb bud are unlikely to be completely responsible for the strain-specific phenotypic differences of *lst* mice, although we can not formally exclude the presence of a *Cart1* allele that functions as a dominant negative in the limb.

Craniofacial patterning by Cart1 and Alx4

Alx4 and *Cart1* are highly expressed in mesenchymal cells of

the craniofacial region and the first branchial arch. Together, these cells will give rise to the cartilages and bones of the face, nose, mandible and base of the the skull. Consistent with the functional redundancy described above, there are prominent defects in these structures in double mutant mice. Because the exencephaly induced by *Cart1* mutation results in secondary defects in craniofacial patterning, the genetic interactions are most easily described in *Cart1*$^{+/-}$ mice. Although both *Alx4*$^{-/-}$ and *Cart1*$^{+/-}$ mice have normal facial development, *Alx4*$^{-/-}$/*Cart1*$^{+/-}$ mice have cleft palate, cleft face and mandibular truncations, which constitute a series of midline fusion defects. Thus, Alx4 and Cart1 display redundant properties in the patterning of craniofacial structures, with an increase in lateral fates and a decrease in medial structures. In addition, the bones of the skull, including the parietal, occipital and temporal bones, are markedly reduced in *Alx4*$^{-/-}$/*Cart1*$^{+/-}$ mutant embryos. Craniofacial development is dependent on the intricate patterning of a large number of bones, and the inductive signals that underlie this complex process are not well described. The developmental basis of the medial-lateral patterning defect in the craniofacial region of *Alx4*$^{-/-}$/*Cart1*$^{+/-}$ mice is not clear, but is of interest with regard to recent suggestions that patterning of structures derived from the branchial arches may occur by similar developmental mechanisms as the patterning of the limbs (Shubin et al., 1997).

Skeletogenesis and paired-type homedomain proteins

Inductive interactions play a central role in skeletal patterning throughout the body. Bones of the cranium and face, which are formed primarily from cranial neural crest progenitors, as well as the skeleton at other sites in the embryo, are patterned by poorly understood intercellular signaling events. Alx4 and Cart1 belong to the subfamily of transcription factors that contain the paired-type homeodomain with a glutamine or lysine at position 50, and therefore are predicted to also bind the P3 sequence. This subfamily contains several other members, including MHox (Prx1), S8 (Prx2), Alx3, goosecoid (Gsc) and goosecoid-like (Gsl), many of which are expressed in overlapping domains during develoment. The biochemical data suggests that the similar DNA-binding activity of these proteins are likely to generate a series of complex genetic interactions among the genes encoding P3-binding proteins. One possible site of genetic interaction among these proteins is in the limb bud, where strain-specific differences in the phenotype of polydactylous mutations are observed not only for *Alx4* mutant mice, but for other genes as well (Masuya et al., 1997). Our data show that quantitative changes in the level of *Cart1* expression can alter Alx4-dependent polydactyly, even though *Cart1* mutant mice have no limb abnormality. By extension, it seems probable that strain-specific genetic changes that alter the expression of P3-binding proteins in the limb bud could function as strain-specific modifiers of other polydactyly mutations. In addition to the limb bud, many of these genes are highly expressed in the craniofacial mesenchyme, as well as mesenchyme of the first branchial arch, and both *MHox* and *Gsc* mutant mice have craniofacial abnormalities that include cleft palate and defects in skeletogenesis of structures derived from the first branchial arch (Opstelten et al., 1991; Cserjesi et al., 1992; Gaunt et al., 1993; Martin et al., 1995; Rivera-Perez et al., 1995; Yamada et al., 1995). Furthermore, both mutants have skeletal phenotypes at other sites (rib defects in *Gsc*$^{-/-}$ mice, limb defects in *MHox*$^{-/-}$ mice). Although these proteins exhibit overlapping DNA-binding activities, it is clear that they are not all functionally equivalent. For example, Alx4 and Cart1 function as transcriptional activators, while Gsc is a transcriptional repressor (Danilov et al., 1998; Mailhos et al., 1998). How these diverse transcriptional regulatory activities are integrated to control developmental processes will require further analysis of both the developmental roles played by these genes, as well as their transcriptional regulatory properties.

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