Regulation of Hoxa2 in cranial neural crest cells involves members of the AP-2 family

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SUMMARY

Hoxa2 is expressed in cranial neural crest cells that migrate into the second branchial arch and is essential for proper patterning of neural-crest-derived structures in this region. We have used transgenic analysis to begin to address the regulatory mechanisms which underlie neural-crest-specific expression of Hoxa2. By performing a deletion analysis on an enhancer from the Hoxa2 gene that is capable of mediating expression in neural crest cells in a manner similar to the endogenous gene, we demonstrated that multiple cis-acting elements are required for neural-crest-specific activity. One of these elements consists of a sequence that binds to the three transcription factor AP-2 family members. Mutation or deletion of this site in the Hoxa2 enhancer abrogates reporter expression in cranial neural crest cells but not in the hindbrain. In both cell culture co-transfection assays and transgenic embryos AP-2 family members are able to trans-activate reporter expression, showing that this enhancer functions as an AP-2-responsive element in vivo. Reporter expression is not abolished in an AP-2α null mutant embryos, suggesting redundancy with other AP-2 family members for activation of the Hoxa2 enhancer. Other cis-elements identified in this study critical for neural-crest-specific expression include an element that influences levels of expression and a conserved sequence, which when multimerized directs expression in a broad subset of neural crest cells. These elements work together to co-ordinate and restrict neural crest expression to the second branchial arch and more posterior regions. Our findings have identified the cis-components that allow Hoxa2 to be regulated independently in rhombomeres and cranial neural crest cells.

Key words: Neural crest, Hoxa2, AP-2, Transgenic mouse, Gene regulation

INTRODUCTION

The vertebrate hindbrain uses a process of segmentation to generate regional diversity along the anteroposterior (AP) axis during head morphogenesis (Lumsden, 1990; Lumsden and Krumlauf, 1996). The consequences of these events in the hindbrain extend beyond the neural tube itself (Köntges and Lumsden, 1996; Lumsden et al., 1991; Sechrist et al., 1993), due to an important role played by cranial neural crest cells in generating much of the bone and connective tissue of the developing head (Couly et al., 1993; Le Douarin, 1983). Understanding the properties of cranial neural crest in mediating the basic pattern for skeletal structures in the head has primarily come from grafting experiments in avian embryos. Early studies revealed that there is a degree of prepatternning or autonomy in the neural crest cells before they migrate into the branchial arches, because they can retain the ability to generate skeletal elements characteristic of their AP origin when placed in ectopic locations (Kuratani and Eichele, 1993; Noden, 1983; Prince and Lumsden, 1994). Recent experiments have also shown that, in addition to these prepatternning influences, cranial neural crest displays a considerable degree of plasticity and is also dependent on appropriate environmental signals in the branchial arches in order to generate structures with the correct pattern (Couly et al., 1998; Hunt et al., 1998; Saldivar et al., 1996). However, very little is known about the underlying molecular mechanisms that control these aspects of neural crest patterning and development.

Gene expression and targeted inactivation studies have demonstrated that Hox genes are one of the key components that play an integral role in specifying regional identity along the AP axis in craniofacial morphogenesis (reviewed in Krumlauf, 1993, 1994; Maconochie et al., 1996). In addition to their roles in patterning neuroectoderm (rhombomeres) and endodermal derivatives, Hox genes are essential for the specification of both neurogenic and mesenchymal cranial neural crest components (Chisaka and Capecchi, 1991; Chisaka et al., 1992; Gavalas et al., 1997, 1998; Gendron-Maguire et al., 1993; Goddard et al., 1996; Lufkin et al., 1991; Rijli et al., 1993; Studer et al., 1996). One of the clearest examples comes from the analysis of phenotypes in Hoxa2 mutant embryos, in which
mesenchymal but not neurogenic crest derivatives of the second branchial arch adopt a first arch identity (Gavalas et al., 1997; Gendron-Maguire et al., 1993; Rijli et al., 1993). As second arch structures are derived from cells migrating from rhombomere (r) 4 and r4 itself appears unaffected by the mutation, this suggests a primary role for Hoxa2 in the crest independent of patterning in the hindbrain. A recent study suggests that some of these Hoxa2 crest defects are mediated by restricting the chondrogenic domain and inhibiting bone formation in skeletal derivatives of the second branchial arch (Kanzler et al., 1998).

Mutations in a number of other transcription factors, including AP-2α, Pax3, Pax6 Mx1, Dlx 2 and Gsc affect neural crest patterning, showing they are part of the cascade of events that control craniofacial morphogenesis. The regulatory relationships between these various transcription factors are, as yet, unclear. Some of these genes, such as the members of the AP-2 family display prominent domains of expression in cranial neural crest that overlap with many of the Hox genes (Chazaud et al., 1996; Mitchell et al., 1991; Moser et al., 1997b). However, it is an open question as to whether and how such genes are linked to Hox-dependent pathways. While transgenic analysis has begun to build a picture of the cis-elements and upstream factors that regulate segmental expression of Hox genes in the hindbrain (Maconochie et al., 1997; Manzanares et al., 1997; Nonchev et al., 1996b; Pöpperl et al., 1995; Sham et al., 1993), similar detailed studies have not been performed with respect to Hox regulation in the neural crest. There are colinear patterns of Hox expression in both the cranial neural crest cells and the hindbrain segments from which they are derived (Hunt et al., 1991a,b) and current data suggests that the regulation in these tissues can be mediated by the same or separate elements. For example, the same bipartite 10 bp Hox/Pbx motifs required to direct the r4-restricted expression of Hoxb1 and Hoxb2 also mediate their expression in r4-derived neurogenic neural crest cells (Maconochie et al., 1997; Pöpperl et al., 1995). However, the elements that regulate expression of these genes in mesenchymal neural crest cells are separate and have not been identified. Furthermore, we have previously isolated a Krox20-dependent enhancer from the Hoxa2 gene, which directs segmental expression in the hindbrain but it also has independent elements capable of mediating reporter expression in cranial neural crest cells (Nonchev et al., 1996b). This pattern closely resembles the endogenous expression of Hoxa2 in the branchial arches and has a similar AP restriction at the junction between the first and second arch.

In light of the functional involvement of Hoxa2 in patterning neural-curt-derived structural elements of the second branchial arch, we have used this Hoxa2 enhancer as a basis to investigate the regulation of Hoxa2 in cranial neural crest cells. In this study, transgenic analysis identified four different cis-elements that participate in crest-specific regulation. One of these contained a binding site for transcription factor AP-2 family members that was required for enhancer activity. In vitro binding analysis and cell culture transfections revealed that the three murine AP-2 family members (AP-2α, AP-2β and AP-2.2) were each capable of binding to this motif and trans-activating reporters carrying the site. Ectopic expression in transgenic embryos showed that in vivo, the enhancer acts as an AP-2-responsive element in a highly cell type-specific fashion. These findings outline the complexity in controlling crest expression and suggest that AP-2 family members have a direct role in Hoxa2 regulation during patterning of the second branchial arch.

**MATERIALS AND METHODS**

**Transgenic mice and in situ hybridization**

For microinjection, inserts were separated from vector DNA by electrophoresis and purified using a gelase method provided by suppliers (Epicentre Technologies). Transgenic embryos were generated by pronuclear injection into fertilized mouse eggs from an intercross of F1 hybrids (CBAxC57B16) and stained for lacZ reporter activity as described (Whiting et al., 1991). In the trans-activation experiments, males homozygous for the construct 1 (Fig. 2) (carrying the 809 bp Hoxa2 enhancer) were mated to superovulated F1 hybrid females to collect fertilised eggs for the subsequent microinjection of various β-actin/cDNA ectopic expression constructs. F0 embryos were then harvested to assay for changes in lacZ expression. Flat-mounted hindbrain preparations were generated by dissecting embryos of the appropriate stage along the dorsal midline in 70% glycerol. The regions anterior of the midbrain and posterior of the otic vesicle were removed and the preparations were mounted under a cover slip for viewing. Paraffin sections (6 μm) of transgenic embryos were generated after staining for reporter expression. In situ hybridization on whole-mount mouse embryos (Xu and Wilkinson, 1998) was carried out with a probe for Hoxa2 (Hunt et al., 1991a).

**Transgenic constructs**

Mutations, mutants and multimerized oligonucleotides were cloned into reporter expression vectors containing either a basal Hoxb4 promoter (construct #8 (Whiting et al., 1991)) or the minimal human β-globin promoter (BGZ40) (Maconochie et al., 1997; Yee and Rigby, 1993) linked to a bacterial β-galactosidase gene (lacZ) and an SV-40 polyadenylation signal. Constructs #1-11 were generated by 5′, 3′ or internal deletions using the relevant restriction enzymes indicated in Fig. 2. These deletions were made using a previously identified 809 bp BglII enhancer (Nonchev et al., 1996b), located 5′ of the Hoxa2 ATG as a starting point. Constructs #12-14, 16 and 17 represent a series of mutated versions of the 809 bp enhancer in which the small deletions and alterations were generated first by site-directed mutagenesis in M13 (Sculptor IVM System, Amersham) and subsequently cloned into BGZ40. Site-directed mutagenesis was carried out precisely as described by the manufacturers, using mutagenic oligonucleotides with clamps of homology of at least 15 bp 5′ and 3′ of the target sites for generating deletions or substitutions. Oligonucleotides were:

- #12 5′-ACAAAAGGCCCTGACAGAACATCTCAGC-3′;
- #13 5′-TGATCAATCCTGTCTGCTTCTGGCCCTTA-3′;
- #14 5′-AGGAGTCCTCGTGCTCAGAAACCTCTGAC-3′;
- #15 5′-CAAGTGATCAGAGTGCGAGCCACATCAGTG-3′;
- #17 5′-TGGAGCCAAGTGATCAGAGTGCCACCATCAGTG-3′

Construct #15 contains 7 copies of the sequence indicated in Fig. 4A inserted into BGZ40 as described (Manzanares et al., 1997). All mutations and multimerized oligonucleotides were verified by sequencing. Ectopic expression constructs were generated by inserting AP-2 (Williams et al., 1988) and AP-2.2 (Chazaud et al., 1996) cDNAs encoding full-length proteins into the same vector containing the human β-actin promoter that we have previously used to stimulate widespread expression (Pöpperl et al., 1995; Zhang et al., 1994).

**Bandshift analysis with transcription factor AP-2 family members**

Murine AP-2, AP-2β and AP-2.2 proteins were synthesised in vitro from cDNA expression plasmids using the TNT Coupled Reticulocyte Lysate System (Promega, WI). A trace amount of 35S [methionine was included in the transcription/translation cocktail to allow molecular weight-sizing and quantitation of relative protein yields.
following PhosphorImager analysis (Molecular Dynamics, Inc.) of samples analyzed by SDS-PAGE. Equivalent amounts of protein were incubated for 45 minutes at room temperature in 20 µl reactions containing 2×10^4 counts/minute 32P-end-labelled double-stranded oligonucleotide probe (5'-TGGGCA TTCGTAAGGCACGTC-3'), representing a sequence from the 3′ end of the Hoxa2 enhancer that includes a consensus binding site for transcription factor AP-2, 1 µg poly(dI-dC) non-specific competitor, 20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-Cl (pH 7.5). Specific competition analysis was performed by including unlabeled binding site oligonucleotide at increasing molar excess relative to labeled probe. The binding reactions were electrophoresed on a 4% acrylamide gel for autoradiography and PhosphorImager analysis.

**Cell culture AP-2 trans-activation assays**

Three copies of the Hoxa2 AP2-binding site were inserted just upstream of the Adenovirus E1b TATA-box in the reporter gene BCAT by annealing the following oligonucleotides: 5′-TCGACTGCCGCGGCGGACATTCGCTGCCGCGGCGCATTCGCTGCGGGCTGCCTTTG-3′ and 5′-TCGAGAAATTGCCAGGGCGGAGGAATGCCCCAGGGCGGAGGAATCCGCCAGGGCGGAGGAATCCGCCAGGGCGGAGG-3′ and ligating them into the Sall site of BCAT. BCAT, A2BCAT, RSVAP2 and pRSV-β-gal have been previously described (Edlund et al., 1985).

Co-transfections were carried out in HepG2 cells using calcium phosphate (Jordan et al., 1996) or Lipofectin reagent (Life Technologies). For the former, the day before transfection, 7×10^5 HepG2 cells were plated in 6 cm tissue culture dishes. The following day, the medium was changed and cultures were transfected 2 hours later with 40 µg of plasmid DNA (Endofree plasmid kit, Qiagen) containing 25 µg of reporter plasmid, 6 µg of expression vector (with or without cDNA insert) and 9 µg of herring sperm carrier DNA. The DNA co-precipitate was incubated for 10 minutes at room temperature and then added to the culture dishes. The cultures were incubated with DNA for 20 hours, then given a 2 minute glycerol shock (15% glycerol in DMEM complete medium), washed twice with DMEM complete medium and incubated a further 44-48 hours prior to harvest. Cell extracts were prepared and CAT protein was quantitated by CAT-Elisa (Roche Diagnostics).

For lipofection, HepG2 cells were grown to 40-60% confluency in 10 cm culture dishes and split 1:2. The following day, transfection mix was prepared: 4.5 ml Opti-MEM, containing plasmids to be transfected, was mixed with 4.5 ml Opti-MEM containing 90 µg Lipofectin reagent and incubated 15 minutes at room temperature. Cultures of HepG2 were washed twice with Opti-MEM and then incubated with the transfection mix for 20 hours at 37°C. The cells were subsequently washed twice in standard media (DMEM + FCS) and incubated a further 48 hours prior to harvest. Transfections were carried out using 2.5 µg pRSV-β-gal, increasing concentrations of activator plasmid (0-1.0 µg RSV-AP2) and 4 µg reporter plasmid (either test plasmid, basal-BCAT or positive control A2BCAT). Cell extracts were prepared by freeze-thaw lysis and β-galactosidase was measured for normalisation of transfections. CAT assays were performed by thin-layer chromatography (Sambrook et al., 1989) and autoradiographs were quantified using a phosphorimager.

**RESULTS**

**Neural-crest-specific expression mediated by a Hoxa2 enhancer**

We have previously used transgenic analysis to scan the Hoxa2-Hoxa3 intergenic region for cis-regulatory elements governing segmental Hoxa2 expression in the hindbrain and identified an 809 bp enhancer that mediates r3- and r5-restricted expression through the direct regulatory influence of Krox20 (Nonchev et al., 1996b). In addition to r3 and r5, this enhancer also directed reporter expression in neural crest of the second branchial arch and more posterior regions.

In order to examine the patterns and timing of neural crest expression in more detail, we generated lines of mice in

![Fig. 1. Time course and patterns of neural crest expression mediated by a Hoxa2 enhancer.](image)
Multiple cis-acting elements are regulation. More precisely define the generated and tested in transgenic mice to required for neural crest regulation which this enhancer is linked to a lacZ reporter gene (constructs #1 and #3). Since the enhancer mediates strong expression in crest cells migrating from r4 into the second branchial arch, we performed a detailed time course to examine if any staining was detectable in premigratory r4 cells. Our analysis of enhancer activity spanning the stages of cranial crest migration (8.25-9.5 dpc) shows that reporter expression is never detected in r4 (Fig. 1). Reporter expression is first detected between 8.0 and 8.25 dpc in cranial crest cells as they delaminate from this segment and begin to migrate laterally into the second branchial arch (Fig. 1A). In subsequent stages between 8.25 and 10.5 dpc, this expression is activated very rapidly and uniformly as virtually all cells adjacent to the neural tube at this level are strongly positive (Fig. 1B-D). This is clearly seen in both flat-mounted hindbrain preparations and transverse sections through the second arch (Fig. 1E,F). In a similar manner, reporter staining is seen in neural crest migrating into the other more posterior branchial arches and the trunk, but is never detected in the neural tube adjacent to these regions (Fig. 1B,C). Staining is not restricted to mesenchymal crest derivatives as transgene expression is also seen in the facial ganglion (VIIg) and in a small number of surface ectoderm cells in the posterior part of the first arch (Fig. 1H). Furthermore, in the trunk, subsets of both lateral and paraxial mesoderm are also positive. These results show that the Hoxa2 enhancer is not simultaneously active in migrating cranial neural crest cells and the CNS domains from which they are derived.

The timing and the spatial restrictions of these reporter expression patterns in the neural crest cells closely parallels the endogenous Hoxa2 gene and correlates with affected structures in targeted Hoxa2 mutants (Gendron-Maguire et al., 1993; Hunt et al., 1991a; Rijli et al., 1993). Since analysis of the Hoxa2 locus has not revealed any other neural crest regulatory elements, it appears that this enhancer contains the components responsible for regulating the restricted domains of Hoxa2 expression in neural crest essential for normal patterning. Therefore, we used this enhancer as a starting point for the molecular dissection of Hoxa2 neural crest regulation.

Multiple cis-acting elements are required for neural crest regulation
A series of deletion constructs were generated and tested in transgenic mice to more precisely define the cis-elements required to mediate reporter expression in neural crest (Fig. 2). The first 3’ deletion construct (#2) lead to a complete loss of transgene staining in neural crest cells migrating into the branchial arches, while expression in trunk derivatives was unaffected (Fig. 3B). Reporter expression also persisted in r3 and r5, emphasising that the neural crest and rhombomeric domains are differentially regulated. This showed that one important cis-component is located at the 3’ end of this enhancer and we refer to this as NC1.

In a similar manner, a set of 5’ deletions (constructs #3-7) identified a second cis-requirement, NC2 (Fig. 2). Removal of the 5’ regions containing the Krox20-binding sites (#3) and flanking sequences (#4) eliminated r3 and r5 expression, but staining in the branchial arches and the trunk was unaffected (Fig. 3C,D). However deleting a further 100 bp (#5) completely abolished all reporter expression in the hindbrain, arches and trunk (data not shown). The loss of enhancer activity was not affected by two additional 5’ deletions (constructs #6 and #7, Fig. 2). Together, this deletion analysis defines the 5’ and 3’ limits of a 550 bp enhancer.

Fig. 2. Constructs for transgenic analysis in mapping the neural crest specific elements in a Hoxa2 enhancer. At the top is a restriction map of this 809 bp enhancer and below are listed the various deletions and mutations that were linked to a lacZ reporter gene for regulatory analysis. Construct numbers are noted on the left and the efficiency of expression in neural crest on the right. The relative position of the previously identified Krox20-binding sites (Nonchev et al., 1996b) and the four neural crest elements (NC1-NC4) uncovered in this study required for enhancer activity are noted at the bottom. AP2 refers to the consensus AP2-binding site in NC4.
minimal enhancer (#4) able to confer expression in neural crest and the trunk.

To address whether additional sequence elements are required for regulatory activity, we generated a series of internal deletion constructs (#8-11 Fig. 2). This analysis confirmed that the 100 bp NC2 region mapped above is essential for enhancer activity (#8; Fig. 3E) and identified two new regions (NC3 and NC4) that are involved in controlling neural crest expression. Construct #9 contains a deletion of a 97 bp fragment that defines one of the additional cis-requirements (NC3) (data not shown), while the second (NC4) is contained in a 160 bp region (#11) near the 3' end of the enhancer (Fig. 3G). Both of these deletions abolished reporter staining in the branchial arches, but not in r3 and r5 or trunk domains. An internal deletion in the middle of the enhancer (#10) had no effect on its activity (Fig. 3F). This suggests a complex picture for regulating Hoxa2, where at least four cis-acting regions are required in concert to direct expression to the branchial arches and some of these also participate in controlling expression in trunk derivatives.

Analysis of elements within the NC2 and NC3 regions

To begin to investigate the elements involved in NC2 and NC3 control of neural crest expression, we examined the sequence of the adjacent deletions that serve as a basis for mapping these domains (#4,5,8,9; Fig. 2). As a part of this analysis, we compared these regions with a previously identified enhancer from the chicken Hoxa2 gene, which directs expression in the hindbrain (Nonchev et al., 1996a) and found several blocks of sequence identity. The sequence in NC3 does not correlate with the consensus-binding site for any known transcription factors. However, there is 28 bp conserved block spanning the NC2/NC3 junction, in which 7 bp were located in NC2 and the remaining 21 bp in NC3. Therefore, to test if this sequence was functionally important, we generated a series of three adjacent 15 bp deletions in the context of the full 809 bp enhancer (#12-14). The deletion closest to the junction (#12) reduced the levels of crest expression, but did not eliminate it entirely (Fig. 3H). Interestingly, this alteration also influenced hindbrain expression (as evidenced by the absence of reporter staining in r3) without affecting trunk expression (Fig. 3H). This suggests that some common motifs might be involved in controlling both crest and rhombomeric expression. The two remaining 15 bp deletions (#13-14) in NC3 completely abolished reporter expression in the branchial arches, but had no effect on r3/r5 (Fig. 3I,J and data not shown). This indicates that the 30 bp conserved block spanning NC2 and NC3 contains separate elements required for both neural crest and rhombomeric expression. The sequences near the 5' end of the conserved block are essential for r3 expression and contribute to levels of neural crest staining, while those at the 3' end of the block have no effect on rhombomeric domains but are critical for expression in second arch neural crest cells. In addition, it is interesting that deletions in #13 and #14 both abolish staining in the branchial arches of all embryos, but expression in trunk neural crest cells is only lost in some cases (compare Fig. 3I and J). This suggests that the cranial and trunk neural crest activities differ slightly in the degree to which they require common components.

Within NC2, in addition to the 7 bp domain at the junction with NC3, there is a 56 bp region which has 82% identity with the chick Hoxa2 enhancer (Fig. 4A). This is located in the middle of the fragment and corresponds to the only region in NC2 containing binding sites related to known transcription factors. There are divergent motifs of consensus sites for members of the Maf and AP-2 families. The MafB consensus sequence is 5'-TGCTGAC-3' and the mouse and chick motifs have diverged from this in their last 2 bp and 3 bp, respectively (Fig. 4A,B). The palindromic sequence 5'-GCCNNNGGC-3' represents the AP-2 consensus site and there is a potential highly divergent site (5'-GCTGGGC-3') (Fig. 4A,C). Since MafB/kreisler has been shown to be involved in regulating Hox expression in rhombomeres (Manzanares et al., 1997), and AP-2 is both expressed in and required for cranial neural crest patterning (Mitchell et al., 1991; Schorle et al., 1996; Zhang et al., 1996), we examined their possible involvement in more detail. To directly determine if these proteins are able to bind to the divergent motifs in NC2, we performed in vitro gel-shift assays and footprint assays with bacterially expressed and purified MafB/kreisler protein and in vitro translated AP-2 family proteins. However, we found that these proteins are not able to bind to this conserved block under the same conditions in which we have shown they successfully interact with their consensus binding sites (data not shown).

As these two sites partially overlap, we wanted to investigate the potential in vivo role of this conserved block and generated multimers of this sequence linked to a lacZ reporter gene. In transgenic embryos, seven copies of this sequence were sufficient to mediate widespread expression in both cranial and trunk neural crest cells (Fig. 4D-F). There was no anteroposterior (AP) restriction to the staining patterns in the head and branchial arches, as expression was seen in the frontonasal mesenchyme and first arch in addition to the more posterior arches where Hoxa2 is normally expressed (Fig. 4D,E). There was no detectable staining in the neural tube in any of the embryos (Fig. 4E,F), supporting the idea that these sequences in NC2 participate in regulating neural crest-specific expression. This widespread pattern of reporter expression in neural crest does not correspond to any of the known Maf members and suggests that this sequence is responding to upstream factors that are broadly distributed in neural crest cells. Although the expression is similar to many aspects of patterns of AP-2 family members in neural crest cells, we find that AP-2 family members do not interact with this sequence in vitro. This suggests that this conserved block integrates the activity of different pan-neural crest components.

An AP-2 site in NC4 is required for enhancer function

Sequence comparisons with NC1 and NC4 revealed no homologies or conserved blocks shared with the chick enhancer. Furthermore screening them against the transcription factor sites database revealed no obvious binding motifs in NC1, while there were a few matches to consensus binding sites in NC4. Therefore, we focused our analysis on NC4, as one motif (5'-GCCTGGGGC-3') corresponds perfectly to the
consensus binding site, 5′-GCCNNNGGC-3′, for the transcription factor AP-2 (Williams and Tjian, 1991a,b). This site is located at the 3′ end of the 160 bp NC4 region only 13 bp from the junction with the NC1 deletion. Due to the roles of AP-2 in neural crest patterning, as noted above, we examined this site in more detail.

Gel-shift assays were used to address whether AP-2 binds to the consensus site in vitro. In addition to AP-2 (AP-2α), two AP-2-related genes, AP-2β and murine AP-2.2 (human AP-2γ, ERF-I), have also been cloned (Bosher et al., 1995; McPherson et al., 1997; Moser et al., 1995; Oulad-Abdelghani et al., 1996; Williams et al., 1988). We therefore also examined the ability of the three murine AP-2 family members to bind to the putative AP-2-binding site in the Hoxa2 enhancer. In gel-shift assays, each of the three AP-2 family members was able to form a stable complex with the Hoxa2 AP-2-binding site probe (Fig. 5A, lanes 2, 8 and 14) and failed to bind appreciably to a mutated version of the probe (2 bp substitutions within the palindromic portion, data not shown). The probe complexes were specifically titrated out with increasing excess of unlabeled probe (Fig. 5A lanes 3-7, 9-13, 15-19). However, although approximately equivalent amounts of protein were used, differences in the amount of shifted probe and in specific competition could be seen for the three AP-2 family members (Fig. 5A,B). These data suggest that the three family members have different relative affinities for this site that are ordered: AP-2α > AP-2β > AP-2.2. Thus, while all three AP-2 family members are capable of binding in vitro, differences in binding affinity as well as other differences in protein structure could affect their abilities to activate transcription through this site in vivo.

We next addressed whether this AP-2 site was necessary for Hoxa2 enhancer activity in vivo. Therefore, we generated two reporter constructs containing either a deletion of the AP-2 site (#16) or the same two base pair substitutions which abrogated in vitro binding (#17) in the context of the entire 809 bp Hoxa2 enhancer (Fig. 6). Both the deletion and point mutations specifically lead to the loss of reporter staining in branchial arch neural crest cells, leaving the r3/5 domain unaffected (Fig. 6B,C). This demonstrates that the site in NC4 capable of binding to AP-2 with high affinity in vitro is also required in vivo for cranial neural crest expression directed by this Hoxa2 enhancer.

**Trans-activation by AP-2**

In light of these findings, we wanted to determine if this site was capable of responding to AP-2 in vivo. First, we utilized a transient transfection cell culture assay (Williams et al., 1988) to compare the ability of the site in Hoxa2 to act as an AP-2 target with that of a well-characterised AP-2 motif (Fig. 7). The human hepatoma cell line HepG2 has been used for co-transfection experiments to assay AP-2-dependent transcriptional activation (Lee et al., 1987) because it does not contain detectable levels of endogenous AP-2 (Williams et al., 1988). Co-transfection with expression plasmids for each of the three AP-2 family proteins failed to activate transcription from the basal CAT reporter (Fig. 7), but AP-2α and AP-2.2 each gave appreciable activation of A2BCAT, a reporter CAT construct carrying three copies of an AP-2 site from the human metallothionein promoter (Fig. 7). In contrast, AP-2β failed to activate via this same binding site in the context of the HSV tk promoter-CAT reporter, although it can activate transcription from this site in the context of another promoter and cell line (Moser et al., 1995). However, all three AP-2 family proteins activated the Hoxa2 CAT reporter, carrying three copies of the Hoxa2 AP-2 motif, to an even higher degree than they activated A2BCAT. Hence this Hoxa2 motif is a bona fide AP-2-responsive element in HepG2 cells.

The Hoxa2 enhancer does not normally mediate reporter expression in neural crest cells anterior to the second arch, despite the fact that AP-2 family members are expressed in a broad pattern in cranial neural crest cells, including the first arch and frontonasal mesenchyme. This illustrates that the enhancer is not simply an AP-2-responsive element that reflects all the endogenous sites of AP-2 family expression. However, we wanted to test the ability of the Hoxa2 enhancer to respond to elevated levels of AP-2 family members in vivo by performing ectopic expression in transgenic mice. For this purpose, we utilized a human β-actin promoter/vector that has previously been used in transgenic **trans-activation or**
ectopic expression studies for Krox20, kreisler, Wnt8 and several Hox genes (Gould et al., 1997; Maconochie et al., 1997; Nonchev et al., 1996a,b; Pöpperl et al., 1995, 1997; Zhang et al., 1994). This construct was used to generate low levels of widespread expression of the AP-2 or the AP-2.2 cDNAs in a transgenic line already containing the 809 bp Hoxa2/lacZ reporter (construct #1; Fig. 2). In over 500 cases spanning six generations, reporter expression was never observed in the first branchial arch of embryos from this transgenic line in the absence of the ectopic AP-2 expression vectors (Fig. 8A and data not shown). In contrast, for each of the two AP-2 family members tested, we reproducibly observed (n=7) trans-activation of the lacZ reporter in the first branchial arch immediately adjacent to r2 (arrowheads; Fig. 8B,C). There were no differences in the pattern of induction between AP-2 and AP-2.2, despite the fact that AP-2.2 displayed the lowest in vitro binding affinity for this site. Hence, it still retains the ability to trans-activate from this enhancer in vivo.

Induction of this reporter was never observed in midbrain neural crest cells, frontonasal mesenchyme or any tissue outside of the neural crest. Therefore with the vector used, the ability of increased AP-2 family member expression to activate the enhancer is restricted to selected neural crest cell populations. The reporter has been activated in a small group of neural crest cells immediately adjacent to r2 and extending in towards the middle of the first branchial arch (Fig. 8B,C). This indicates that only a subset of these cells are sensitive to the levels of ectopic AP-2 expression under these conditions. The position of the reporter staining in first arch cells adjacent to and often touching r2 suggests they are derived from r2 and that this represents an induction rather than an altered migration of second arch cells. Consistent with this, we have found in mouse grafting experiments that second arch neural crest cells expressing this Hoxa2 reporter are unable to maintain reporter expression when placed in the first branchial arch (Paul Trainor and R. K.; unpublished). Therefore, in agreement with the cell culture transfection analysis, these results demonstrate that the enhancer can be trans-activated in response to AP-2 family members in vivo and that other components serve to restrict the nature of this response.

**Hoxa2 expression in AP-2 null mutants suggest compensation by other AP-2 family members**

To begin to address how many of the AP-2 family members are actually involved in regulating this enhancer activity, we examined Hoxa2 expression patterns in AP-2 targeted loss-of-function mutants (Schorle et al., 1996). Initially, we performed whole-mount in situ hybridisation on AP-2 null mutant embryos with a Hoxa2 probe. It appeared that there were no widespread changes in the neural crest expression of Hoxa2 (data not shown). In an alternative and more sensitive approach, we also crossed the Hoxa2 lacZ reporter line (construct #1) into the AP-2 mutant background. In homozygous AP-2 mutant embryos, the general or global patterns of reporter expression in neural crest migrating into the second branchial arch was normal (Fig. 9B,D). The only difference that we observed was fairly subtle and corresponded to more diffuse staining in a continuous stream of neural crest cells migrating from r4 in mutant embryos, compared to wild type. However, this could be an indirect affect related to the neural crest cell abnormalities in the AP-2 mutants.

This analysis in the AP-2 mutant background illustrates that endogenous AP-2 is not the only AP-2 family member essential for global enhancer activity. This is not surprising, in view of our results showing that the other AP-2 family members can bind to the Hoxa2 motif and can trans-activate this enhancer in cell culture and transgenic embryos. It is likely that other members of the family could be compensating for the loss of AP-2 or provide more of the major regulatory input to this enhancer. Consistent with this idea, analysis of the expression patterns of AP-2.2 and AP-2β have revealed that they are also expressed in a broad pattern of early premigratory and postmigratory cranial neural crest cells (Chazaud et al., 1996; Moser et al., 1997b), indicating that

**Fig. 4.** Conserved sequences in NC2 are capable of directing widespread reporter expression in the neural crest. (A) The sequence of a region in NC2 with a high degree of homology shared between mouse and chick. The bracket above the sequence refers to the region multimerized for transgenic analysis in D-F. The two boxed regions indicate divergent sequences related to consensus binding sites for AP-2 and Kreisler/Maf proteins. (B,C) Alignment of the kreisler/Krm1 (B) and the AP-2 (C) consensus sites with the respective mouse and chick sequences. (D-F) Seven copies of the 30 bp sequence bracketed in A are sufficient to mediate widespread reporter expression in cranial and trunk neural crest at 9.5 dpc. There is no expression in the neural tube. White arrows indicate expression in segmentally migrating trunk crest. lb, limb bud; ba, branchial arch; ov, otic vesicle; nt, neural tube.
are required in combination with the AP-2 site to both potentiate enhancer activity and regionally restrict expression in neural crest cells of the second branchial arch and more posterior regions. This reveals a considerable degree of complexity in the regulatory mechanisms underlying control of Hoxa2 expression in neural crest and raises a number of interesting questions as to how the different components are integrated to generate the highly restricted domains of expression.

**DISCUSSION**

In this paper, we have investigated the regulation of Hoxa2 in neural crest cells. Using transgenic analysis, we identified multiple cis-elements all of which are required by a Hoxa2 enhancer to direct expression in cranial neural crest cells. One of these contains a binding site for the transcription factor AP-2 and our trans-activation studies in cell culture and transgenic embryos suggest that this enhancer is a direct target for members of the AP-2 family. The upstream factors that bind to the other elements are not known, but these motifs they are present at the appropriate time and place to contribute to enhancer activity.

**Multiple components regulate neural crest expression**

The complex nature of the enhancer is reflected by the fact that deletions or mutations in each of four different cis-regulatory elements results in a complete loss of reporter expression in neural crest cells. This raises the question as to how activity of the enhancer is restricted to neural crest cells and at specific axial levels. All the AP-2 family members are broadly expressed in premigratory and/ or migratory neural crest cells in cranial and trunk regions, although there are some differences in the relative timing between genes (Chazaud et al., 1996; Mitchell et al., 1991; Moser et al., 1997b). This combined with our demonstration that the AP-2 site is functionally required and responds to AP-2 family members in vivo, suggests that AP-2 is a part of the mechanism that restricts expression to neural crest. Furthermore, the broad neural crest regulatory activity of the conserved block in NC2 when multimerized, suggests that it has a similar role in crest-specific expression. Therefore, these two cis-acting components appear to integrate activities that underlie the general ability of the Hoxa2 enhancer to mediate expression specifically in neural crest cells.

The other two cis-acting regions (NC1 and NC3) could participate in restricting broad neural crest cell expression to the second brachial arch and posterior domains. However, deletions of these regions do not result in the expansion of reporter expression, and instead lead to the loss of activity in neural crest (Fig. 3). This indicates that they do not simply function in the enhancer to repress the activities of the AP-2 and NC2 regions. They are essential for potentiating enhancer activity, but they could also have a role in limiting the axial domains. A similar type of co-operativity has been described for an enhancer in the Hoxb3 gene, whereby MafB/kreisler-responsive elements are dependent upon factors working through a region containing an ETS-related motif to both potentiate and restrict its activity to r5 (Manzanares et al., 1997).

NC1 and NC3 could be involved in controlling the relative level or efficiency of enhancer-mediated expression, stimulated through the AP-2 and NC2 elements. As a part of this, restricted expression or activity of the factors working through these sites might contribute to spatial constraints. Consistent with this idea, one of the small deletions in NC3 (construct #12, Fig. 3H) leads to a marked reduction in the level of reporter staining specifically in the cranial neural crest. Furthermore our trans-activation experiments demonstrate that increasing the relative levels of AP-2 family members can alter the spatial domains of enhancer activity. Ectopic expression of AP-2 and AP-2.2 induce reporter staining, only in a subset of cells in the first branchial arch,
where both genes are already normally expressed. This illustrates that, in at least one neural crest context, the enhancer is sensitive to the levels of AP-2 family member expression. The lack of ectopic activation of the reporter in other anterior regions or outside the neural crest could reflect the fact that even higher levels of AP-2 might be required to stimulate expression. However, our analysis in the paper also clearly showed that other *cis*-elements, besides the AP-2 site, are normally required for enhancer activity in neural crest. Therefore, low levels or the absence of the factors acting on these other sites might also serve to limit the ability of the enhancer to respond to normal or elevated AP-2 in anterior region implying that there is still a need for other cofactors. Nevertheless, the transgenic experiments do show that, in at least one context, having more AP-2 or AP-2.2 can activate enhancer activity. This might occur through a reduced dependence on the cofactors or an ability to overcome cofactor-dependent repression as a consequence of higher levels of AP-2, and/or that there are limiting amounts of AP-2 family member expression in this part of the first arch. This suggests that correct enhancer activity is dependent upon the proper levels and balance of upstream factors co-ordinated by the four *cis* regions.

The number of different *cis*-elements required for enhancer activity may reflect the diverse processes that pattern the properties of cranial neural crest itself. The evidence for plasticity of *Hox* expression in cranial neural crest, and its dependence upon environmental signals in the branchial arches (Coully et al., 1998; Hunt et al., 1998; Saldivar et al., 1996), suggests that the control of gene expression in migrating crest cells receives inputs from a number of different signals and transduction pathways. Therefore, each of the regions in the enhancer could be responsible for mediating the readout of distinct sets of cascades. For example, cell culture experiments have shown that the transcription factor AP-2 mediates induction by pathways involving, SV40 T antigen, N-ras, retinoids, Protein kinase C and cAMP (Doerksen et al., 1996; Hyman et al., 1989; Imagawa et al., 1987; Kannan et al., 1994; Lüsch et al., 1989; Mitchell et al., 1987).

We have used an r3/r5 enhancer from the chick *Hoxa2* gene for sequence comparisons to show that there are conserved blocks in both NC2 and NC3. However, within the chick enhancer, we could find no sequences related to NC1 and NC4 despite the fact that we have shown the AP-2 site in NC4 is essential for the mouse enhancer activity. It is important to note that the chick enhancer used for the comparisons does not mediate expression in neural crest (Nonchev et al., 1996a) indicating that other regions are required for its ability to direct reporter expression in crest cells. Hence, there may still be sequences related to NC1 and NC4/AP-2 that are required for regulation of neural crest cell expression and they might be organized or positioned in a different manner in the chick *Hoxa2* locus.

**Enhancer activity and AP-2 family members**

Our results demonstrate that the *Hoxa2* enhancer can act as a response element for multiple members of the AP-2 family presumably through direct interaction with the AP-2-binding site required for enhancer activity. Even though AP-2 binds in vitro to the site in the enhancer with the highest affinity and AP-2.2 with the lowest, there is no detectable difference in their in vivo ability to *trans*-activate the reporter. The similarity or overlaps in both timing and spatial expression patterns of AP-2-related genes in cranial neural crest, as well as their ability to *trans*-activate the enhancer, implies that there is a potential redundancy in their function. Hence, while our results show that AP-2 genes are involved, they do not formally demonstrate which members of this family are essential for regulating *Hoxa2*. Mouse mutants for AP-2β do not display defects in neural crest cells despite strong expression in these cells, but they do have kidney phenotypes (Moser et al., 1997a). There is a severe craniofacial phenotype in AP-2 null mutants correlated with some of the AP-2 expression domains in neural crest cells (Schorle et al., 1996; Zhang et al., 1996). These studies demonstrate that some of the functions for different family members are non-redundant or fail to be compensated by other AP-2 members.

AP-2 null mutants display a generalised defect in multiple embryonic fields, including cranioabdominoschisis and multiple craniofacial malformations. Neural crest migration is however not affected in the AP-2 mutants and although underdeveloped, middle ear primordia are all present. Clearly, that such a severe phenotype is present in AP-2 null mutants demonstrates a wide spectrum of craniofacial non-redundant function for AP-2, but there are other sites of AP-2 expression in second branchial arch crest mesenchyme that appear normal and might be compensated for by other AP-2 family members. *Hoxa2* null mutants display a homeotic transformation of second arch skeletal elements towards first arch phenotypes (Gendron-Maguire et al., 1993; Rijli et al., 1993). Of particular relevance is that second arch mesenchymal-crest-derived structures, such as Reichter’s cartilage, the stapes and part of the hyoid bone are all missing, and are replaced with a duplication of Meckel’s cartilage. This leads to duplicated primordia for the malleus and incus. These *Hoxa2* phenotypes only partially overlap with those of the AP-2 mutants, suggesting that AP-2 is not the only component involved in mediating *Hoxa2* expression in the second arch neural crest cells. In light of this comparison between mutant phenotypes, it is not surprising that enhancer activity is not lost or seriously reduced in an AP-2 null background. This confirms that AP-2 itself is not the only AP-2-related activity regulating the *Hoxa2* enhancer, but does not exclude the possibility that other AP-2 members along with AP-2 could be involved.

Our own studies and published analysis have shown that all three AP-2 family members are expressed in early premigratory and postmigratory neural crest cells at 8.0-9.0 dpc, and it is only in later stages that their patterns diverge or become restricted to different subsets of neural crest populations (Chazaud et al., 1996; Mitchell et al., 1991; Moser et al., 1997b). Since these overlaps correlate with the time when the *Hoxa2* enhancer is active, all of the AP-2 members could be contributing to *Hoxa2* regulation and analysis in double and triple mutants will be required to fully define how many members contribute to and are critical for *Hoxa2* enhancer regulation.

We have recently identified DAP-2, the *Drosophila* homolog of AP-2 family genes (Monge and Mitchell, 1998). The gene is expressed in discrete regions of the procephalic neuroectoderm, the brain, ventral nerve cord and maxillary
might represent an evolutionarily conserved regulatory relationship.

**Alternative mechanisms for neural crest expression of Hox genes**

Our temporal analysis of the Hoxa2 enhancer activity has demonstrated that there is never any reporter expression in r4, but that r4-derived neural crest cells shows strong staining immediately after delamination from the neural tube and migration into the second branchial arch. Regulatory regions capable of directing r2- and r4-restricted expression were previously mapped in the intron and 3' flanking region of the Hoxa2 gene (Frasch et al., 1995). Together with our results, this shows that the regulation of Hoxa2 expression in rhombomeres is regulated by independent elements from those used to control expression in cranial neural crest cells.

In contrast, small 10 bp motifs in Hoxb1 and Hoxb2 are able to simultaneously mediate expression in r4 and r4-derived neurogenic neural crest derivatives (Maconochie et al., 1997; Pöpperl et al., 1995). This reveals that common elements can be used for controlling hindbrain and neural crest expression, and suggests that neurogenic crest and rhombomeres may have similar regulatory requirements. It is important to note that one of the 15 bp deletions in NC3 eliminated r3 expression and reduced the levels of staining in second arch crest (construct #12; Fig. 3H). Hence, even though there are different requirements for Hoxa2 expression, some of the cis-elements can contribute to both hindbrain and crest expression. In the case of Hoxb1 and Hoxb2, regions controlling endogenous expression in mesenchymal cranial neural crest have not been isolated. This implies that additional or independent elements are utilised for controlling expression in mesenchymal versus neurogenic derivatives of neural crest and this might be a general property of Hox gene regulation in craniofacial morphogenesis. Consistent with this idea of independent regulation in different subsets of neural crest, several of the

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**Fig. 6.** An AP-2-binding site in the NC4 region is essential for Hoxa2 enhancer activity in the neural crest cells in vivo. Lateral views of β-gal stained 9.5 dpc transgenic embryos are shown. (A) Expression in r3, r5 and the second branchial arch crest in an embryo carrying construct #1 as a wild-type control. (B) A 9 bp deletion covering only the AP-2 site within this enhancer (ΔAP-2), specifically results in the loss of expression in second branchial arch neural crest cells. (C) In a similar manner, point mutations in the AP-2 site (mAP-2) also abolish reporter staining in cranial neural crest cells. The sequence of the wild-type, deleted and mutated versions of the AP-2 site in the enhancer are shown below. r, rhombomere; ba2 nc, second branchial arch neural crest cells; ba2, second branchial arch.

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**Fig. 7.** The AP-2 site from the Hoxa2 enhancer functions as an AP-2-responsive element in cultured cells. (A) Relative increase in transcription of reporter plasmids (indicated in B), co-transfected with the three murine AP-2 family members. The histogram indicates amounts of CAT protein produced in HepG2 cells co-transfected with mixtures containing 25 μg of the reporter plasmid with or without 6 μg of a designated AP-2 family member expression plasmid. All three AP-2 family proteins transactivated the reporter with three copies of the AP-2 site from the Hoxa2 enhancer (Hoxa2CAT) but failed to transactivate the basal reporter lacking these sites (BCAT). AP-2 and AP-2.2 also transactivated the A2BCAT reporter carrying three copies of an AP-2 site from the human metallothionein promoter, but to a lesser extent than Hoxa2CAT. AP-2β was unable to transactivate A2BCAT. The data represent two transfection experiments each with duplicate samples for all tests. (B) Diagram of the constructs for the three reporter genes and three expression vectors for murine AP-2 family proteins used in the transfection analysis in A are depicted.
phenotypes in Hox mouse mutants display abnormalities restricted to either the neurogenic or mesenchymal crest derivatives (Chisaka and Capecchi, 1991; Chisaka et al., 1992; Gavalas et al., 1998; Gendron-Maguire et al., 1993; Luftin et al., 1991; Rijli et al., 1993).

In conclusion, the identification of multiple control elements required for neural crest expression, is an important first step in beginning to define the upstream factors and the mechanisms integrating information from signalling cascades critical for patterning neural crest cells. Our findings suggest that Hoxa2 is a direct target for trans-activation by AP-2 family transcription factors and that roles of these proteins in neural crest cells of the second branchial arch are mediated in part through Hox-dependent pathways.

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Fig. 8. The Hoxa2 enhancer is trans-activated in vivo by AP-2 family members. (A) Expression in r3, r5 and neural crest cells of the second branchial arch in an embryo carrying construct #1 as a control. (B,C) Ectopic expression of AP-2 (B) and AP-2.2 (C) driven by a human β-actin promoter in embryos from a transgenic line carrying construct #1 results in the ectopic induction of Hoxa2 enhancer activity in the first branchial arch. The arrowheads in B and C indicate the new site of reporter trans-activation in the first branchial arch, which is never seen in control embryos (A). ba1 and ba2, first and second branchial arches; r, rhombomere; ov, otic vesicle. All embryos are 9.5 dpc and shown in a dorsolateral view.

Fig. 9. Comparison of Hoxa2 enhancer activity in wild-type and AP-2 mutant backgrounds. (A,B) Dorsal views of transgene expression (constructs #1) at 9.5 dpc in a wild-type (A) and a homozygous AP-2 null (B) background. The arrowheads indicate that despite the morphological changes in the AP-2+/+ embryos the pattern of staining in second arch neural crest cells is little affected. (C) Lateral views of the same embryos again showing similar reporter expression in wild-type and mutant backgrounds. +/+ , wild type; −/−, AP-2 homozygous mutant; ba2, second branchial arch; r, rhombomere.


