The transcription factor AP-2α has been implicated as a cell type-specific regulator of gene expression during vertebrate embryogenesis based on its expression pattern in neural crest cells, ectoderm, and the nervous system in mouse and frog embryos. AP-2α is prominently expressed in cranial neural crest cells, a population of cells that migrate from the lateral margins of the brain plate during closure of the neural tube at day 8–9 of embryonic development. Homozygous AP-2α mutant mice die perinatally with cranio-abdominoschisis, full facial clefting, and defects in cranial ganglia and sensory organs, indicating the importance of this gene for proper development. By using a subtractive cloning approach, we identified a set of genes repressed by AP-2α that are described to retard cellular proliferation and induce differentiation and apoptosis. We show that these target genes are prematurely expressed in AP-2α mutant mice. One of the genes isolated, the Krüppel-box transcription factor KLF-4 implicated in induction of terminal differentiation and growth regulation, is found expressed in mutant embryonic fibroblasts. We show that fibroblasts lacking AP-2α display retarded growth but no enhanced apoptosis. Based on these data we suggest that AP-2α might be required for cell proliferation by suppression of genes inducing terminal differentiation, apoptosis, and growth retardation.

The family of AP-2 transcription factors consists of four different genes known as AP-2α, -β, -γ, and -δ, respectively (Tcfap2a, Tcfap2b, and Tcfap2c-Mouse Genome Informatics) (1–7). AP-2 transcription factors homodimerize using a unique C-terminal helix-span-helix motif. Sequence-specific binding to the consensus GCCN(3/4)GGC is mediated by a basic domain overlapping with the dimerization domain. The transcriptional activation is mediated by a proline/glutamine-rich cassette at the N terminus (6). These domains are highly conserved in all three known AP-2α genes.

During development, expression of AP-2α initiates around day 8 of embryonic development (E1) 8.0 in premigratory neural crest cells (8). During organogenesis expression of AP-2α can be detected mainly in neural crest-derived tissue, kidney, and skin. Gene knockout experiments have shown that disruption of the AP-2α gene leads to a very severe body wall and neural tube closure phenotype with homozygous animals dying perinatally (9, 10). The mutants show a hypoplasia of the cranial ganglia and high degree of apoptotic cranial neural crest cells indicating the importance of AP-2α for craniofacial development (9). Although a variety of genes have been proposed as targets for transcriptional regulation of AP-2α (reviewed in Ref. 11) based on studies in cell culture systems, the pathways regulated by AP-2α in the process of craniofacial morphogenesis are poorly understood.

In the study presented here we used a combination of suppression-subtractive hybridization (12) and high throughput differential screening to identify target genes of transcription factor AP-2α. By using a single head of an E8.75 knockout and a control animal, we generated cDNA, which was subjected to suppression-subtractive hybridization. The success of the subtraction was demonstrated by virtual Northern blots with different known genes. We subjected 4800 recombinant clones to two rounds of differential screening using reverse Northern blots, and we isolated a total of 52 recombinant clones. Four genes from the screen for repressed genes, which we analyzed further, are known to be involved in growth control, differentiation, and apoptotic processes.

The genes are KLF-4 (Krüppel-like factor 4 (13, 14), mEFEMP-1 (epidermal growth factor-containing fibulin-like extracellular matrixprotein 1, (15)), Mtd (Matador (16)), and Stra13 (Stimulated by retinoic acid 13 (17)). RT-PCR analyses confirmed that the genes were derepressed in the mutants. Most notably KLF-4 could be detected as early as E 8.5 in the mutant mesenchyme, whereas in wild-type animals KLF-4 can first be detected at E 10.5. Expression of the KLF-4 gene had been shown to induce cell cycle exit and terminal differentiation (18). In fact, fibroblasts derived from AP-2α mutant animals were shown to express KLF-4 and displayed reduced proliferation. We conclude that AP-2α might repress a set of target genes, which suppress cellular proliferation and induce terminal differentiation and apoptosis. The work outlined in this paper helps in understanding the molecular processes controlled by transcription factor AP-2α.

EXPERIMENTAL PROCEDURES

Isolation of RNA—Poly(A)⁺ RNA was prepared from a single embryo head at E 8.75 (exactly 18 somites) from a wild-type and an AP-2α-deficient specimen according to the manufacturer’s instructions (Invitrogen) in control of proliferation and differentiation.*

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§ The abbreviations used are: E, embryonic development; GAPDH, glyceraldehyde phosphate dehydrogenase; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; RT, reverse transcriptase.

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vitrogen FastTrack 2.0) with the exception that the material was homogenized using a 16-ga syringe before adding the lysing buffer. The quality of the isolated RNA was determined via RT-PCR with GAPDH and AP-2a (sequences of the primers available upon request).

**Subtractive Hybridization**—Isolation of AP-2a up-regulated target genes was achieved by performing suppressive-subtraction hybridization protocol between wild type (“tester”) and AP-2-deficient (“driver”) cDNA using the PCR-select cDNA subtraction kit (CLONTECH) with modifications as described (19) (referred to as forward screen). For genes repressed by AP-2a, the same procedure was used except that cDNA from AP-2a-deficient embryonic heads served as tester, and the wild type material was used as driver (referred to as reverse screen).

For the first hybridization the mixture of driver and tester, cDNA was denatured at 100°C for 20 s and then cooled over 1 min to 68°C, and temperature was maintained for 8 h. For the second hybridization, driver cDNA was denatured at 100°C for 20 s and then added to the pooled mixture of the previous hybridization and incubated at 68°C for 20 h. Modifications of the manufacturer’s protocol were done according to published procedures (19).

**Subtraction Efficiency**—The PCR-amplified cDNA was digested with 

**Different Screening via Reverse Northern**—A total of 4800 individual clones was picked and used to inoculate 96-well microtiter plates containing LB media supplemented with 100 μg/ml ampicillin. After inoculation on a gyratory shaker for 4 h, a 10-μl aliquot was transferred to 50 μl of distilled water in PCR tubes. The bacteria were lysed by heating to 100°C for 5 min, and then 40 μl of distilled water was added. Samples of 10 μl were used to amplify the cDNA insertions using M13 standard primers under standard conditions. (Note: clones containing no insert also did produce a band on the gel equal to the multiple cloning site.) The PCR products were run in duplicate on high density agarose gels (Centipede; Owl Scientific, Woburn, MA). PCR-amplified cDNA from AP-2a-deficient embryonic heads was digested with the appropriate primers in standard buffer (Amersham Biosciences AB) with 2 units of Taq polymerase (30 cycles at 94°C, 20 s; 52°C, 20 s; and 72°C, 2 min). For the subtracted cDNA, a nested PCR approach was used with the following conditions: 1st PCR, 27 cycles at 94°C, 20 s; 68°C, 20 s; and 72°C for 2 min; 2nd PCR, 12 cycles at 94°C, 20 s; 69°C, 20 s; and 72°C for 2 min. Equal amounts of the amplified material was run on an agarose gel (visualized under a UV transilluminator after staining with ethidium bromide), blotted, and spotted to hybridization under stringent conditions.

**Virtual Northern**—Virtual Northern blots were performed according to the manufacturer’s instructions (SMART and PCR-Select subtraction manuals, CLONTECH). 500 ng of unsubtracted SMART cDNA and 500 ng of SMART cDNA after PCR-Select subtraction derived from both wild type and AP-2a-deficient mRNA was separated on an agarose gel, transferred with 3% EBA and transferred onto a nylon membrane (Hybond-N, Amersham Biosciences). The blots were then hybridized to [32P]CTP-labeled PCR fragments of GAPDH and neomycin resistance gene under Church buffer conditions.

**Isolation of Full-length cDNA**—For isolation of full-length cDNAs, two mouse embry library (E 8.5 and E 10.5, Invitrogen) were pooled and plated. Filter lift assays were performed with pools of four recombinant fragments each. Colonies, which gave a positive signal, were picked using 1 ml of sterile Eppendorf tips, diluted in LB, and streaked onto 10-cm bacterial dishes. Filter lifts of these were cut into quadrants and probed with individual fragments. Positive clones were picked, and the insert verified with restriction digests.

**Whole Mount in Situ Hybridization**—Digoxigenin-labeled probes were generated using recombinant clones containing full-length cDNAs with T7 and SP6 RNA polymerase. E 8.5–10.5 embryos were hybridized according to standard protocols as previously described (20) (stratus.lifesci.ucla.edu/hhmi/derob).

**RESULTS**

**Generation of cDNA Libraries of a Single Head of Wild-type and AP-2a Mutant Embryos at E 8.75**—AP-2a mutant animals fail to close the cranial neural tube, an event that takes place between E 8.5 and 9.5 of murine development. The expression of AP-2a is first detected at E 8.0 in the apical neural fold and the lateral head mesenchyme. To isolate genes involved in the initial steps of the developing phenotype, we isolated embryos at age E 8.75. To ensure that we were using same age material, the somites were counted. Each embryo was genotyped using yolk sac DNA and PCR primers specific for the AP-2a locus. By using 18-somite embryos, RNA was generated from a single head of a wild-type as well as a knockout specimen. Then cDNA was generated and amplified (SMART cDNA synthesis kit, CLONTECH). These cDNAs were then used for a PCR-based subtraction, the suppressive-subtractive hybridization protocol (12) (PC-Select, CLONTECH). The screen for genes induced by AP-2a is referred to as “forward” screen (RNA from wild type was used as driver and RNA from AP-2a-deficient as tester). Genes repressed by AP-2a were identified using RNA from AP-2a mutants as driver and RNA from wild type as tester, termed “reverse” screen.

After the forward- and reverse-subtracted cDNA libraries were generated, they were tested for the efficiency of the subtraction. The degree of subtraction can be determined by monitoring the enrichment of sequences specific to one population after subtraction and depletion of transcripts common to both populations. The G418 resistance gene, introduced to activate the AP-2a locus, is under ubiquitinous expression driven by
the phosphoglyceraldehyde transferase promoter and thereby serves as an excellent internal positive control that should be found enriched in the cDNA of the forward screen. Conversely, glyceraldehyde phosphate dehydrogenase (GAPDH, a housekeeping gene) should be depleted from the subtracted material. GAPDH was shown to be reduced 1000-fold in the subtracted compared with the unsubtracted material of both the forward and the reverse subtraction (Fig. 1A), whereas the neomycin cDNA was found enriched 50-fold in the forward direction (Fig. 1B). Taken together, the results indicate that the population of cDNA had been successfully subtracted, and the highly expressed housekeeping genes like GAPDH had been eliminated or greatly reduced. So we could conclude that both subtractions of the cDNA worked to a high efficiency, and the subtracted cDNA libraries could be transformed into a cloning vector for the screening procedure.

**Screening of 4800 Colonies Yields 52 Differentially Expressed Genes**—For the identification of genes regulated by AP-2α, 2400 colonies of each subtracted cDNA library were picked and the cDNA fragments amplified by colony PCR. Each cDNA fragment was then separated on two high density gels in parallel (Fig. 1C), blotted, and hybridized with radiolabeled cDNA derived from wild-type (Fig. 1D, upper panel) and AP-2α-deficient embryos (Fig. 1D, lower panel). In the first round we identified 254 clones in the forward and 294 clones in the reverse screen as being differentially expressed. We re-screened these clones in a second round and used only the clones with the most striking differences in signal. Taken together, we performed two rounds of reverse Northern to screen 4800 clones from both the forward and reverse direction, and we found 52 clones being differentially expressed. Due to extreme limitation of mRNA material, we performed virtual Northern blots to verify that a given clone is in fact differentially expressed. Thirty clones were tested this way (data not shown), and 95% showed the expected differential expression pattern.

Many of the Clones Represent Novel Sequences—All 52 clones were sequenced and analyzed using the BLAST server to determine the nature of the sequence. As depicted in Table 1, sequence analysis showed that the 52 clones encode for 46 different genes (25 known and 21 novel cDNAs) indicating that we had isolated some genes several times.

**Isolation of Full-length cDNA Clones**—Generation of cDNA pools with SMART (CLONTECH) results in average fragment lengths of 300–600 base pairs. To clone full-length cDNA of the clones, we screened a commercial cDNA library stage E 8.5 and E 10.5 (Invitrogen). 500,000 clones were hybridized with pools of four fragments. Positive signals were picked and rescreened with the individual fragment. After determination of the insert size the clones were used for sequence analysis.

The full-length clones obtained were subjected to a whole mount in situ screen to detect genes expressed in structures affected by the AP-2α knockout phenotype. Fig. 1 (E–H) shows a panel of genes that are derived from the screen for repressed genes, Stra13 (Fig. 1E), mEFEMP-1 (epidermal growth factor-containing fibulin-like extracellular matrix protein-1, Fig. 1F), Mtd (Matador 1, Fig. 1G), and KLF-4 (Krüppel-like factor 4, Fig. 1H).

The transcriptional repressor Stra13 had been described to be expressed at day E 9.5 in Rhombomere 5 (17). We were able to reproduce these data and moreover detected Stra13 RNA in tip of the tail and in the mesenchyme surrounding the dorsal aorta at E 9.5 (Fig. 1E, arrowheads). Stra13 had been reported to induce growth arrest and terminal differentiation in cell culture (17).

Our screen resulted in cloning of the murine homologue of EFEMP-1, an extracellular matrix protein that was found over-expressed in senescent and quiescent fibroblast cultures and a patient with Werner syndrome, a genetic disorder characterized by accelerated aging (15). We see mEFEMP-1 in cells of the paraxial mesenchyme in the hindbrain and in a fine, distinct line in the trunk (Fig. 1F, arrow).

Mtd (or bok1), a member of the Bcl-2 family, had been de-
### Table I

**Result of the screen for genes regulated by AP-2**

Clones found in the screen for genes induced and repressed are shown. Results from sequencing and Blast analysis are shown as “known” (sequence obtained produced an alignment with sequences deposited in GenBank™). A sequence was termed “unknown” when there is no annotation found in the GenBank™ DataBank or the alignment was to a sequence of unknown function. Further categories show known homologies and the GenBank™ accession number.

| Clone | cDNA size | Gene name | Homologies to | Remarks | GenBank™ accession no. |
|-------|-----------|-----------|---------------|---------|-----------------------|
| Genes induced by AP-2α | | | | | |
| fl01 | 799 bp | Cytochrome c oxidase (Polypeptide I) | Three times isolated | 13841 | |
| fl02 | 716 bp | Unknown | Isolated from *in vitro* fertilized eggs cDNA; zinc (RING) finger domain | AK018364 | |
| fl03 | 1559 bp | Glutathione peroxidase 3 | Human CGI-18 *Drosophila* Dp 1 | Small homologies | NM 008161 |
| fl04 | 3.0 kb | Unknown | | | |
| fl05 | 3.6 kb | | Kinase and/or zinc finger | | |
| fl06 | 1208 bp | GrpE-like 1, mitochondrial (GrpE1), mRNA | Human cDNA clone p586c1620 | cDNA clone | NM_024478 |
| fl07 | 2.0 kb | Unknown | | Probably zinc finger domain | BC005647 |
| fl08 | 1312 bp | Unknown | | | |
| fl09 | 2.5 kb | SNAP 23 | | | |
| fl10 | 1.5 kb | Prolactin-related protein | | | |
| fl12 | 2508 bp | | Rat dihydriopside succinyltransferase (d04011) | Three times isolated | AF011385 |
| fl13 | 1.5 kb | 26 S proteosomal ATPase | | | |
| fl14 | 889 bp | Mouse homologue of MCT-1 | Human MCT-1 (93%) | | |
| fl15 | 1095 bp | Unknown | Isolated from mouse early blastocyst cDNA; Gly- and His-rich, zinc finger | | |
| fl16 | 826 bp | Unknown | Rat hypertension-related protein (u41803) | Transmembrane protein | |
| fl17 | 2.1 kb | Selenoprotein P | | | |
| fl18 | 1.9 kb | Poly(A)-binding protein II | Human EFEMP-1 | | |
| fl19 | 630 bp | 60 S ribosomal protein L18 | | | |
| fl20 | 1730 bp | Unknown | Isolated from male kidney cDNA | | |
| fl21 | 485 bp | Unknown | Isolated from mouse cDNA, probably phosphatase | | |
| Genes repressed by AP-2α | | | | | |
| fl22A | 3.0 kb | Annexin, Lipocortin 1 | | | |
| fl22B | 2.9 kb | Krüppel-like factor 4 | | | |
| fl23 | 1404 bp | Lipocortin 1 | | | |
| fl24 | 2164 bp | Syntaxin-7 | | | |
| fl25 | 2821 bp | Sca-1 (stem cell antigen-1) | | | |
| fl32 | 1880 bp | Unknown | | | |
| fl53 | 1837 bp | Unknown | | | |
| fl54 | 2337 bp | Unknown | | | |
| fl55 | 1894 bp | Unknown | | | |
| fl56 | 1573 bp | Stra13 | (AF362729) *Mus musculus* ion transport regulator CHIF (*Fxyd4*) gene | | |
| fl57 | 2067 bp | Bc 10 | (bHLH, RA inducible) | | |
| fl58 | 1457 bp | Mtd | (Apoptosis activator) | | |
| fl60 | 2375 bp | Unknown | | | |
| fl62 | 928 bp | OSF-3/pag-1 | Various mouse ESTs | | |
| fl64 | 470 bp | Unknown | Ubiquitin/60 S ribosomal fusion protein | | |
| fl65 | 570 bp | Unknown | Receptor or zinc finger | | |
| fl66 | 580 bp | Surf-3 | Various mouse ESTs | | |
| fl67 | 770 bp | Saposin | | | |
| fl68 | 1076 bp | Acidic ribosomal phosphoprotein | | | |
| fl69 | 535 bp | Ribosomal subunit S5 | | | |
| fl70 | 2620 bp | mCDC46 | | | |
| fl71 | 2187 bp | Unknown | Various mouse ESTs | | |
| fl72 | 718 bp | TRBP 107 | Surface glycoprotein | | |
scribed to be expressed in the brain, liver, and lymphoid tissues and to activate apoptosis (16, 21, 22). Our in situ analysis revealed expression in limb bud and paraxial mesenchyme in the trunk and craniofacial mesenchyme at E 10.5 (Fig. 1G, arrowheads).

Kruppel-like factor-4, KLF-4, is published to be expressed in the epithelial cells of the mucosa of the gut (23). However, we see expression in a highly regionalized pattern in the frontal-nasal area, the first and second branchial arch, the apical ectodermal ridge of the limb buds, and the dorsal root ganglia in the trunk (Fig. 1H, arrowheads). Many lines of evidence suggest that KLF-4 expression is indicative for growth arrest and differentiation. For example, NIH 3T3 cells are found to express KLF-4 under serum deprivation or upon contact inhibition (18). PMT-3 cells, which are transfected with a plasmid expressing KLF-4, show reduced thymidine uptake as well as greatly diminished DNA synthesis as determined with bromodeoxyuridine labeling (14).

Taken together, the genes repressed by AP-2α retard the cell cycle and induce terminal differentiation and apoptosis.

Four Target Genes Are Expressed Prematurely in the Mutant Animals—We had subjected a subtracted library from E 8.75 wild type and mutant material to an in situ screen, which was aimed to detect genes that display a distinct and overlapping expression pattern compared with AP-2α. For a target gene to be involved in the cranio-abdominoschisis, it had not only to be expressed in the similar structures but also at the gestational time the mutant embryo would start to display phenotypic alterations. Hence, we decided to concentrate the next analysis around stages E 8.5–10.5. We performed RT-PCR analyses of pools of three embryonic heads stage E 8.5–10.5 of wild type and mutants, respectively (Fig. 2A). Three of the four genes (KLF-4, Mtd, mEFEMP-1, Fig. 2A) tested were found to be expressed prematurely in the mutant animals; one gene (Strα13, Fig. 2A) seemed to be expressed more strongly in the mutants. This result demonstrates that loss of AP-2α leads to a derepression of the target genes in the mutant embryo.

In Mutants Premature Expression of KLF-4 Is Localized to the Mesenchyme—To be considered as a direct target of a transcription factor, the gene in question has to be expressed in the same cells. At E 8.5 AP-2α mRNA is found in mesenchymal parts of the embryo, mainly in the cranial mesenchyme of the head folds (8, 9). Because the RT-PCR analysis had already shown the temporal differences, we performed a series of whole mount in situ hybridizations to determine the spatial expression of KLF-4 in the mutants compared with the wild-type controls. We found KLF-4 expressed in mesenchymal structures of mutants at stage E 8.5 (Fig. 2B, right), whereas no signal could be detected in wild type (Fig. 2B, left). These results indicate that loss of AP-2α leads to misexpression of KLF-4 as early as E 8.5.

Embryonic Fibroblasts Express KLF-4—We next established primary fibroblast cultures from E 13.5 to 15.5 embryos to substantiate the findings from the RT-PCR and in situ analyses. RNA was prepared from control and mutant cultures that had been passaged three times to obtain a pure population of fibroblast cells. We could not detect signals for Mtd or mEFEMP-1 (not shown), and the signal of Strα13 was comparable from wild type to mutant animal (Fig. 2A), but KLF-4 was found to be induced in mutant fibroblast cells (Fig. 2A). This result demonstrates that the derepression could also be found in the in vitro system tested.

AP-2α-deficient Embryonic Fibroblasts Are Growth-retarded—We decided to utilize this cell system to exploit further the functional consequences of this altered gene expression. Transfection of KLF-4 cDNA in NIH 3T3 fibroblast culture had been described to result in reduced proliferation (18). Based on these results, we reasoned that fibroblast cultures from AP-2α-deficient animals expressing KLF-4 might display different growth rates compared with wild-type control cultures. To test this hypothesis, we performed a cell growth assay. 2 × 10⁴ cells were seeded in a tissue culture dish and counted after 24, 48, and 72 h according to standard procedures (24). As seen in Fig. 3B, cell numbers increased steadily in control cultures (Fig. 3B, wt), in contrast cells lacking AP-2α were clearly growth-retarded (Fig. 3B, ko).

This difference in growth may be due to enhanced apoptosis or reduced proliferation. We performed annexin V staining of wild type and mutant cultures and found comparable levels of apoptosis in both cultures (not shown). This result is in agreement with our finding that Mtd, the target gene inducing apoptosis, is not found expressed in fibroblast cells of either genotype.

To test for proliferating cells, we stained wild type and mutant cultures with the PCNA antibody. We found that only a fraction of the mutant cells were PCNA-positive, whereas in the wild type controls nearly all of the cells were positive for this marker (Fig. 3, C and D). This result shows that the

| Clone  | cDNA size | Gene name                  | Homologies to                      | Remarks                  | GenBank accession no. |
|-------|-----------|----------------------------|------------------------------------|--------------------------|-----------------------|
| fl73  | 3012 bp   | Unknown                    | Homo sapiens ring finger protein 11 | kinase domain            | AK002219              |
| fl74  | 3844 bp   | Unknown                    | (AF205855) M. musculus serum and glucocorticoid-dependent protein kinase (Sgk) | isolated from M. musculus adult male kidney cDNA |                     |
| fl75  | 1466 bp   | Unknown                    | (BC015399) H. sapiens, similar to glutathione peroxidase 3 | isolated from human cDNA |                     |
| fl76  | 1565 bp   | Unknown                    | Various mESTs                      |                          |                       |
| fl77  | 2085 bp   | Unknown                    | Human cDNA clone                   |                          |                       |
| fl78  | 891 bp    | Prolactin-related protein  |                                    |                          |                       |
| fl79  | 1660 bp   | Unknown                    |                                    |                          |                       |
growth retardation seen in mutant cell lines originates from reduced proliferation but not enhanced apoptosis.

**Analysis of Promoter Sequences Reveals Potential Binding Sites for AP-2**

To determine the presence of possible AP-2-binding sites in the promoter regions of KLF-4, Mtd, Stra13, and mEFEMP-1 we performed an in silico search for GCCN(3–4)GGC, the bona fide AP-2-binding site on genomic fragments spanning the promoters of the respective genes using the MatInspector version 2.2 program (25). Analysis of the promoter of KLF-4 (GenBank™ accession number AF117109) showed that there are at least three half-sites that fulfill the criteria for AP-2 binding. The promoter of the human Stra13 gene (GenBank™ accession number AC090955) shows four AP-2 consensus sequences in close proximity to the transcriptional start site. A 1.5-kb fragment upstream of the first exon of Mtd shows at least four AP-2-binding sites (GenBank™ accession number AC027147). The promoter of EFEMP-1 (GenBank™ accession number AC096549) encodes for one AP-2-binding sequence 750 base pairs 5′ to exon 1. Taken together, all promoter regions analyzed contain AP-2-binding elements. Hence a direct regulation of these genes by AP-2 is possible.

**Expression of AP-2a Promotes Proliferation and Cell Survival via Repression of Genes Involved in Terminal Differentiation and Apoptosis**

Based on the data presented, we would like to present a model whereby transcription factor AP-2 acts as a suppressor of KLF-4 (and other target genes such as Mtd, Stra13, and mEFEMP-1). Because the genes suppressed are described to induce cell cycle exit and terminal differentiation, AP-2 might serve as a gatekeeper in controlling the proliferation versus differentiation of cranial neural crest cells (Fig. 4).
As long as AP-2α is expressed during the migratory phase of neural crest migration, the cells are able to proliferate. As soon as the cells reach their respective targets, AP-2α expression ceases and genes inducing differentiation would be derepressed (Fig. 4A). Lack of AP-2α leads to premature expression of AP-2α target genes, which induce cell cycle arrest, terminal differentiation, and apoptosis (Fig. 4B).

**DISCUSSION**

We have been isolating target genes of transcription factor AP-2α involved in craniofacial development. We utilized a combination of elements of suppression-subtractive hybridization and high throughput differential screening which permitted the rapid cloning of rarely transcribed differential genes. We started with a single head of a wild-type and AP-2α-deficient embryo (stage 18 somites) and constructed cDNA libraries, which were subjected to suppression-subtractive hybridization (12, 26). Control hybridization showed that the subtraction was successful in that the housekeeping gene GAPDH was reduced — 1000-fold. This method has several important features allowing for the successful isolation of AP-2α target genes, which are involved in craniofacial development. Transcripts that differ more than 5-fold in their abundance between the samples to be compared have a higher likelihood to be isolated compared with transcripts that show only moderate differences in expression (12). The representation of different mRNA species in the respective pools is being normalized, which increases the relative representation of rare transcripts (27). This was most likely the important step because master regulatory genes, such as transcription factors, are expressed at very low levels compared with housekeeping genes. We subjected 4800 recombinant clones to two rounds of differential screening using reverse Northern blots and isolated a total of 52 recombinant clones. After the screening procedure we came up with 52 differentially expressed clones, which were then analyzed for their specific expression pattern in whole mount in situ hybridizations. We show that the technique allows for inexpensive large scale screen of gene expression programs affected by loss of AP-2α, beginning from minute amounts of tissue. Four genes from the screen for repressed genes, which we analyzed further, are known to be involved in cell cycle control, differentiation, and apoptotic processes. They are discussed below.

The cranial closure defect in AP-2α mutants becomes evident first around closure of the neural tube at E 8.5–9.5 (9, 10). Therefore we decided to look at the expression of these potential target genes of AP-2α in this time window. We performed RT-PCR of these genes at E 8.5–10.5 from poly(A)^+ RNA of wt and mutant embryos. The experiment could clearly show that the expression of these genes is altered in the mutant embryos. Stra13, expressed at E 8.5 in control embryos, was found to be more strongly expressed from E 8.5 on in the mutants. MEFEMP-1, Mtd, and KLF-4 show a derepression leading to premature expression starting from day E 8.5 in mutants. The transcription factor KLF-4 shows the most tremendous effect. In control embryos its expression is first detectable at E 10.5, but in embryos we observe expression already at E 8.5. This finding was further substantiated in whole mount in situ hybridizations of wt and ko embryos at E 8.5. We show that lack of AP-2α leads to up-regulation of KLF-4 in cranial mesenchyme and in fibroblast cultures. As a consequence the proliferation of the fibroblast cells is reduced as determined by
PCNA staining and cell growth assay. Thus, AP-2α might serve as a gatekeeper in promoting proliferation by suppression of differentiation in neural crest cells during migration.

The role for proliferation is further supported by the fact that many human mammary tumors and cell lines derived from tumors display overexpression of AP-2 genes (28). Furthermore, AP-2 is able to activate c-erb-B2, a receptor tyrosine kinase implicated in cellular proliferation (1). In fact, AP-2α had been described as being an oncogene, and transgenic experiments addressing this issue are underway. Furthermore, AP-2α is expressed in the mitotically active basal cell layers of the skin but not the terminally differentiating, nondividing suprabasal layers (29). It is tempting to speculate that it is in fact repression exerted by AP-2 transcription factors that keeps the cells of the basal layer in the cell cycle and prevents premature differentiation. Although AP-2α-deficient mice do not display defects in the cells of the skin, loss of its function might be compensated by AP-2γ, which is expressed strongly in basal cells (30).

With the results of the screen for target genes of AP-2α, the failure of the cranial neural tube to close in mice lacking AP-2α might be explained. Based on a model put forward by Schoenwolf and co-workers, neural tube, surface ectoderm, and cranial mesenchyme act in an orchestrated way to bring about the closure of the neural tube (31–33). Failure of one component results in failure of the neural tube to close. We speculate that due to the lack of AP-2α, cells of the cranial mesenchyme express differentiation and growth retarding genes prematurely. As a consequence the proliferation at the time of cranial neural tube closure (E 8.75) is reduced resulting in hypoplastic mesenchyme. This hypothesis is supported by the fact that the cranial ganglia resulting from the cranial mesenchyme area are highly hypoplastic, and the branchial arches are underdeveloped in AP-2α-deficient animals at E 10.5 (9). Due to the hypoplastic mesenchyme, the cranial neural tube would not get the physical support needed resulting in insufficient bending of the tube at the lateral hinge regions (31). This lack in bending finally leads to the neural tube closure defect observed.

Data from Drosophila AP-2 (dAP-2 (34, 35)) support the function of AP-2 as being a regulator of cell growth. DAP-2 is expressed in the cells of the presumptive leg joints, and flies mutant for dAP-2 show a severe reduction in leg length. They fail to produce joint structures, indicating that dAP-2 expressed in joint cells is able to influence growth of leg segments (36, 37). Overexpression of dAP-2 does not induce overgrowth of the limb. In the model put forward by Kerber et al. (36) dAP-2 would be needed to promote cell growth and cell survival. They speculate that dAP-2 might activate a cellular survival factor. Thus dAP-2 and AP-2α appear to have conserved functional roles.

The question remains whether KLF-4, Mtd, Stra13, and mEFEMP-1 are directly regulated by AP-2α. Our data demonstrate that KLF-4 is expressed in mutant mesenchymal cells at E 8.5 as well as fibroblasts lacking AP-2α. Furthermore in silico analysis of the promoter regions of KLF-4, Mtd, Stra13, and mEFEMP-1 indicate that there are sites that fulfill the criteria for AP-2α binding. However, further experiments will be necessary to test directly the binding and transactivation of AP-2α on the promoter of KLF-4 as well as the other target genes reported.

The role of the requirement of KLF-4 deregulation in AP-2α mutants can be tested by genetic complementation. The KLF-4 null mouse line had been reported to complete embryonic development and die up to 1 week after birth due to insufficient barrier function of the skin (38). Generating a double mutant mouse deficient for AP-2α and KLF-4 will serve to test this hypothesis.

Taken together, the family of AP-2 transcription factors might play a role in the well orchestrated phases of proliferation of various tissue types where they function as control factors that inhibit the expression of differentiation associated genes and enable the expression of growth-promoting genes.

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