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ARTICLE

Identification and Regulation of Tissue-Specific *cis*-Acting Elements Associated With the Human AP-2α Gene

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Mice lacking transcription factor AP-2α exhibit defects in the formation of the head, body wall, heart, neural tube, eye, and limbs, reflecting important sites of AP-2α expression in the developing embryo. AP-2α is also expressed in the postnatal mammary gland and has been linked to tumor progression and defects in growth regulation in the breast. We have used a transgenic mouse approach to identify tissue-specific *cis*-acting sequences associated with expression of the human AP-2α gene. Our analysis indicates that multiple elements located throughout the gene contribute to expression in the trigeminal ganglia, spinal cord, mammary gland, and epidermis. A discrete *cis*-element located within the fifth intron is required for expression in the face and limbs, and we have derived a permanent line of AP-2α::*lacZ* transgenic mice to assess expression of this latter enhancer throughout morphogenesis. We also introduced this transgene into an AP-2α–null mouse background and detected subtle alterations of its expression within the progress zone and apical ectodermal ridge of the forelimbs. Similar changes in *lacZ* expression were observed within the zeugopod, and these correlated with defects in radius condensation in AP-2α–knockout mice. Taken together, these findings indicate that cell:cell communication within the forelimb is altered in the absence of AP-2α and reveal novel regulatory potential for AP-2α in limb development. *Developmental Dynamics 228:194–207, 2003.*

Key words: *lacZ* reporter; transgenic mouse; AP-2α; transcription factor; limb; face; mammary gland; spinal cord; gene expression; regulatory elements

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INTRODUCTION

The human AP-2α gene is the archetypal member of the AP-2 family of "basic-helix-span-helix" transcription factors (Williams et al., 1988; Moser et al., 1995; Bosher et al., 1996; Oulad-Abdelghani et al., 1996; Hilger-Eversheim et al., 2000; Zhao et al., 2001; Cheng et al., 2002; Feng and Williams, 2003). This gene family regulates important aspects of vertebrate development and is linked with tumor progression in both breast and skin cancer (Schorle et al., 1996; Zhang et al., 1996; Moser et al., 1997a; Nottoli et al., 1998; Turner et al., 1998; Gee et al., 1999; Bar-Eli, 2001; Auman et al., 2002; Brewer et al., 2002; Werling and Schorle, 2002; Zhang et al., 2003). During mouse and chick embryogenesis, AP-2α expression has first been reported in premigratory neural crest cells, and is maintained in these cells as they migrate throughout the trunk and cranial regions of the embryo (Mitchell et al., 1991; Shen et al., 1997). Several tissues derived from the neural crest continue to express AP-2α during their growth and morphogenesis, including the frontonasal process, branchial arches, and peripheral nervous system ganglia

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(Mitchell et al., 1991; Shen et al., 1997). An additional prominent site of AP-2α expression is in the developing limb bud. AP-2α transcripts can be detected soon after the limb bud appears, and expression persists in the mesenchyme of the progress zone during subsequent outgrowth. Significant AP-2α expression is also observed in the developing epidermis, kidney, cerebellum, spinal cord, and eye. AP-2α levels decline later in embryogenesis, although expression in some tissues, such as the ductal epithelium of the mammary gland, persists into adulthood (Zhang et al., 2003). Indeed, altered regulation of AP-2α expression within the mature mammary gland has been linked to the progression of human breast cancer (Turner et al., 1998; Gee et al., 1999).

Gene targeting experiments have shown that the AP-2α gene is required for multiple developmental processes in vertebrates. Mice lacking both copies of the AP-2α gene die perinatally and exhibit severe malformation of the head and trunk, including exencephaly and thoracoabdominoschisis (Schorle et al., 1996; Zhang et al., 1996). Further analysis has shown that AP-2α is independently required for at least six major morphogenetic events during embryogenesis: formation of the neural tube, face, eye, body-wall, cardiovascular system, and forelimbs (Nottoli et al., 1998; West-Mays et al., 1999; Brewer et al., 2002). The absence of AP-2α can also affect cell fate determination and gene expression in the skin and nervous system (Maytin et al., 1999; Kramer et al., 2000).

The range of phenotypes observed in the absence of AP-2α displays a significant overlap with those caused by teratogenic levels of retinoic acid (Ross et al., 2000). Moreover, the expression of AP-2α can be altered by the application of retinoic acid both in vivo and in vitro (Williams et al., 1988; Lüscher et al., 1989; Shen et al., 1997), strongly suggesting that AP-2α is an important component of this morphogen's mechanism of action. Indeed, with respect to craniofacial development, the AP-2α gene may provide an important link between the environmental and genetic causes of human birth defects. Experiments in the chick indicate that alterations in AP-2α expression in the developing face correlate with the teratogenic effects of retinoic acid in this tissue (Shen et al., 1997). In addition, genetic mapping studies indicate that the human AP-2α gene (*TFAP2A*) is located at chromosome 6p24, a locus that is associated with craniofacial abnormalities, including orofacial clefting (Davies et al., 1995, 1999; Topping et al., 2002). This latter finding is significant, given that mice with a single functional copy of the AP-2α gene frequently display facial dysplasia characterized by a twisted snout and dental malocclusion (Nottoli et al., 1998). Moreover, mice lacking both copies of AP-2α, and chimeric mice containing a mixture of wild-type and AP-2α–null cells, have severe facial clefts (Schorle et al., 1996; Zhang et al., 1996; Nottoli et al., 1998). The orofacial defects commonly associated with the AP-2α gene are classified as cleft lip with or without clefting of the primary palate (CL/P) and are distinct from clefts of the secondary palate (Wilkie and Morriss-Kay, 2001). CL/P pathologies result from defective fusion of the facial prominences during a critical period of embryogenesis, i.e., ~embryonic day (E) 9.5–E12.5 in the mouse. The observation that the AP-2α gene is highly expressed in the facial prominences during this time period suggests that this transcription factor directly regulates growth and fusion of the face from within these tissues. Therefore, it is likely that identification and characterization of the *cis*-acting sequences responsible for AP-2α expression in the facial prominences would advance our understanding of the regulation of craniofacial development.

Data obtained in chick and mouse experimental systems also demonstrate that AP-2α may have an important role in patterning the forelimb. In the chick, removal of the apical ectodermal ridge (AER) causes a rapid loss of AP-2α expression in the mesenchyme, and this loss precedes the severe reduction in the size of the limb bud (Shen et al., 1997). Treatment with fibroblast growth factor (FGF) 4 rescues limb bud outgrowth and leads to a recovery of AP-2α expression in the limb mesenchyme. Taken together, these data indicate that AP-2α in the mesenchyme may act downstream of regulatory signals emanating from the AER in regulating outgrowth and patterning of the limb bud. A patterning function for AP-2α in the limb is supported by the finding that mice in which there is a loss or alteration of AP-2α expression frequently exhibit forelimb defects. The majority of AP-2α-null mice exhibit forelimb phocomelia, characterized by loss of the radius (Schorle et al., 1996; Zhang et al., 1996). More striking phenotypes are seen in chimeric mice in which the juxtaposition of wild-type and AP-2α–null cells can lead to forelimb duplications (Nottoli et al., 1998). These data indicate that cross-talk between populations of cells may be critical for the expression and function of AP-2α. Recently, studies on the single *Drosophila* AP-2 gene (*dAP-2*) have shown that it may also regulate leg morphology through both cell-autonomous and nonautonomous mechanisms (Kerber et al., 2001; Monge et al., 2001).

Given the importance of AP-2α during mammalian development, and its possible role in breast cancer, we wish to understand the regulatory mechanisms responsible for its specific expression pattern. We previously have identified the AP-2α proximal promoter and mapped conserved initiator and octamer sequences within this region that are responsible for the basal expression of this gene (Creaser et al., 1996). Here, by using reporter gene analysis in transgenic mice, we have extended these studies by identifying *cis*-regulatory elements associated with the human AP-2α gene that direct expression to tissues including the limb, face, and mammary gland. Furthermore, we have used these AP-2α::*lacZ* transgenes to analyze changes in the distribution of cells that are capable of using these AP-2α regulatory sequences in the AP-2α-null background. These studies indicate that communication between the progress zone and

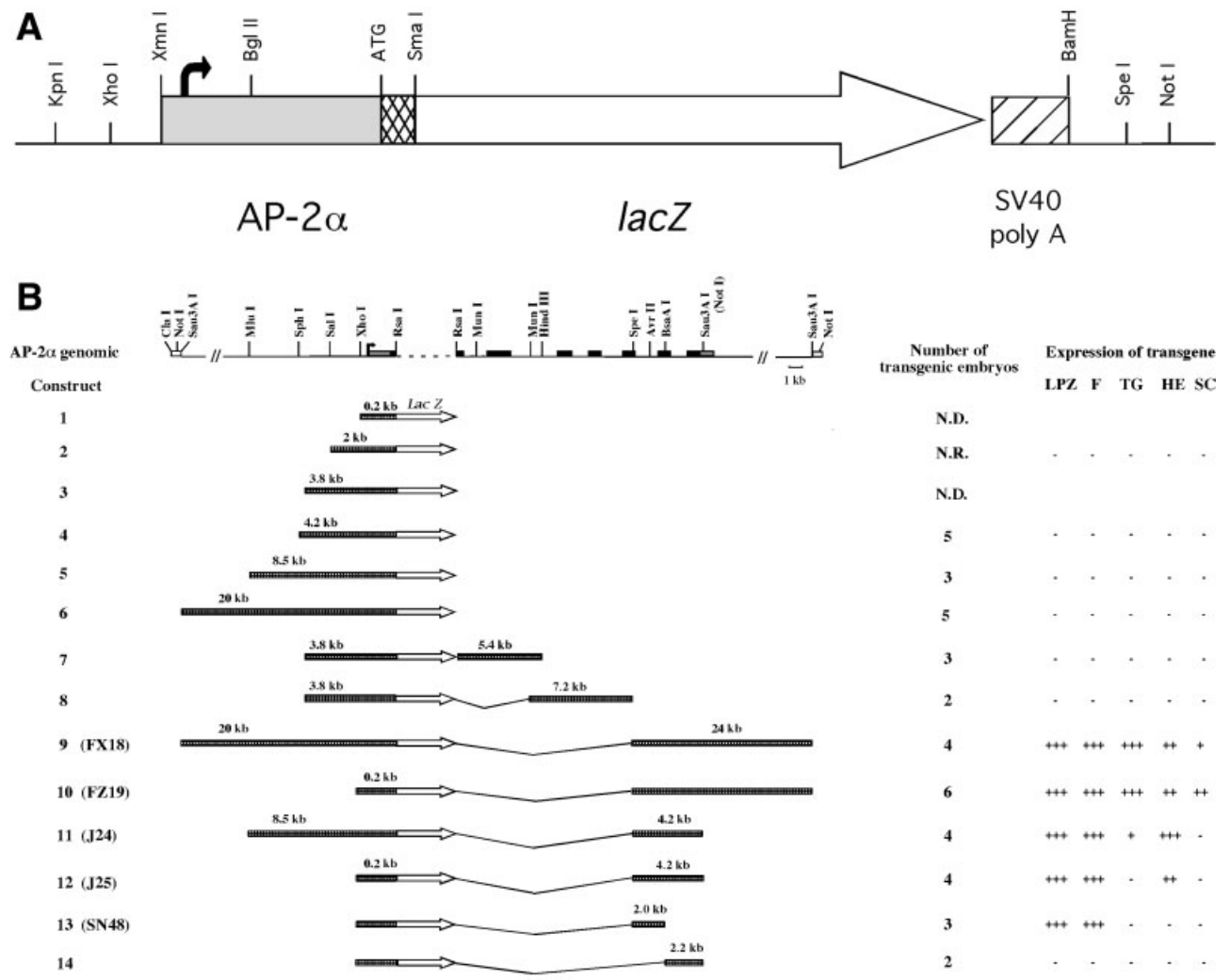

Fig. 1. Schematic diagram of the human AP-2α gene and the AP-2α::*lacZ* fusion constructs and summary of transgene expression. **A:** The C1 *lacZ* 42 plasmid showing the minimal AP-2α promoter as well as the 5′ untranslated leader sequence (gray box) that contains an IR3 repetitive element, the major transcriptional initiation site (bent black arrow), and the first 13 codons of AP-2α (hatched box), which are fused in-frame to the *lacZ* coding region. **B:** The organization of the human AP-2α genomic clone, illustrating the position of the exons (black boxes), 5′ and 3′ untranslated regions (gray boxes), and the relevant cosmid sequences (open boxes) is shown at the top. The dashed line has been introduced to account for the position of the *lacZ* gene insertion in the first intron. Below are shown the various constructs referred to in the text, along with their pattern of expression. LPZ, limb bud progress zone; F, frontonasal prominence; TG, trigeminal ganglia; HE, head ectoderm; SC, spinal cord. The relative intensity of β-galactosidase activity is also shown ranging from no staining (-) to intense staining (+++). The number of transgenic embryos obtained for each construct that generated either specific or nonspecific β-galactosidase activity is also indicated. N.D., not done. N.R., performed but not recorded. Permanent lines were obtained using construct 11.

apical ectodermal ridge is altered in the absence of AP-2α, and reveal that the AP-2α gene may be subject to autoregulation.

RESULTS

Multiple AP-2α *cis*-Regulatory Sequences Are Located Downstream of the Transcriptional Start Sites

We conducted a transient transgenic analysis in the mouse to identify the sequence elements responsible for the expression of AP-2α in the developing mammalian embryo. Previously, in vitro analysis of the AP-2α basal promoter indicated that it does not contain a classic TATA box but instead relies on an initiator element that works in association with a conserved octamer motif just upstream (Creaser et al., 1996). Given the atypical nature of the AP-2α initiation sites, we decided to identify tissue-specific *cis*-regulatory elements required for the in vivo expression of AP-2α in the context of its own promoter. For these purposes, a basic construct was made, C1 Xho β gal, which contained approximately 200 nucleotides of the human AP-2α promoter upstream of the start sites. This construct possesses all the aforementioned sequence elements, the translational start site, and the first 13 codons of AP-2α fused in frame to the *lacZ* coding region (Fig. 1A). By using this basic plasmid, we then de-

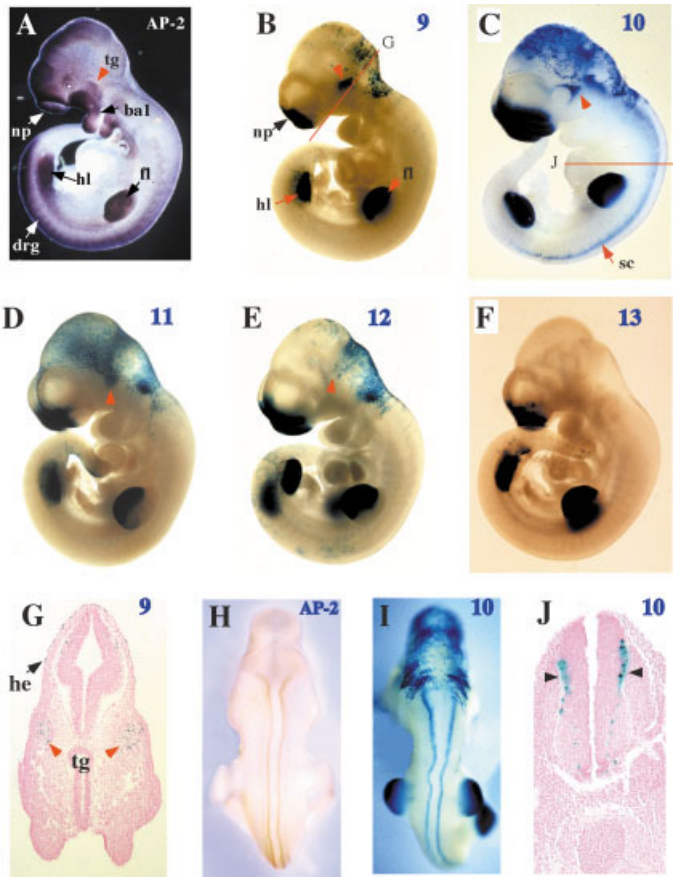

Fig. 2. Expression of the human AP-2α::*lacZ* reporter constructs in transgenic mouse embryos. The transgene construct numbers, or staining corresponding to the endogenous mouse AP-2α gene (AP-2), are indicated in blue lettering at the top right of each panel. **A:** Lateral view of an embryonic day (E) 10.5 nontransgenic mouse showing expression of the endogenous AP-2α protein. Expression is highlighted in the nasal processes (np), branchial arches (ba1), dorsal root ganglia (drg), forelimbs (fl) and hindlimbs (hl). Red arrowhead marks position of the trigeminal ganglion (tg). **B:** Lateral view of E10.5 mouse harboring construct 9. Reporter activity was detected in the nasal processes, trigeminal ganglia, head epidermis, forelimbs, and hindlimbs. **C:** Lateral view of E10.5 mouse harboring construct 10, FZ19. Reporter activity was detected in essentially the same sites as those for construct 9, but higher activity was detected in the spinal cord (sc). **D:** Lateral view of E10.5 J24 transgenic mouse (construct 11). **E:** Lateral view of E10.5 mouse harboring construct 12. No expression was detected in the trigeminal ganglion. **F:** Lateral view of E10.5 mouse harboring construct 13. **G:** Cross-section of the hindbrain of an E10.5 mouse bearing construct 9, in the plane of section diagrammed in B. Note staining in the head ectoderm (he) and trigeminal ganglia. **H:** Dorsal view of an E10.5 nontransgenic mouse showing expression of the endogenous AP-2α gene in the spinal cord by in situ hybridization. **I:** Dorsal view of E10.5 mouse bearing construct 10 (same embryo as shown in B). **J:** Cross-section of embryo shown in C, at the plane diagrammed.

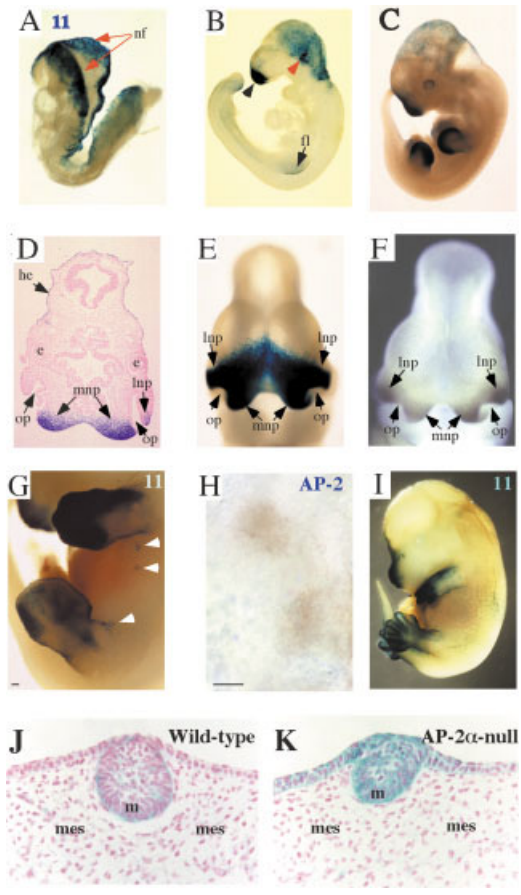

Fig. 3. The expression pattern of construct 11, the J24 transgene, between E8.5 and E13.5. Lateral views of embryos are shown at embryonic day (E) 8.5 (**A**), E9.5 (**B**), E11.5 (**C**). Note, an E10.5 J24 embryo is shown in Figure 2D. The neural folds (nf, red arrows), trigeminal ganglion (red arrowhead), facial prominence (black arrowhead), and forelimb bud (fl, black arrow) are highlighted. **D:** Cross-section through head of an E10.5 transgenic mouse. lnp, lateral nasal process; mnp, medial nasal process; he: head epidermis; e: eye; op: olfactory pit. **E:** Frontal view of E10.5 head expressing the J24 transgene. **F:** Frontal view of E10.5 nontransgenic mouse head stained by whole mount immunohistochemistry with an AP-2α-specific monoclonal antibody. Lateral views of embryos at E12.5 (**G**), and E13.5 (**I**). G is an enlargement of the flank to illustrate the β-galactosidase activity observed in the mammary gland rudiments (white arrowheads). **H:** In situ hybridization on skin that has been removed from the flank of a nontransgenic E12.5 mouse to demonstrate expression of the endogenous AP-2α gene in the mammary gland rudiments. **J,K:** Cross-section of E12.5 wild-type (J) and AP-2α–null (K) mammary bud. M, epithelial component of mammary rudiments; mes, mesenchyme. Scale bar = 250μm in G, 100 μm in H.

veloped a series of constructs in which up to 20 kb of upstream AP-2α sequences were attached to the *lacZ* gene (constructs 1–6, Fig. 1B). Members of this series were next injected into one-cell mouse embryos, and the transgenic founder mice were analyzed subsequently by whole-mount β-galactosidase staining at E10.5. This developmental time point was chosen for the initial analysis because it enables AP-2α expression to be assayed in both the limb bud and in neural crest derivatives, such as the face and cranial ganglia. Despite the importance of the promoter proximal region for basal level expression in vitro, we did not detect any strong *lacZ* expression in vivo from constructs containing this element when it was placed solely in association with upstream sequences. Even the presence of 20 kb of upstream AP-2α genomic sequences (construct 6) failed to produce any strong and consistent pattern of *lacZ* expression in transgenic mice at E10.5.

We next examined if *cis*-regulatory sequences might reside in the regions downstream of the AP-2α start sites. The human AP-2α gene consists of seven exons and six introns that span 18 kb (Bauer et al., 1994). A large transgene was made containing 20 kb of upstream sequences in association with a 24 kb fragment of downstream sequences. These latter sequences encompassed the fifth and sixth introns and the 20-kb region immediately downstream of the stop codon (construct 9, Fig. 1B). At E10.5, this construct was able to direct strong expression of the transgene to the mesenchyme of the frontonasal process and limb buds (Fig. 2B), which are major sites of endogenous AP-2α expression at this stage of development (Fig. 2A and Mitchell et al., 1991). Additional staining was observed in the trigeminal ganglia, and in dorsolateral cells within the brain and spinal cord (Fig. 2B,G, and data not shown), which again correspond to sites of endogenous AP-2α expression. Transgene expression was also observed in files of cells that emanated from the dorsal surface of the head. In cross-section, these cells occurred within the head ectoderm and were not components of the neural crest (Fig. 2G).

The 24-kb downstream fragment was next tested in the context of the minimal 0.2-kb human AP-2α promoter. The pattern of *lacZ* activity directed by this shorter transgene, (Fig. 1B, construct 10; Fig. 2C,I) was similar to that seen with the aforementioned transgene that contained 20 kb of upstream sequence (Fig. 1B, construct 9; Fig. 2B). These findings indicate that major *cis*-regulatory elements responsible for AP-2α expression occur downstream of the start site. However, one noticeable difference in the activity of the two transgenes was that construct 10 was more strongly expressed within the spinal cord (Fig. 2C,I,J) than construct 9 (Fig. 2B). This latter observation may indicate that negative regulatory sequences reside upstream of the basal promoter, and their removal allows for higher levels of spinal cord expression from the shorter transgene.

Because regulatory sequences appeared to be concentrated downstream of the start site, we generated and tested additional transgenes containing sequences from the more promoter proximal intron sequences within introns 1–4. However, a construct composed of the first intron and part of the second intron attached to 3.8 kb of AP-2α upstream regulatory sequences (Fig. 1B, construct 7), failed to produce any specific staining. Similarly, a fragment containing the majority of the second intron and all of the third and fourth introns (Fig. 1B, construct 8) did not produce any consistent β-galactosidase staining pattern. Therefore, we concentrated our further transgenic analysis on the more downstream sequence elements, from intron 5 onward, that recapitulated multiple aspects of the endogenous AP-2α expression pattern.

Regulatory Elements Responsible for Facial Mesenchyme and Limb Bud Expression Reside Within the Fifth Intron

Two constructs were made in which the 4.2-kb genomic region that spans exons 5 through 7 was attached to either 8.5 kb or 0.2 kb of upstream sequence (Fig. 1B, constructs 11 and 12, respectively). The shorter transgene, construct 12 (J25), directed β-galactosidase expression to the limb, face, and head ectoderm (Fig. 2E), indicating that major regulatory sequences lie within the fifth and sixth introns. The addition of 8.3 kb of further upstream sequences in the plasmid J24 (construct 11) gave the same pattern of expression as construct 12 but also restored expression at a low level to the trigeminal ganglia (Fig. 2D, arrowhead). This latter finding indicates that there are regulatory sequences responsible for AP-2α expression upstream of the start site, although they may need to cooperate with downstream sequences to yield a high level of expression. Unlike construct 10, neither construct 11 nor 12 produced expression in the spinal cord, suggesting that the elements responsible for the expression in spinal cord were located downstream of the coding region. The 4.2-kb fragment that directed ex-

Fig. 4. Analysis of embryonic day (E) 12.5 spinal cord expression in transgenic mice bearing construct 10 (FZ19). **A,B:** Dorsal and lateral views of an E12.5 transgenic mouse, respectively. **C,D:** Cross-sections of the E12.5 transgenic mouse shown in B, in the planes of section diagrammed.

Fig. 5. Comparison of J24 *lacZ* transgene expression between wild-type (WT) and AP-2α-null (Null) embryos. **A,B:** Lateral view of wild-type (A) and AP-2α–null (B) J24 embryos at embryonic day (E) 10.5. **C,D:** Frontal view of a wild-type (C) and an AP-2α–null (D) embryonic head at E10.5. **E,F:** Lateral view of wild-type (E) and AP-2α–null (F) embryo at E12.5. **G,H:** Frontal view of a wild-type (G) and an AP-2α–null (H) embryonic head at E12.5.

Fig. 7. Alterations in J24 *lacZ* transgene expression in the forelimb buds of AP-2α–null embryos correlate with agenesis of the radius. Forelimbs from wild-type (A–C) and AP-2α–null (D,E) embryos were analyzed for the expression of the J24 *lacZ* transgene. **A,D:** Dorsal view of embryonic day (E) 12.5 wild-type (A) and AP-2α-null (D) forelimbs. **B,E:** Cross-section of the forelimbs shown in A and D at the planes diagrammed. The positions of the condensing radius (r) and ulna (u) are shown. The mutant forelimb in E lacked a radius (arrow). **C:** Cross-section of E13.5 wild-type forelimb in the region of the digits.

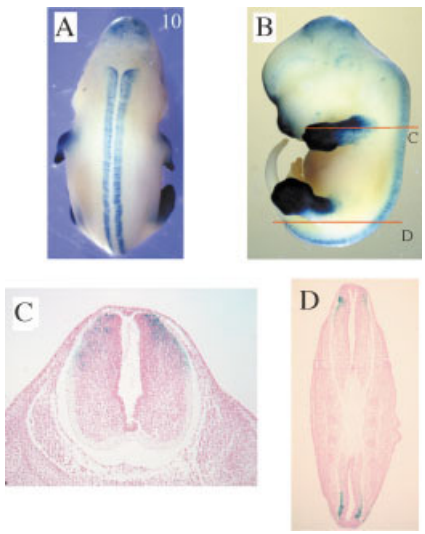

Fig. 4.

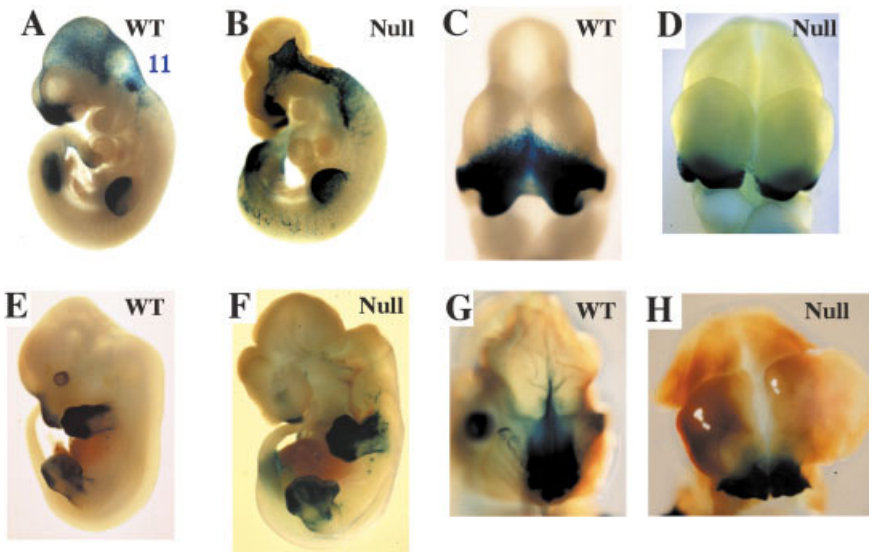

Fig. 5.

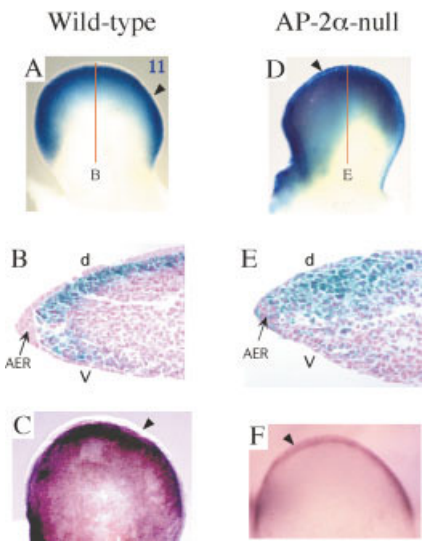

Fig. 6. J24 *lacZ* transgene expression is altered in the forelimb buds of AP-2α–null embryos. Forelimbs from wild-type (A–C) and AP-2α–null (D–F) embryos were analyzed either for the expression of the J24 *lacZ* transgene (A,B,D,E) or for the presence of endogenous AP-2α transcripts (C,F). **A,D:** Dorsal iew of E11.5 wild-type (A) and AP-2α–null (D) transgenic forelimbs. The arrowhead indicates the position of the apical ectodermal ridge (AER). **B,E:** Sagittal section of limb buds shown in A and D, respectively, at the levels indicated. The dorsal (d) and ventral (V) axis is shown, and the AER is indicated by an arrow. **C,F:** Dorsal view of E10.5 nontransgenic forelimbs from wild-type (C) and AP-2α–null (F) embryos, in which expression of the endogenous AP-2α transcript has been visualized using whole-mount in situ hybridization. The arrowhead indicates the position of the AER. Note, the limb in C has been overstained to illustrate more clearly the lack of expression in the AER.

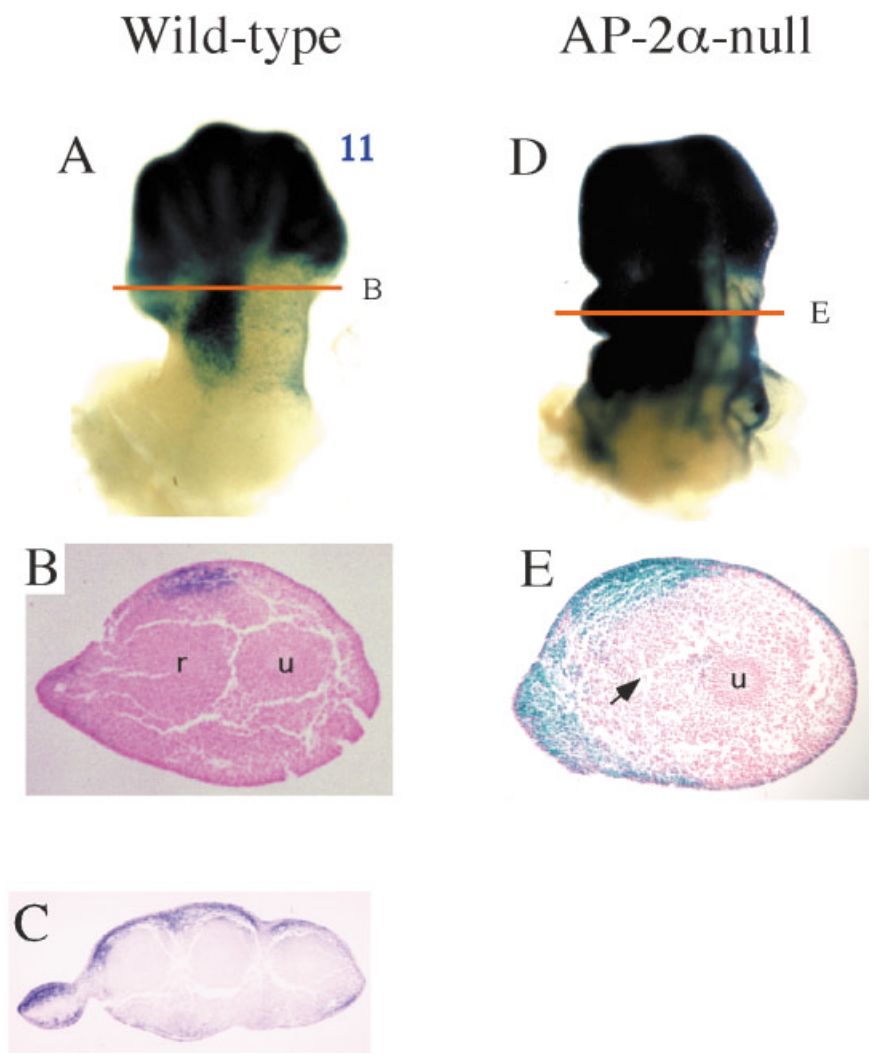

Fig. 7.

pression to the face, limbs, and ectoderm was further divided into two pieces, corresponding to introns five or six, and tested in association with the minimal promoter (Fig. 1B, constructs 13 and 14, respectively). The 1.95-kb fifth intron was able to direct expression to the face and limbs (construct 13; Fig. 2F), whereas the sixth intron did not possess any activity in this context (construct 14). Thus, an enhancer element specific for the mesenchyme of the limb and frontonasal prominence resides within the fifth intron of the human AP-2α gene.

Spatiotemporal Analysis of J24 Transgene Expression Reveals *cis*-Regulatory Sequences Directing Mammary Gland and Epithelial-Specific Gene Expression

To perform a more comprehensive analysis of the spatiotemporal regulation of AP-2α transgene expression in the limb, face, and trigeminal ganglia, we generated stable transgenic mouse lines with construct 11, J24 (Fig. 3). Two lines were obtained, which both gave identical patterns of *lacZ* expression, and one of these lines was analyzed in more detail. During embryogenesis, expression of this transgene was first detected between E8.0 and E8.5, in the cranial neural folds (Fig. 3A). The onset of transgene expression in the embryo and its location surrounding the neural plate approximated to the expression pattern of the endogenous AP-2α gene. Between E9.5 and E11.5, expression associated with the neural folds became concentrated in files of ectodermal cells that emanated from the dorsal surface of the head (Fig. 3B–D).

By E9.5, β-galactosidase activity became apparent in the frontonasal prominence (Fig. 3B) and persisted through E13.5, becoming more restricted to the midline of the face over this time period. Histologic examination at E10.5 indicated that transgene expression was confined to the mesenchyme of the medial and lateral nasal processes and was not present in the facial ectoderm at this stage (Fig. 3D). The timing and spatial distribution of β-galactosidase activity within the mesenchyme of the medial and lateral nasal processes during this period recapitulates that of the endogenous AP-2α protein (Fig. 3E,F). These findings suggest we have identified an enhancer element in the human AP-2α gene that can direct a very similar spatiotemporal pattern of craniofacial expression as the endogenous mouse gene. The J24 transgene also contained sequences that were capable of directing β-galactosidase activity to the trigeminal ganglia, another site of endogenous AP-2α expression. Transgene expression in these ganglia was apparent from E9.5 to E10.5 but was not observed at subsequent stages (Figs. 2D, 3B, and data not shown).

The J24 *lacZ* transgene also recapitulated the expression pattern of the endogenous AP-2α gene in the limb bud progress zone. Expression could first be detected in a patch of mesenchyme in the nascent forelimb bud at E9.5 (Fig. 3B). By E10.5, expression was readily apparent in a region corresponding to the progress zone in both fore- and hindlimb buds (Fig. 2D). Expression was then maintained in the distal limb mesenchyme from E11.5–13.5, becoming progressively confined to the autopod during these stages (Fig. 3C,G,I). A second, more limited, expression domain could be observed at E12.5 and E13.5 as a dorsal stripe in the mesenchyme of the zeugopod (Fig. 3G,I). We will discuss the histologic analysis of transgene expression in wild-type limbs in the final part of the Results section, in a comparison to the data obtained in AP-2α–null limbs.

J24 transgene expression also became evident in two further tissues by E12.5: the developing genital tubercle (data not shown) and the epithelial compartment of the mammary gland rudiments (Fig. 3G,J). Mammary-specific transgene expression was observed in all five pairs of mammary buds at this time point. In Figure 3G, arrowheads indicate three visible buds that are discernible as blue dots on the flank, whereas the other two buds on this side are obscured by the limbs. Because AP-2α expression was not previously noted in the embryonic mammary glands, in situ hybridization was used to extend this analysis. In agreement with the transgenic data, we determined that endogenous AP-2α transcripts were also expressed in the mammary gland rudiments at E12.5 (Fig. 3H). Transgene expression was confined to the epithelial component of the mammary gland bud, and no reporter activity was detected in the surrounding mesenchyme (Fig. 3J). Extensive ectodermal expression of the endogenous AP-2α gene has also been reported in the developing hair follicles of the epidermis (Byrne et al., 1994). The J24 transgene also directed widespread punctate β-galactosidase staining corresponding to this aspect of AP-2α expression by E13.5 (Fig. 3I). This age was the last time point analyzed by whole-mount analysis since we and others have noted that stain penetration problems occur in mice from E13 onward (Hogan et al., 1994). The AP-2α gene is not extensively expressed during the postnatal period; therefore, we have not performed a detailed analysis of J24 expression in the adult at this juncture. Nevertheless, we have noted that J24 transgene activity occurred within the ductal epithelia of the mammary gland at postnatal time points (data not shown) in common with the expression of the endogenous AP-2α gene (Zhang et al., 2003). This latter finding suggests that the enhancer we have identified may be critical for regulating AP-2α function in normal breast physiology and during the progression of breast cancer (Bosher et al., 1995; Turner et al., 1998; Gee et al., 1999; Zhang et al., 2003).

Analysis of Sequences Required for Expression in the Mammary Gland, Genital Tubercle, and Spinal Cord

Given the potential importance of the sequences required for mammary-specific AP-2α expression, further studies were undertaken to determine their location. The embryonic mammary buds are not apparent at the E10.5 time point we had used previ-

ously for transient analysis. Therefore, a new set of E12.5 transient transgenic embryos were generated and analyzed by using either construct 5, which contains only the upstream sequences present in J24, or construct 12, which has the minimal promoter and all of the J24 downstream elements. Unlike J24, neither of these constructs were capable of generating expression in the E12.5 mammary gland rudiments (data not shown). Thus, expression in the embryonic mammary gland required cooperation between *cis*-regulatory elements located both upstream and downstream of the transcriptional initiation sites, a situation analogous to that observed for expression within the trigeminal ganglia. In contrast, the sequences required for expression within the genital tubercle at E12.5 were more localized. *LacZ* expression was obtained in transgenic mice harboring construct 12 but was not seen with construct 5 (data not shown). These findings position the regulatory sequences required for genital tubercle expression downstream of the start site within introns five and six.

Construct 10, which contained more extensive downstream sequence than J24, directed expression to the face, limbs, trigeminal ganglia, head ectoderm, and spinal cord at E10.5 (Fig. 2C). We also analyzed this transgene in a transient analysis at E12.5 to determine whether there were any changes in the expression pattern (Fig. 4). β-galactosidase activity remained robust in the limbs, face, and spinal cord (Fig. 4A,B). However, expression in the trigeminal ganglia and head ectoderm were no longer apparent at E12.5, and no new expression domains were observed. The continued expression in the limb and face and loss of expression in the head ectoderm and trigeminal ganglia were consistent with the data obtained with the J24 transgene at E12.5. With respect to the spinal cord, construct 10 expression was more dorsal in aspect at E12.5 when compared with E10.5 (Fig. 4C, compare with 2J). Moreover, there was a change in the dorsoventral position of the *lacZ*-positive cells along the rostral caudal axis of the spinal cord. Cells expressing the transgene were restricted to more dorsolateral positions toward the head (Fig. 4C), in contrast to their more mediolateral positions toward the tail (Fig. 4D). Together, these findings indicate that we have identified the appropriate regulatory sequences necessary for spinal cord expression, because the spatiotemporal expression pattern of this human *lacZ* transgene corresponds to that of the endogenous mouse AP-2α gene in presumptive spinal sensory interneurons (compare Fig. 2H and 2I; Mitchell et al., 1991). Further mapping studies suggest that the spinal cord regulatory sequences occur within the most 3′ 18 kb of the AP-2α sequences that we have tested (data not shown).

Expression of the Transgene in the Forelimb Buds Is Expanded in AP-2α–Null Mice

We wished to determine whether cell populations that would normally express AP-2α were specifically altered in the AP-2α– knockout mouse background. Therefore, we crossed the J24 transgene into the AP-2α–null background so that the *lacZ* marker could be used to follow the distribution of such cell populations. In general, we found that significant expression of the *lacZ* transgene still occurred in the appropriate tissues in the absence of AP-2α, although such tissues—including the face and mammary buds—were frequently displaced due to the dysmorphology (Fig. 5). These results indicated that the loss of AP-2α gene expression did not necessarily lead to a reduced population size for the cells in which it was usually expressed, a situation we have also noted with a *lacZ* knock-in allele of AP-2α (Brewer et al., 2002). However, there were two exceptions to this general trend. The first change was a decrease in transgene expression in the trigeminal ganglia, a result that was expected because these structures are greatly reduced in size in AP-2α–null mice (data not shown; Schorle et al., 1996; Zhang et al., 1996). More striking and unexpected were the alterations of J24 transgene expression in the forelimbs of AP-2α–null mice when compared with their wild-type littermates.

Whole-mount preparations of wild-type limbs at E11.5 showed that J24 was expressed in distal limb tissue (Fig. 6A). Sagittal sections through the forelimb at this stage showed that the β-galactosidase activity was present in the mesenchyme and not in the overlying epidermis or in the apical ectodermal ridge (Fig. 6B). Within the mesenchyme of the progress zone, transgene activity was more pronounced toward the dorsum (Fig. 6B). In marked contrast, for the E11.5 AP-2α–null forelimb, additional *lacZ* reporter activity was detected in the AER and the epidermis, as well as in its normal mesenchymal location (Fig. 6D,E). Furthermore, with respect to the progress zone, expression in the AP-2α–null background now encompassed the entire dorsoventral axis of the limb bud, although as in the wild-type it was still more concentrated on the dorsal aspect (Fig. 6E). To ascertain if these alterations were a peculiarity of the transgene or represented bona fide changes in AP-2α gene regulation, we examined the expression of the nonfunctional targeted AP-2α–knockout allele. Although this allele is not capable of producing a functional protein, it is still transcribed in the null background. In situ hybridization studies confirmed that the endogenous AP-2α gene was also activated in the AER of the mutant limbs (Fig. 6F, arrowhead).

Whole-mount and histologic analysis of J24 transgene expression was also performed at E12.5 and E13.5 for wild-type forelimbs. At these stages, the transgene was still heavily expressed in the distal mesenchyme of limb bud, although it was less prevalent in the regions corresponding to digit formation (Fig. 7A; also Fig. 3G,I). In histologic sections, expression was again most pronounced in the dorsal region of the mesenchyme, adjacent to the regions of digit condensation (Fig. 7C). Patches of expression could also be seen in the limb epidermis beginning at E13.5 (Fig. 7C), in common with the activation of epidermal expression in other regions of the embryo at this stage (Fig. 3I).

At E12.5 and E13.5 a second domain of expression occurred in the

zeugopod, specifically in a stripe of mesenchyme that resides dorsal to the position of radius condensation (Fig. 7A,B). When J24 was introduced into an AP-2α–null background, alterations in transgene expression were also apparent at E12.5 in this region of the mouse forelimb bud. First, expression of the transgene was activated in the epidermis of the knockout mice (Fig. 7E) in common with the E11.5 time point (Fig. 6E). Second, the discrete domain of expression normally seen in the mesenchyme dorsal to the radius was often, but not always, replaced by an expanded region of reporter activity that encompassed the lateral and ventral regions on the posterior side of the limb bud (Fig. 7D,E). The partial penetrance of this altered expression pattern was intriguing given that 65–70% of AP-2α–knockout animals normally lacked a radius (Zhang et al., 1996). Further investigation revealed that there was a significant correlation between the two phenomena: of 18 forelimbs, 12 had the expanded domain of reporter activity, and these also lacked areas of condensation corresponding to the position of the radius (Fig. 7E, and data not shown). The aberrant expression of AP-2α specifically affects the patterning of the forelimb and does not produce gross alterations of hindlimb morphology. Intriguingly, in contrast to the forelimb, the expression of the J24 *lacZ* transgene in the hindlimb was essentially identical in both the wild-type and AP-2α–knockout backgrounds. In particular, no expression was observed in the apical ectodermal ridge of the hindlimb in either genetic background, and there were no differences in the mesenchymal domains of expression (data not shown).

DISCUSSION

The AP-2α gene performs important functions in several morphogenetic processes critical for mammalian development. It will be necessary to determine how expression of this gene is controlled before we can fully understand the mechanisms by which AP-2α participates in the regulatory network that governs embryogenesis. In this report, by using a series of AP-2α::*lacZ* transgenes, we have demonstrated that the tissue-specific expression of AP-2α relies on multiple regulatory elements. Furthermore, we have discovered that the pattern of gene expression generated by these sequences can be altered in the forelimb in the absence of the AP-2α protein, indicating that this gene may have a hitherto unsuspected negative autoregulatory function.

We had originally determined that the basal promoter of the human AP-2α gene encompassed the transcriptional initiation sites and the adjacent upstream region of ~100 bp (Creaser et al., 1996). This sequence was highly conserved between human, mouse, and chicken and was able to direct accurate transcriptional initiation in tissue culture cells. However, in the current study, we demonstrate that this sequence by itself does not possess the requisite *cis*-regulatory elements to direct appropriate tissue-specific expression in vivo. Moreover, the addition of up to 20 kb of genomic sequence 5′ to the start site failed to produce any consistent pattern of *lacZ* gene expression. In contrast, the addition of 24 kb of genomic DNA extending downstream from exon five produced robust β-galactosidase activity in multiple sites in the developing embryo. Specifically, the downstream sequences contain *cis*-regulatory elements directing expression to the neural folds, frontonasal prominences, limb bud progress zone, trigeminal ganglia, central nervous system, skin, mammary gland, and genital tubercle. These tissues correspond to important sites of endogenous AP-2α expression, and the results indicate that we have identified bona fide regulatory sequences responsible for many aspects of AP-2α gene transcription within the developing embryo (Mitchell et al., 1991). Our analysis further illustrates that the individual *cis*-regulatory sequences are dispersed over a large region of the AP-2α gene. Indeed, although we analyzed ~45 kb of human AP-2α genomic sequence the *cis*-acting elements responsible for AP-2α expression in the developing eye, migrating neural crest and branchial arches were not identified. This finding indicates that the *cis*-acting sequences required for AP-2α expression in the mesenchyme of the facial prominences are distinct from the regulatory elements needed in the migratory NCC that give rise to the facial prominences. Similarly, distinct regulatory networks must be responsible for directing AP-2α expression in the mesenchyme of the facial prominences as opposed to the branchial arches. An alternative possibility is that our findings represent a fundamental difference in regulation of the human and mouse AP-2α genes in their respective species. Thus, the human gene may have lost or gained some expression potential with respect to the mouse gene that could reflect differences in the utilization of AP-2α to sculpt body plan in these distinct mammalian species. Alternatively, more subtle differences in the *cis*-regulatory elements between the human and mouse genes could alter the ability of the cognate transcription factors to bind to the human gene in the transgenic mouse system with consequent effects on the expression pattern. Future experiments on both the mouse and human AP-2α genes will be necessary to determine whether there are species-specific functional differences between the respective *cis*-regulatory sequences and/or whether additional sequence elements reside even more distal to the promoter and enhancer elements analyzed in this study.

Our analysis indicates that the interaction of multiple enhancer elements is responsible for the appropriate tissue-specific expression of the AP-2α gene. The most defined enhancer region resides within the fifth intron, and this sequence directs strong *lacZ* expression to the frontonasal prominence and limb bud progress zone in association with the core promoter. An examination of the sequence of the fifth intron enhancer element did not reveal any clearly defined *cis*-acting motifs. In particular, even though AP-2α expression is responsive to retinoic acid application in the developing chick face (Shen et al., 1997), we were unable to identify a conserved retinoic acid responsive element based

solely on the primary sequence. Therefore, a detailed molecular dissection of this 1.95-kb intronic region will be required to pinpoint the crucial *cis*-acting motifs required for limb and face-specific AP-2α expression. Such an analysis will be especially pertinent with respect to craniofacial development because the AP-2α gene maps to a chromosomal locus associated with human orofacial clefting (Davies et al., 1995, 1999; Topping et al., 2002). Thus, it is possible that alterations within the AP-2α coding sequences, or within the associated *cis*-regulatory elements, will contribute to human craniofacial abnormalities. Indeed, the reason we chose to study the human rather than mouse AP-2α *cis*-regulatory sequences was the potential for understanding the genetic causes of human craniofacial defects.

Sequence elements within the fifth intron can contribute to other aspects of AP-2α expression in addition to those within the face and limb. In conjunction with the sixth intron, these sequences produce expression in the ectoderm of the head. Moreover, the region encompassing introns five and six is also involved in directing gene expression to the trigeminal ganglia and mammary buds. In these two tissues, the downstream elements need to cooperate with *cis*-regulatory sequences located in an 8.5-kb region upstream of the basal promoter to produce robust tissue-specific expression. At least one additional downstream region of the AP-2α gene is involved in driving expression within the trigeminal ganglia. This conclusion is based on the finding that construct 10 (FZ19), which consists mainly of the 24-kb downstream sequence, is expressed in the trigeminal ganglia even though it lacks the 8.5-kb upstream fragment discussed above. Based on the structure of construct 10, the pertinent *cis*-regulatory elements must be located 3′ to the AP-2α coding region, specifically downstream of exon seven. Thus, the expression of the endogenous AP-2α gene within the trigeminal ganglia may result from the concerted action of multiple, dispersed, *cis*-acting elements. Also note that sequences located downstream of the AP-2α coding region are not only involved in producing tissue-specific gene expression within the trigeminal ganglia but also within the central nervous system. Further analyses will be required to determine the extent and importance of synergistic interactions for AP-2α expression, especially with respect to any regulatory elements that exist in the more distal sequences that were outside the scope of the current analysis. Finally, given the preponderance of enhancer sequences we have located downstream of the AP-2α transcriptional start site, it will be interesting to determine whether a similar arrangement of regulatory sequences occurs in the related mammalian AP-2β and AP-2γ genes (Moser et al., 1995; Bosher et al., 1996; Chazaud et al., 1996). The genomic organization of these three genes is very similar, each consisting of seven exons and six introns (Bauer et al., 1994), and they are also expressed in overlapping patterns in the neural crest, central nervous system, limb, and face (Mitchell et al., 1991; Chazaud et al., 1996; Moser et al., 1997b).

In the course of mapping AP-2α *cis*-regulatory sequences, we also generated permanent lines of AP-2α–null mice that carry the J24 AP-2α::*lacZ* transgene. This approach enabled us to follow the distribution of cells that express this AP-2α::*lacZ* marker in the face and limbs of AP-2α-knockout mice. The outgrowth and patterning of the limb bud relies on continued communication between the mesenchymal cells in the progress zone and the apical ectodermal ridge (Tickle, 2003). Signaling molecules of the FGF and hedgehog families and their cognate receptors are key participants in this process. However, the transcriptional mechanisms by which these signaling pathways are established and interpreted are largely uncharacterized. Previous studies in the chick have indicated that AP-2α expression in the progress zone responds rapidly to signals emanating from the AER (Shen et al., 1997). The current data strongly suggest that the AP-2α gene can also respond to signaling information passing in the opposite direction, i.e., from the progress zone to the AER. For such a cell nonautonomous mechanism, we would postulate that when AP-2α protein is removed from the progress zone, a cell:cell signaling cascade is disrupted so that repression of the AP-2α gene no longer occurs within the AER. This hypothesis is supported by studies in *Drosophila*, which indicate that the *dAP-2* gene can act in a nonautonomous manner to regulate leg outgrowth (Kerber et al., 2001; Monge et al., 2001). Alternatively, a cell autonomous mechanism may be responsible for the activation of the transgene in the AER. In this instance, a very low level of AP-2α expression would occur normally within the AER and this expression would repress AP-2α promoter activity. In the absence of functional AP-2α protein, repression of the AP-2α gene would be relieved. Supporting this latter hypothesis, AP-2 binding sites have been identified within the AP-2α promoter, and it has been shown previously that AP-2α can regulate its own expression in vitro (Bauer et al., 1994; Creaser et al., 1996). Furthermore, we did note that the level of J24 transgene activity was consistently higher in the mammary glands of AP-2α–null mice, suggesting that AP-2α may normally autoregulate its own expression in this tissue (compare Fig. 3J and K). In the future, detailed mapping of the sequences responsible for AP-2α promoter activation within the limb will aid in distinguishing between these cell-autonomous and non–cell-autonomous signaling mechanisms. From our analysis of the expression of the endogenous AP-2α–null allele in the AP-2α–knockout background (Fig. 6F), we can deduce two further mechanistic insights into this gene's transcriptional control in the limb. Although the endogenous gene is activated in the AER of the knockout forelimbs, there is a concurrent loss of expression in the progress zone. The absence of expression in the progress zone may actually reflect the nature of the AP-2α–null allele used in this analysis (Zhang et al., 1996). The knockout allele we have used lacks a large part of intron five, and this is the intron we have now

shown drives expression in the progress zone. The finding that expression of the knockout allele is absent in the progress zone suggests that this enhancer is indeed critical for the normal expression of the AP-2α gene in this region. We are currently testing this idea by generating a new allele of the AP-2α gene that is lacking this enhancer. The second conclusion that can be made from this analysis is that the sequences required for activation of the null allele in the AER are distinct from those present in intron 5, which drive expression in the mesenchyme, because these latter sequences have been deleted from the mouse genome.

The expression pattern of the *lacZ* transgene in the limb buds of wild-type and AP-2α– knockout mice also provides an explanation for the agenesis of the radius observed in the majority of AP-2α–null animals (Schorle et al., 1996; Zhang et al., 1996). Normally, the endogenous gene and the AP-2α::*lacZ* transgene are expressed as a stripe in the dorsal mesenchyme of the zeugopod. The timing and location of this expression domain precedes condensation of the radius in the underlying mesenchyme. In mice lacking AP-2α, this domain of expression frequently expands and eventually encompasses more dorsolateral tissue within the forelimb. Significantly, for mice in which this alteration in expression occurs, condensation of the radius does not happen. Conversely, AP-2α–null mice that maintain a discrete stripe of expression are still able to initiate radius condensation. These findings support a model in which the expression of AP-2α in the dorsal mesenchyme generates an important cell:cell signaling center for radius condensation in the underlying tissue. In the absence of AP-2α, this signaling center is either entirely lost, or possibly rendered ineffective by being dispersed over a wider area. An alternative explanation for this phenomenon is that agenesis of the radius and the altered expression pattern of AP-2α in the zeugopod are not linked directly but that both are indicative of earlier defects within the developing limb bud. In this regard, it is apparent that in E12.5 AP-2α–null mice the handplate is frequently misshapen (see Fig. 7, and J.Z. unpublished observations). Experiments in murine, avian, and amphibian systems have demonstrated that the formation of anterior limb structures can be affected by manipulating the interaction between the AER and the progress zone, which may act by lowering the amount of mesenchyme in the developing limb bud (Alberch and Gale, 1983; Ohuchi et al., 1997; Lettice et al., 1999). Thus, inappropriate proliferation and/or cell:cell signaling within the progress zone and AER of AP-2α–null mice could eventually result in the observed abnormalities in the pattern of gene expression and bone formation in the zeugopod region. Defective early limb bud patterning has also been proposed for the problems with radius formation that are seen in both RAR$\alpha\gamma$ double-mutant mice and in mice lacking *hoxa-11/hoxd-11* (Lohnes et al., 1994; Davis et al., 1995). These particular RAR and *hox* mutant combinations cause development defects in the forelimb to a greater extent than in the hindlimb. Similarly, all the effects of AP-2α so far documented are forelimb-specific: alterations were not previously reported in the hindlimbs of either AP-2α-null mice or chimeric animals (Schorle et al., 1996; Zhang et al., 1996; Nottoli et al., 1998). The finding that the activity of the AP-2α promoter is only altered within the forelimb of the AP-2α–null mouse when compared with the fore- and hindlimbs of wild-type mice may provide a mechanistic link to explain this discrepancy. Finally, we note that the action of AP-2α in the forelimb must be somewhat redundant, because radius formation still occurs at a low frequency even in the absence of AP-2α. Because the AP-2γ gene is also expressed in the developing limb bud, this related gene may compensate for the loss of AP-2α with respect to limb patterning (Chazaud et al., 1996).

In summary, we have identified distinct regulatory elements responsible for directing AP-2α expression to important sites of AP-2α function throughout embryogenesis. The data obtained from our current studies also indicate that AP-2α can regulate epithelial:mesenchymal interactions in the limb. It is at present uncertain whether AP-2α acts by means of similar tissue interaction mechanisms to regulate other morphogenetic processes, such as development of the facial prominences, eye, and/or nervous system. Alternatively, in these systems AP-2α may act in a cell autonomous manner to control proliferation, differentiation, or cell survival (Schorle et al., 1996; Zhang et al., 1996; West-Mays et al., 1999; Kramer et al., 2000). Whether or not there is a unified cellular basis for the influence of AP-2α on developmental decisions, it remains possible that AP-2α still exerts its effects through a common set of target genes, such as cell adhesion molecules, cell surface receptors, and cell-cycle regulated proteins (Mitchell et al., 1987; Leask et al., 1991; Fini et al., 1994; Zutter et al., 1994; Gaubatz et al., 1995; Bosher et al., 1996; Hennig et al., 1996; Zeng et al., 1997). Further analysis of the regulatory network responsible for controlling AP-2α expression will complement studies on these downstream targets and lead to greater insight into many aspects of development.

EXPERIMENTAL PROCEDURES

Construction of Transgenes

The isolation of human cosmid clones spanning the AP-2α gene locus was described previously (Creaser et al., 1996). Two of these cosmid clones were used to make the AP-2α::*lacZ* reporter constructs: the Cosh1 clone extends more 5′ of the AP-2α– coding region, whereas Cosh7 contains more 3′ sequence. The construct C1 *lacZ* 42 was the parent plasmid for the generation of the AP-2α::*lacZ* fusions (Fig. 1A). The sequence between nucleotides −10 and +321 of the AP-2α gene was amplified by polymerase chain reaction (PCR) using the forward primer TWY 4 (5′-GCC TAC TCG AGA AGC TTA TGC ATG TCG ACG GAA AAG TTT CTA CCA TTA GAG-3′) and reverse primer TWY 2 (5′-GCC TAC CCG GGG TCC TCG TAC TTG ATA TTA TCC-3′). The sequences corresponding to AP-2α are underlined in these primers, and the flanking nu-

cleotides contain recognition sequences for the restriction enzymes *Xho*I, *Hind*III, *Nsi*I, and *Sal*I in TWY4, and *Sma*I in TWY 2. PCR was performed on Cosh1 that had been linearized with *Cla*I. PCR conditions were 2 cycles at 94°C for 1 min 20 sec, 52°C for 30 sec, 72°C for 3 min; 29 cycles at 94°C for 40 sec, 72°C for 3 min; and 1 cycle at 72°C for 12 min. Subsequently, the 360-bp PCR fragment was digested with *Xho*I and *Sma*I and cloned into the corresponding sites of the plasmid pLZFSV (Bradshaw et al., 1996). The integrity of the resulting C1 *lacZ* 42 plasmid was confirmed by DNA sequencing.

A series of constructs containing greater amounts of genomic DNA fused to *lacZ* were made by using standard cloning procedures and available restriction enzyme sites (see Fig. 1B). First, plasmids were made that contained the indicated amount of sequence upstream of the transcriptional start site: C1 Xho β gal, 190 bp; C1 Sal β gal, 2 kb; C1 Acc β gal, 3.8 kb; C1 Sph β gal, 4.2 kb; and C1 Mlu β gal, 8.5 kb (constructs 1–5, Fig. 1B). The C1 FL β gal plasmid (construct 6) contains the maximum amount of 5′ flanking sequence from the cosmid Cosh1. This plasmid was made by subcloning an approximately 18-kb *Hin*dIII–*Sal*I fragment of Cosh1 into the corresponding sites of C1 Sal β gal. Note that the *Hin*dIII site is located within the pWE15 vector sequences, 60 bp from the junction with the human genomic DNA. The actual upstream limit of the AP-2α genomic sequence is an *Sau3A I* site.

Standard cloning procedures were also used to generate a series of plasmids in which sequences located downstream of the AP-2α transcriptional initiation sites were placed 3′ to the *lacZ* gene. In the plasmid pBS/ACC/M12 (construct 7), a 5.4-kb fragment of AP-2α genomic sequences, from the *Rsa*I site in the first exon to the *Hin*dIII site in the second intron, was inserted into the 3′ polylinker of the plasmid C1 Acc β gal. The plasmid pBS/ACC/MS (construct 8) was made in a similar manner, using a 7.5-kb fragment of Cosh1 that extends from an *Mun*I site in the second intron to the *Spe*I site in the fifth exon. The plasmid FX18 (construct 9) is a derivative of C1 FL β gal (construct 6) that contains an additional 24 kb of downstream sequence extending from the *Spe*I site in the fifth exon until the pWE15 vector boundary of Cosh7. The plasmid FZ19 (construct 10) contains the same downstream fragment placed into C1 Xho β gal. The plasmids J24 and J25 (constructs 11 and 12) were generated by cloning a downstream 4.2-kb *Spe*I–*Not*I fragment of Cosh I into the corresponding sites of C1 Mlu β gal (construct 5) and C1 Xho β gal (construct 1), respectively. The downstream genomic fragment spans from the *Spe*I site in the fifth exon to a *Sau3A I* site in the 3′ untranslated region of the seventh exon, and then on to the adjacent *Not*I site in the pWE15 vector. Constructs 13 and 14 are derivatives of J25 (construct 12). The plasmid SN48 (construct 13) contains a 1.95-kb insert of AP-2α extending from the *Spe*I site in the fifth exon to the *BsaA I* site in the sixth exon. Plasmid SN25 (construct 14), contains a 2.2-kb fragment which spans from the *BsaA I* site in the sixth exon to the *Sau3A I* site in the 3′ untranslated region of the seventh exon.

Generation of Transgenic Mice

Before microinjection, vector and insert sequences were separated by treatment with appropriate restriction enzymes, followed by sucrose density centrifugation. The fragments corresponding to the AP-2α::*lacZ* insert fragments were dialyzed extensively against 10 mM Tris · Cl; 1 mM EDTA, pH 7.5. Transgenic mice were generated by microinjecting DNA into pronuclei of fertilized oocytes of inbred FVB mice (Taconic). The embryos surviving the microinjection were transferred into oviducts of pseudopregnant CD-1 fosters (Charles River). The concentration of DNA used in the microinjection was adjusted to 1–5 μg/ml, based on the length of the constructs. The founder embryos were either analyzed for the expression of the transgene at the embryonic time points indicated in the text, or were allowed to develop to term. Transgenic mouse lines derived in the latter instance were used to analyze the expression of the *lacZ* marker in subsequent generations of FVB embryos. Only transgenes that gave a reproducible pattern of *lacZ* expression were considered to harbor specific AP-2α *cis*-regulatory elements. The derivation of AP-2α–knockout mice on a Black Swiss background has been described previously (Zhang et al., 1996). The analysis of transgene activity in the AP-2α–null background was performed on a mixed FVB/Black Swiss background.

Isolation of Genomic DNA, Southern Blot, and PCR Analysis

Identification of transgenic mice was performed by Southern blot or PCR analysis. Genomic DNA was isolated from either the placentas of embryos or tails of 3- to 4-week-old mice, as described (Laird et al., 1991). A total of 12 μg of genomic DNA was digested with the restriction enzyme *Bam*HI, electrophoresed on a 0.8% agarose gel, and transferred to nitrocellulose (Schleicher & Schuell) or Hybond N filters (Amersham Biosciences). The filter was hybridized with a 3-kb *Bam*HI fragment of the bacterial *lacZ* gene, which was either radioactively labeled with ^{32}P by random priming or labeled nonradioactively with the "Genius Nonradioactive Nucleic Acid Labeling and Detection Kit" (Roche Applied Science). Detection was then performed by autoradiography or light-emission, respectively. PCR was performed using the forward primer *LacZ*-F1 (5′-TAC AAC GTC GTG ACT GGG AA-3′) and reverse primer *LacZ*-R1 (5′-CCA GAT AAC TGC CGT CAC TC-3′), which are equivalent to nucleotides 45-65 and 606-586 of the plasmid pMC1871 (Amersham Biosciences). The PCR conditions were 1 cycle at 94°C for 1 min 20 sec; 32 cycles at 94°C for 45 sec, 60°C for 45 sec, 72°C for 2 min; and 1 cycle at 72°C for 10 min. A fragment of 562 bp was amplified from mice containing the transgene.

Detection of β-Galactosidase Activity

Embryos at specific stages of development were obtained by timed

matings. The day of the vaginal plug was considered 0.5 days post coitum (E0.5). The embryos were dissected free from the maternal tissues into cold PBS, fixed for 30 min in 0.25% glutaraldehyde, rinsed with PBS, and stained for β-galactosidase activity as described previously (Bieberich et al., 1990). Stained embryos were post-fixed in 4% paraformaldehyde at 4°C overnight and embedded in paraffin for sectioning. Six- to 8-μm sections were cut and mounted on poly-L-lysine–coated slides. The sections were counterstained with nuclear fast red.

In Situ Hybridization and Immunohistochemistry

Whole-mount in situ hybridization was performed essentially as described (Wilkinson, 1992) by using the plasmid TRIP1, which contains the mouse AP-2α cDNA sequences corresponding to amino acids 97 to 437 inserted between the *Hin*dIII and *Eco*RI sites of pBluescript II KS- (Stratagene). The plasmid was digested with *Hind*dIII, and then transcribed with T7 RNA polymerase in the presence of digoxigenin-UTP, by using conditions described by the manufacturer (Roche Applied Science). Whole-mount immunohistochemistry was performed as described (Mark et al., 1993) by using the 3B5 AP-2α-specific monoclonal antibody (Zhang et al., 1996).

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