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Functional interaction between a RARE and an AP-2 binding site in the regulation of the human *HOX A4* gene promoter

Lesah Fry Doerksen, Anuradha Bhattacharya, Perry Kannan, David Pratt and Michael A. Tainsky*

Department of Tumor Biology, Box 79, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

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ABSTRACT

***HOX A* genes are induced in a temporal fashion after retinoic acid (RA) treatment in non-N-*ras*-transformed PA-1 human teratcarcinoma cells. However, in N-*ras*-transformed PA-1 cells, RA-induced expression of *HOX A* genes is delayed. The mRNA for the transcriptional activator AP-2 is overexpressed in these *ras*-transformed cells, but AP-2 transcriptional activity is inhibited relative to non *ras*-transformed PA-1 cells. Constitutive expression of AP-2 mimics the effect of *ras* by transforming cells and inhibiting differentiation in culture. We analyzed 4 kb of the human *HOX A4* gene promoter and identified seven putative AP-2-binding sites in the DNA sequence. Transcription assays with variably sized *HOX A4* promoter reporter constructs revealed that a 365 bp region of the promoter, –2950 to –3315 relative to the mRNA start, controls RA responsiveness and *ras*-mediated inhibition of *HOX A4* activity. This region contains an AP-2 binding site and a RARE. Elimination of the AP-2 site by site-directed mutagenesis demonstrated that the AP-2 site is involved in RA-mediated transcriptional activation of the human *HOX A4* promoter in combination with the RA receptor response element (RARE). In N-*ras*-transformed cells, low *HOX A4* promoter activity results from *ras* inhibition of AP-2 transactivation.**

INTRODUCTION

Cellular differentiation and pattern formation during embryonal development are effected via a complex system of regulated gene expression. Homeobox genes and the mechanisms by which they are regulated are important contributors to the system. Homeobox genes contain a 180 bp DNA sequence (homeobox) that encodes the homeodomain (1,2), a four-α-helix protein structure that binds DNA with sequence specificity as part of a transcription factor (3). Homeobox genes are present in the genome of eukaryotes ranging from *Arabidopsis* and snapdragons (4) to humans (5). Vertebrate homeobox genes, the *Hox* genes, encode proteins containing a homeodomain highly homologous to the *Drosophila* class I *Antennapedia* homeodomain. In vertebrates, there are 38 genes organized into four different chromosomal clusters, *Hox A*, *B*, *C* and *D*. The *Hox* genes are expressed with regional and temporal specificity during embryonic development, particularly in the central nervous system, the axial skeleton, and the limbs (6–8). Each cluster is ~120 kb in length, and the genes in each cluster are oriented in the same 5′ to 3′ direction of transcription with respect to other genes in the cluster (7–10).

The *AP-2* gene is regulated in tissue culture by the developmental morphogen, retinoic acid (RA) (11). The AP-2 protein is a sequence-specific transcriptional activator (12) which is important in the regulation of keratin gene expression during epidermal development (13) and whose expression pattern is regulated both spatially and temporally in neural crest-derived cell lineages (14,15). AP-2-binding sites exist in the SV40 transcriptional control region and the regulatory regions of the human metallothionine II A gene, the murine major histocompatibility complex gene, the collagenase gene, the human growth hormone gene, the human proenkephalin gene, the human keratin K14 gene, and the c-*erbB-2/HER2* and c-*erbB-3/HER3* genes (16–24). AP-2 is a 52 kDa protein (25) containing a glutamine- and proline-rich activation domain, a DNA-binding domain, and a dimerization domain that is necessary for DNA binding.

One mechanism used by oncogenic forms of *ras* to effect cellular transformation is the targeting of the activation of transcription factors. For example, when a *ras* oncogene is expressed, c-Jun is activated by increased phosphorylation of the c-Jun activation domain (25,26). Also, Tandem Ets binding sites were shown to be necessary for the rat stromelysin promoter to maximally respond to *ras* (27). AP-2 expression is increased in cells transformed by *ras* (28).

RA is a key morphogen in vertebrate development (29–31) and a potent regulator of both adult and embryonic cell differentiation (32,33). Retinoids induce body axis formation in different animal systems, while specifically regulating the *Hox* genes (34). The level and timing of RA induction of *Hox* genes is colinear in cell lines and embryos with the position of each gene within a *HOX* cluster (35–38). During RA-induced differentiation, specific activation of homeobox genes has been reported in a variety of human and mouse teratocarcinoma cell lines (39–41). For example, the expression of human *HOX* genes was specifically induced by RA in NT2/D1 and

* To whom correspondence should be addressed

PA-1 teratocarcinoma cells with temporal specificity (42,43). RA-induced differentiation activates the expression of many genes, some of which contain the consensus DNA-binding sites for AP-2. In NT2 (11) and PA-1 human teratocarcinoma cells (28), AP-2 mRNA levels are transiently elevated after RA treatment (44), and this induction is regulated at the level of transcription.

The PA-1 cell line was derived from a female ovarian germ cell tumor (45). The PA-1 subclones provide a cell culture model for the study of developmental gene regulation in which the temporal induction of homeobox genes is induced by RA treatment in non-*ras*-transformed PA-1 subclones. However, in N-*ras*-transformed subclones, RA-induced expression of these homeobox genes is delayed (43). RA induction of AP-2 mRNA is transient and peaks between 24–48 h of RA treatment in non-*ras*-transformed PA-1 cells, with a concomitant increase in AP-2 transactivation activity (28). Paradoxically, N-*ras*-transformed PA-1 cells have a high basal level of AP-2 mRNA but a low level of AP-2 transactivation activity, and RA treatment does not significantly alter AP-2 mRNA levels or the activity of AP-2. There is a direct link between N-*ras*-induced transformation and AP-2-mediated transcriptional activity in PA-1 cells. AP-2 inhibits gene expression by an auto-interference mechanism in N-*ras*-transformed PA-1 cells (28). Interestingly, constitutive expression of *HOX A4* cDNA under the control of an SV40 promoter in N-*ras*-transformed cells resulted in differentiation of the PA-1 cells (43). However, overexpression of AP-2 inhibited differentiation in PA-1 cells (28). It is possible that AP-2 may be part of a feedback loop to regulate the *HOX A4* promoter. In addition, these results indicated that AP-2 might play a role in downstream signal transduction of N-*ras*-induced cellular transformation.

As *HOX A4* is a key regulator of RA-induced differentiation in PA-1 cells (43), identifying regulatory mechanisms of the expression of *HOX A4* is essential to understanding the process of cellular differentiation. Computer analysis of a 4 kb section of the *HOX A4* promoter DNA sequence indicated seven potential AP-2-binding sites, implicating AP-2 as a possible regulator of the *HOX A4* gene. In differentiation-resistant, *ras*-transformed PA-1 cells, the failure of AP-2 to transactivate *HOX A4* gene expression may be a factor in the disregulation of the *HOX A4* gene. Alternatively, the disregulation of the *HOX A4* gene may be a general effect of *ras* on a subset of transcription factors in the cell. Here we show that at least one of the AP-2 sites contained in the *HOX A4* gene promoter is involved in the mechanism of RA induction of the *HOX A4* gene promoter and in the *ras*-mediated inhibition of the promoter.

MATERIALS AND METHODS

Cell culture

PA-1 cells were grown in modified Eagle's medium (MEM) (GIBCO Laboratories, Gaithersburg, MD) supplemented with 5% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) and antibiotics at 37°C in a 5% CO_2 incubator.

Calcium phosphate transfection

Non-*ras* (9117) and *ras*-transformed (9113) PA-1 subclones were seeded at a density of 8×10^5 cells/100 mm plate/8 ml of MEM. Twenty-four hours later, the cells were fed with 6 ml of MEM. Equal moles of reporter plasmid DNA were used in transfections based on the sizes of the constructs used in the transfection. In cotransfection experiments, increasing amounts of the AP-2 expression vector, pSAP2, were cotransfected into non-*ras*-transformed PA-1 cells with *HOX A4* promoter constructs. The control vector, pSG5, was used to maintain a total DNA concentration of 2.5 μg in the cotransfections. In addition, each plate was cotransfected with 1 μg of a constitutive β-galactosidase expression plasmid (pCH110) driven by an SV40 promoter to control for transfection efficiency. Precipitates were then allowed to incubate for 30 min at room temperature before being distributed evenly onto the cells. After an incubation period of 16 h at 37°C in 5% CO_2, the medium was removed. Duplicate transfected plates of cells were then fed with 8 ml of MEM supplemented with 10^{-5} M RA (Sigma Chemical Co., St. Louis, MO) or with non-RA-supplemented MEM. The cells were cultured for an additional 24 h to allow the cells to respond to the RA and induce homeobox gene promoter activity. The transfected cells were then harvested while on ice. The cell pellets were resuspended in 100 μl Tris buffer (0.25 M, pH 7.8) and subjected to three cycles of freeze-thawing [10 min for each freeze (–70°C) and thaw (37°C)] to lyse the cells. The cell extracts were then cleared by centrifugation at 4°C for 5 min. β-galactosidase (β-gal) activity in the transfected cells was used as an internal control for transfection efficiency.

The volume of cell extract containing 0.2 U β-gal activity was used in each chloramphenicol-acetyl transferase (CAT) assay. CAT assays were performed using standard procedures. Each silica gel plate was then analyzed using the Betascope Blot Analyzer (Betagen, Framingham, MA) to determine the percent conversion of acetylated forms of chloramphenicol.

Site-directed mutagenesis and polymerase chain reaction

A 39 bp oligonucleotide primer, 5′-GCGC(AAGCTT)GTTTACATT**TCCTTTGGC**TTTTCCCCTTG-3′, identical to the sequence surrounding and containing the AP-2 response element (indicated in bold) starting at –3250 in the *HOX A4* promoter; a 39 bp oligonucleotide primer, 5′-CGC(AAGCTT)GTTTACATT**TttTTTGGC**TTTTCCCCTTG-3′, containing two mutated nucleotides (as lower case); and a 24 bp reverse primer, 5′-TAGCCGACATTTTCATATTTGTTA-3′, were synthesized. The bases enclosed in parentheses are *Hin*dIII sites, and the AP-2 sites are in bold-face type. The terminal GCGC bases are intended to stabilize the oligonucleotide. Polymerase chain reaction (PCR) with the primers described was used to create a 167 bp fragment spanning the region from –3267 to –3101 containing a wild-type AP-2-response element and another 167 bp fragment containing a mutant AP-2-response element. The PCR reactions included 1× PCR buffer, 2.5 mM $MgCl_2$, 80 ng of each primer, 0.4 mM dNTP mix, 100 ng of template DNA and 2.5 U *Taq* DNA polymerase. The PCR program follows: 95°C for 3 min while adding *Taq* DNA polymerase to reactions, then 94°C for 1 min, 58°C for 2 min and 72°C for 3 min. (35 cycles). The TA Cloning System (Invitrogen Corp., San Diego, CA) was employed to clone the PCR fragments containing the RARE and either the wild-type AP-2 response element or the mutant AP-2 response element. After obtaining clones that contained the correct insert by restriction enzyme analysis, the sequence of the clones was confirmed by DNA sequence analysis. The PCR fragments were then subcloned into the pBLCAT2 vector and resequenced.

Electrophoretic mobility shift assays

Purified AP-2 proteins or nuclear extracts from HeLa cells or PA-1 9117 cells were prepared as previously described (28) and

incubated with 2.5 × 10^4 cpm of labeled double-stranded oligonucleotides in 1× GSA buffer (10 mM HEPES pH 7.9, 2.5 mM DTT, 50 mM KCl, 6 mM $MgCl_2$, 100 µg/ml BSA, 0.01% NP-40, 10% glycerol) supplemented with 1 µg BSA and 1 µg dI-dC at 30°C for 30 min. The wild-type human metallothionine AP-2 binding sequence 5′-AGGAACTGACC**GCCCGCGGC**CCG-TGTGCAGAG-3′ or the mutated sequence 5′-AGGAACTGAC-C**GaCCGCtGC**CCGTGTGCAGAG-3′, containing two mutated nucleotides (as lower case) were used as a control. The AP-2 DNA binding site 5′-ACATT**TCCTTTGGC**TTTT-3′ from the *HOX A4* promoter at position –3150 was also used to show AP-2 binding and competition of binding. A mutant *HOX* A4 DNA binding site was also used, 5′-ACATTT**tt**TTTGGCTTTT-3′, containing two mutated nucleotides (as lower case). The AP-2 sites are indicated in bold type. Native gel electrophoresis was performed on 4% polyacrylamide gels (29:1) at 10 V/cm.

DNA sequence analysis

Sequencing of the mutant and wild-type AP-2 clones was accomplished by using the Sequenase Kit from USB (United States Biochemical, Cleveland, OH) following the recommended protocol from the manufacturer. The upstream DNA sequence of the human *HOX A4* gene is in the GenBank accession file U41755.

RESULTS

N-*ras* inhibits activity of human *HOX A4* gene promoter

By searching the Transcription Factor Sites Version 7.3 database, we identified within 4 kb of DNA sequence upstream of the *HOX A4* mRNA start site many potential transcription factor binding sites. In particular, the sequence showed seven putative AP-2 binding sites (16), a RARE (46), *ras*-response elements (47) and *HOX*-binding sites (48) (Fig. 1A). To determine the role of any of these AP-2 sites in the effect of *ras* transformation on the activity of variably sized *HOX A4* promoter CAT constructs in the pBLCAT3 vector (Fig. 1A), we transfected the constructs into subclones of the PA-1 cell line. The PA-1 cell line provided us with a model for *ras*-mediated inhibition of differentiation through an N-*ras*-transformed subclone of the PA-1 cell line containing a spontaneously activated N-*ras* that causes an inhibition of differentiation in response to RA (43). Another tumorigenic PA-1 subclone that is not N-*ras*-transformed and is differentiation competent in response to RA was used to compare the effects of *ras*. Transcription assays showed that the transcriptional activity of the 4.0 kb promoter was induced ~10-fold in the non-*ras* cells but was only induced 2-fold in the *ras*-transformed cells, a 5-fold inhibition in the *ras* cells (Fig. 1B). The 2.8 and 0.6 kb promoter constructs, however, were not highly inducible by RA treatment in either cell line and had activities similar to the basal level activity of the pBLCAT2 vector, which contains the herpes simplex virus thymidine kinase minimal promoter. The transcription assays revealed, therefore, that the RA induction of the 4.0 kb *HOX A4* promoter activity is reduced, similar to mRNA expression, in the *ras*-transformed cells relative to its activity in the non-*ras*-transformed cells. However, the effect of *ras* transformation on both the 2.8 and 0.6 kb promoters is minimal. This is consistent with the fact that these promoters do not contain the RARE at –3148 to –3132 from the mRNA start site and are therefore only minimally affected by RA. These results indicate that the region of the *HOX A4* promoter regulated by RA and *ras* is between –4052 and –2810

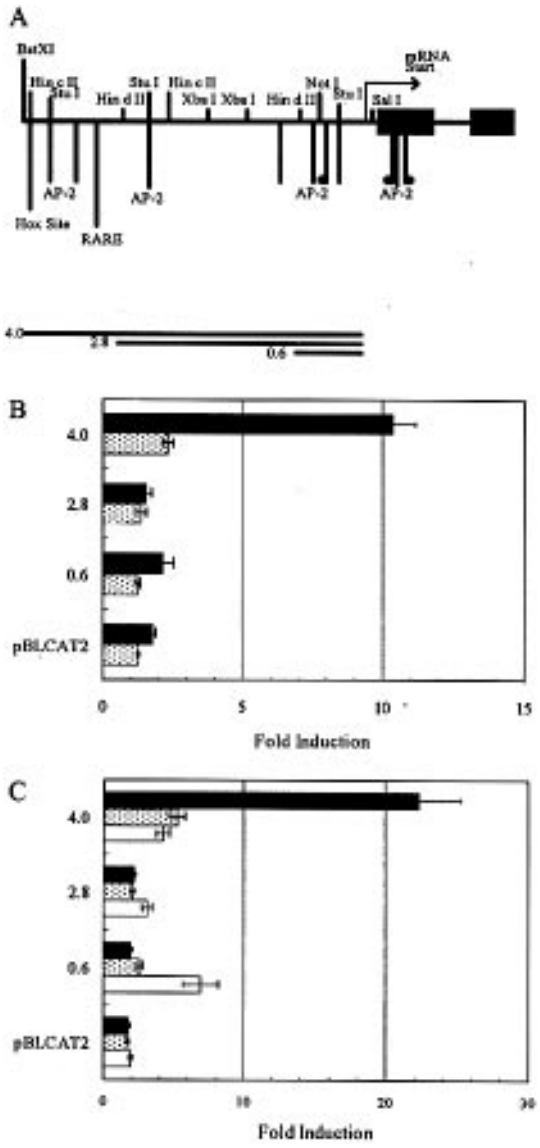

Figure 1. N-*ras* and AP-2 overexpression inhibit the activity of the *HOX A4* gene promoter. (**A**) Schematic representation of the *HOX A4* gene promoter. Lines extending down from the horizontal line represent AP-2 binding sites, a RARE and a *HOX* binding site, as labeled. The d-lines labeled 'AP-2 site' have the consensus sequence GCCNNNGGC. The straight lines labeled 'AP-2 site' have the sequence CCNNNGGC, which binds DNA but with less affinity. The 4.0, 2.8 and 0.6 kb *HOX A4* promoter constructs are shown in relation to their position on the *HOX A4* gene promoter. (**B**) Effects of RA and *ras* on the *HOX A4* promoter constructs in transient transcription assays. Each of the promoter constructs were transfected into non-*ras*-transformed 9117 cells (black) and *ras*-transformed 9113 cells (shaded) (see Materials and Methods). (**C**) Effect of AP-2 overexpression on *HOX A4* promoter constructs in non-N-*ras*-transformed PA-1 cells. Non-*ras*-transformed PA-1 cells were transiently cotransfected with 0 (black), 1.0 (shaded), and 2.5 (open) µg of the AP-2 expression plasmid pSAP2 and one of the *HOX A4* promoter constructs (see Materials and Methods). The pSG5 vector was added to the control (0) or each AP-2 cotransfection to make the total amount of pSG5 2.5 µg.

from the mRNA start site. AP-2 sites between –2810 and the mRNA start do not appear to significantly enhance transcription even though AP-2 activity is activated by RA-treatment.

AP-2 has been shown to inhibit gene expression by an auto-interference mechanism in N-*ras*-transformed PA-1 cells (28). Therefore, we attempted to determine whether AP-2 plays

a role in the mechanism of inhibition of *HOX A4*-promoter activity in these cells. AP2-*tk*-CAT, a reporter plasmid, contains three AP-2-response elements from the distal basal level element of the human metallothionine gene IIA. The level of endogenous AP-2 activity is measured by this reporter plasmid. In our transcription assays, which were optimized for RA induction of the *HOX A4* promoter, a 2-fold decrease of the AP2-*tk*-CAT reporter activity was observed in *ras* cells compared with the activity of the reporter in non-*ras* cells (data not shown).

AP-2 overexpression inhibits the activity of the human *HOX A4* gene promoter

N-*ras* transformation results in the overexpression of the transcription factor AP-2, and AP-2 inhibits transcriptional activation by an auto-interference mechanism in PA-1 cells (28). We wanted to test whether overexpression of AP-2 inhibits differentiation-specific gene expression, as measured by the activity of the *HOX A4* reporter plasmid. It is possible that the down-regulation of the *HOX A4* gene promoter by AP-2 is part of an overall mechanism of differentiation inhibition.

To determine whether overexpression of AP-2 inhibits the transcriptional activity of the 4.0, 2.8 and 0.6 kb *HOX A4* promoters, these *HOX A4* promoter constructs and varying amounts of an SV40-driven AP-2 expression plasmid (pSAP2) were cotransfected into non-N-*ras*-transformed cells (Fig. 1C). All AP-2 cotransfections contained a constant amount (2.5 µg) of the pSG5 vector. The 4.0 kb promoter was induced 22-fold with no pSAP2 cotransfected, and its induction was maximally inhibited 5-fold with 2.5 µg of pSAP2. The 2.8 kb promoter, however, was not significantly affected by AP-2 overexpression. The activity of the 0.6 kb promoter seemed to be stimulated rather than inhibited by the overexpression of AP-2. The reason for this stimulation is likely to be due to the three AP-2 sites contained in this promoter construct. Overexpression of AP-2 inhibited the AP2-*tk*-CAT reporter by 25% (data not shown). These results demonstrate that the 4.0 kb promoter but not the 2.8 and 0.6 kb promoters contains the region of DNA where AP-2 inhibits *HOX A4* promoter activity.

N-*ras* inhibits activity of *HOX A4* constructs with enhancer activity

The results of the 4.0, 2.8 and 0.6 kb promoter construct transcription assays indicated that the region of the *HOX A4* promoter regulated by AP-2 and *ras* is –4052 to –2810 from the mRNA start site (Fig. 1A). Therefore, constructs in this 1.25 kb region of the *HOX A4* promoter were analyzed for enhancer activity in the presence and absence of RA in *ras*-transformed PA-1 cells. The constructs included a 1.25 kb fragment of the *HOX A4* promoter, –2810 to –4052, containing the remainder of the *HOX A4* promoter 5′ of the 2.8 kb fragment, a 0.691 kb fragment, –2809 to –3500, containing the AP-2 site at –3253 and the RARE (at –3148 to –3132), a 0.544 kb fragment, –3500 to –4044, containing the AP-2 site at –3590, and a 0.365 kb fragment, –2950 to –3315, that eliminates additional sequence 5′ of the AP-2 site at –3150 and 3′ of the RARE (Fig. 2A). Transient transcription assays showed that the transcriptional activity of the 1.25, 0.691 and 0.365 kb *HOX A4* constructs was inducible by RA and inhibited in the *ras* cells. However, the activity of the 0.544 kb construct was very similar to that of the parent vector pBLCAT2, which contains no AP-2 sites (Fig. 2B). The activity of the 1.25 kb construct was increased to ~45-fold in the non-*ras*-transformed cell line. In the *ras*-transformed cell line its activity was

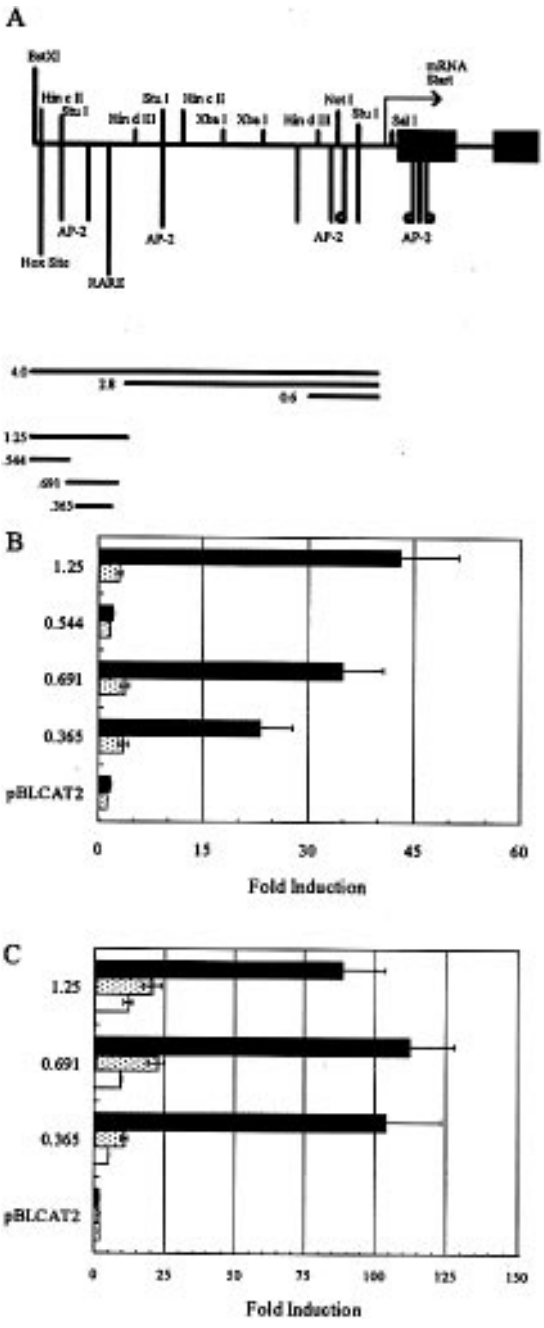

Figure 2. N-*ras* and AP-2 overexpression inhibit the activity of the *HOX A4* constructs with enhancer activity. (**A**) Schematic representation of the *HOX A4* gene promoter. The 4.0, 2.8 and 0.6 kb *HOX A4* promoter constructs are shown in relation to their position on the *HOX A4* promoter. The 1.25, 0.691, 0.544 and 0.365 kb *HOX A4* constructs cloned into pBL-CAT2 are shown in relation to their position on the *HOX A4* promoter. (**B**) Effects of RA and *ras* on *HOX A4* constructs with enhancer activity in transient transcription assays. The 1.25, 0.691, 0.544 and 0.365 kb constructs were transiently transfected into non-*ras*-transformed 9117 cells (black) and *ras*-transformed 9113 cells (shaded) (see Materials and Methods). (**C**) Effect of AP-2 overexpression on *HOX A4* constructs with enhancer activity in non-N-*ras*-transformed PA-1 cells. The constructs were transiently cotransfected with 0 (black), 1.0 (shaded), and 2.5 (open) µg of the AP-2 expression plasmid pSAP2 (see Materials and Methods). The pSG5 vector was added to the control (0) or each AP-2 cotransfection to make the total amount of pSG5 2.5 µg.

decreased 9-fold. Similarly, the 0.691 and 0.365 kb constructs were 7- and 5-fold less active, respectively, in the *ras*-transformed cells. That the overall magnitude of transcriptional activation is decreasing as sequences are removed from the 1.25 kb construct indicates that additional regulatory elements are contained in these regions. However, the 0.544 kb construct showed very little RA-mediated induction in either cell line. Much like the 2.8 and 0.6 kb promoters, the 0.544 kb construct which does not contain a RARE is minimally regulated by RA. Furthermore, the 1.25, 0.691 and 0.365 kb constructs include the same AP-2 site and RARE. This indicates that the AP-2 site and RARE are the important sites of regulation for the human *HOX A4* gene promoter.

AP-2 overexpression inhibits activity of *HOX A4* constructs with enhancer activity

To evaluate the role of various AP-2-binding sites in the mechanism of *ras*-mediated inhibition of the *HOX A4* promoter, the 1.25, 0.691 and 0.365 kb *HOX A4* constructs were used in transcription assays. Specifically, the constructs were cotransfected with 1.0 and 2.5 µg of pSAP2 to determine the effect of AP-2 overexpression on each construct (Fig. 2C). The 0.544 kb *HOX A4* construct was not included in the cotransfection experiments as there was no detectable *ras* inhibition of the promoter activity within this construct in the N-*ras*-transformed cells. The 1.25 kb construct was induced ~90-fold by RA treatment with no pSAP2. As the amount of pSAP2 was increased, the RA induction of the 1.25 kb construct was reduced to 10-fold with 2.5 µg of pSAP2, an inhibition of 9-fold. RA induction of the 0.691 kb construct was ~110-fold with no pSAP2. With the addition of 2.5 µg of pSAP2, the 0.691 kb construct's activity was decreased 11-fold. The 0.365 kb construct was induced ~105-fold when no pSAP2 was transfected, but when AP-2 was overexpressed the induction level of the 0.365 kb construct was inhibited 21-fold. These results demonstrate that overexpression of AP-2 inhibits the 1.25, 0.691 and 0.365 kb constructs through sites contained within a 365 bp region. The results obtained to this point indicated that AP-2 is involved in the regulation of the *HOX A4* promoter.

AP-2 could regulate the transcriptional activation of the *HOX A4* promoter directly or indirectly. Possibly, AP-2 could regulate the promoter directly by binding the DNA at the AP-2-binding site in the 0.365 kb construct. Alternatively, AP-2 could regulate the *HOX A4* promoter indirectly by interacting with the general transcription machinery through its activation domain or through interaction with another cofactor, independent of the AP-2 site in the 0.365 kb construct.

To determine a possible mechanism of regulation of the *HOX A4* promoter by AP-2, we studied the effect of plasmid expression vectors of AP-2 with deletion of either the activation domain or the DNA binding domain (data not shown). Our analysis showed that the overall effect of each domain is far less than that of the total AP-2 protein, indicating that AP-2 inhibitory function may be dependent on the whole protein. Because the results from the AP-2 domain deletion constructs were inconclusive, we employed site-directed mutagenesis as our next approach.

AP-2-binding site is necessary for RA-mediated induction of the *HOX A4* gene promoter

The results from transfections of the *HOX A4* promoter and enhancer constructs implicated the 0.365 kb construct as the region of the *HOX A4* gene promoter regulated by *ras*. The 0.365 kb construct contains one AP-2 site and a RARE (Fig. 2A). To determine whether *ras* affects the promoter through that AP-2 site, we next targeted, by site-directed mutagenesis, the AP-2 site starting at position –3250 to –3258 in the promoter found in the 0.365 kb construct for inactivation. Two 167 bp fragments, –3267 to –3101, containing the AP-2 and RARE within the *HOX A4* upstream enhancer region were constructed by PCR (Fig. 3A); one contained a wild-type AP-2 site and the other contained a mutant AP-2 site, TCCTTTGGC→TTTTTTGGC. The clones were analyzed by DNA sequence analysis to verify the correct *HOX A4* sequence.

If *ras* were inhibiting the *HOX A4* promoter through the AP-2 site in the 167-wt promoter region, one would expect that elimination of the AP-2 site would increase the activity of the 0.167-mt promoter in the *ras*-transformed cells relative to the normal 167-wt promoter in the same cells. Alternatively, if RA-inducible AP-2 activity acts in cooperation with the RARE, we would expect the activity of the 167-mt promoter to be reduced in non-*ras* cells and to be equally low in *ras*-transformed cells relative to the activity of the 167-wt normal promoter. Transcription assays of the 167-wt and 167-mt constructs demonstrated that, like the 0.365 kb promoter, the RA-induced activity of the 167-wt construct was lower in the *ras*-transformed cells than in the non-*ras* cells. RA-inducible activity of the 167-wt *HOX A4* construct was inhibited 6.5-fold in the *ras*-transformed cells. However, the RA-inducible activity of the 167-mt *HOX A4* construct was only 2.5-fold more active in the non-*ras* cells. The activity of the 167-mt construct was lower in the non-*ras* cells than the normal 167-wt construct in the *ras* cells (Fig. 3B). Interestingly, the RA-inducible activity of both constructs was almost identical in the *ras*-transformed cells. Thus the loss of the AP-2 site in the 167-mt construct demonstrated the need for AP-2 binding for RA-inducible activity of *HOX A4* in the non-*ras*-cells. The results indicated that this AP-2 site is a positive regulator in the mechanism of RA inducibility in the non-*ras* cells. In addition, in *ras* cells there is a lower capacity to induce this promoter at the RARE, and mutation of the AP-2 site does not affect promoter activity in the *ras* cells. Therefore, the RARE seems to act as the qualitative regulator of *HOX A4* promoter activity, and AP-2 acts as a quantitative regulator to modify the effect of the RARE.

AP-2 overexpression inhibits activity of both wild-type and mutant 167 bp human *HOX A4* constructs

The 167-wt and 167-mt *HOX A4* constructs were cotransfected with varying amounts of pSAP2 to determine whether AP-2 overexpression inhibits the RA-inducible activity of human *HOX A4* through this AP-2 site. Both the 167-wt and 167-mt constructs were inhibited ~4-fold when AP-2 was overexpressed in non-*ras* cells (Fig. 3C). However, the initial RA-mediated induction of the 167-wt construct was 2-fold higher than that of the 167-mt construct. Therefore, the elimination of this AP-2-binding site inhibits RA inducibility of the 167-mt *HOX A4* construct but has no effect on the mechanism of inhibition of the 167-mt construct activity by AP-2 overexpression, indicating that AP-2 overexpression inhibits the *HOX A4* promoter in this region by a mechanism independent of DNA binding at the AP-2 site.

As this AP-2 site differed from the consensus AP-2 site by one base, we verified that it is functional and can bind to AP-2 in human cells. To show this we used electrophoretic mobility shift analysis of a labeled *HOX A4* AP-2-binding site and HeLa cell nuclear extract,

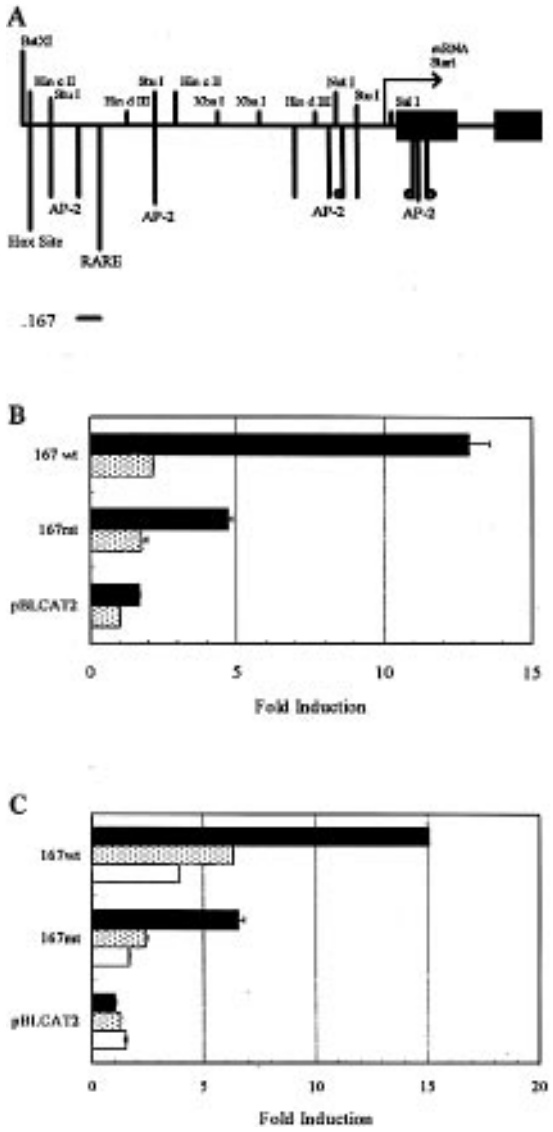

Figure 3. The AP-2 site is necessary for full RA induced *HOX A4* promoter activity. (**A**) Effect of *ras* on 167 bp enhancer constructs 167-wt and 167-mt of the *HOX A4* promoter. The 167-wt (contains a wild-type AP-2 site) and 167-mt (contains a mutant AP-2 site) constructs were transiently transfected into non-*ras*-transformed 9117 cells (black) and *ras*-transformed 9113 cells (shaded) (see Materials and Methods). (**B**) Effect of AP-2 overexpression on wild-type and mutant 167 bp *HOX A4* promoter constructs. Non-*ras*-transformed PA-1 cells were transiently cotransfected with 0 (black), 1 (shaded), and 2.5 (open) μg of the AP-2 expression plasmid pSAP2 and the 167-wt or 167-mt *HOX A4* enhancer constructs (see Materials and Methods). The pSG5 vector was added to the control (0) or each AP-2 cotransfection to make the total amount of pSG5 2.5 μg.

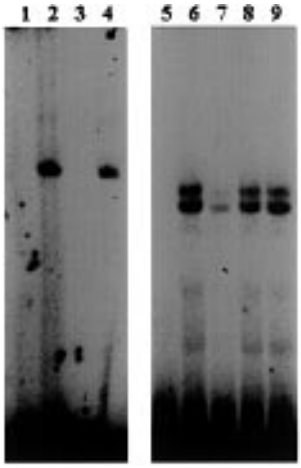

Figure 4. Electrophoretic mobility shift assay of the AP-2 sites from the *HOX A4* and human metallothionine genes. In lanes 2–4, nuclear extract from HeLa cells was incubated with ^{32}P-labeled AP-2 binding site from the *HOX A4* gene ACATT**TCCTTTGGCT**TTT (see Materials and Methods). Lane 1 contained the ^{32}P-labeled *HOX A4* gene AP-2 binding site double-strand oligonucleotide without any nuclear extract. Lane 2 contained a reaction of the ^{32}P-labeled *HOX A4* gene AP-2 binding site oligonucleotide with HeLa cell nuclear extract showing a single shifted DNA–protein complex. The reaction in lane 3 contained a 100-fold excess of the unlabeled oligonucleotide. The reaction in lane 4 contained a 100-fold excess of a mutant *HOX A4* gene AP-2 binding site oligonucleotide in which two C residues in the AP-2 were altered to two T residues, ACATT**TttTTTGGCT**TTT. In lanes 6–9, nuclear extract from 9117 non-*ras* PA-1 cells was incubated with ^{32}P-labeled oligonucleotide containing the AP-2 binding site (indicated in bold) from the human metallothionine gene 5′-AGGAACTGACC**GCCCGCGGC**CCGTGTGCAGAG-3′. Lane 5 contained the ^{32}P-labeled oligonucleotide but no nuclear extract. The reaction in lane 6 contained the ^{32}P-labeled oligonucleotide and nuclear extract from 9117 cells. The reaction in lane 7 contained the same as lane 6 but with the addition of a 100-fold excess of an unlabeled double stranded oligonucleotide AP-2 site from the *HOX A4* gene. The reaction in lane 8 contained the same as lane 6 but with the addition of a 100-fold excess of an unlabeled double stranded oligonucleotide with a mutant AP-2 site from the *HOX A4* gene in which two C residues in the AP-2 were altered to two T residues, ACATT**TttTTTGGCT**-TTT. The reaction in lane 9 contained the same as lane 6 but with the addition of a 100-fold excess of human metallothionine gene mutant AP-2 site (5′-AGGAACTGACC**GaCCGCtGC**CCGTGTGCAGAG-3′).

a cell known to be rich in AP-2 protein. The ^{32}P-labeled oligonucleotide containing the *HOX A4* AP-2-binding site (bold type), ACATT**TCCTTTGGC**TTTT, produced a single band on non-denaturing polyacrylamide gels which was not seen in the absence of HeLa cell nuclear extract (Fig 4, compare lanes 1 and 2). This band was specifically competed away by a 100-fold excess of unlabeled wild-type *HOX A4* AP-2-binding site (Fig. 4, lane 3) but not by a mutant binding site in which two C residues on one side of the palindromic AP-2 site were changed to T residues, ACATT**TttTTTGGCT**TTT (Fig. 4, lane 3). In addition, when we used a ^{32}P-labeled oligonucleotide containing the consensus AP-2 DNA binding site (indicated in bold type) AGGAACTGACC**GC-CCGCGGC**CCGTGTGCAGAG from the human metallothionine gene, we found that two mobility shifted bands were observed after coincubation with nuclear extract from 9117 non-*ras* PA-1 cells that were not present in the absence of nuclear extract, (Fig. 4, compare lanes 5 and 6). Both bands could be competed away by a 100-fold excess of unlabeled wild-type *HOX A4* AP-2-binding site (Fig. 4, lane 7) but not by either a mutant *HOX A4* AP-2 binding site (Fig. 4, lane 8) or a mutant human metallothionine gene AP-2 site AGGAACTGACC**GaCCGCtGC**CCGTGTGCAGAG (Fig. 4, lane 9). The changes in the AP-2 site from the human metallothionine gene were generated in a different pattern of the essential palindromic sequences than those chosen for the *HOX A4* AP-2-binding site, yet both failed to compete. Based on the DNA sequence specificity of these gel shift competitions, these data indicate that the AP-2 site in the *HOX A4* promoter could be specifically bound by cellular proteins that bind a consensus AP-2 site.

In summary, AP-2 is involved in the *ras*-mediated inhibition of the *HOX A4* gene promoter. Lower AP-2 transcriptional activity

and the failure of RA to induce AP-2 activity results in a decreased transcriptional activation of the *HOX A4* promoter in *ras*-transformed cells. However, the AP-2 site in the 167-wt *HOX A4* construct does not seem to be involved in the mechanism of inhibition by overexpression of AP-2. This AP-2 site is involved in the mechanism of RA-mediated induction of the *HOX A4* promoter at the RARE in the process of RA-induced differentiation.

DISCUSSION

Interaction of *HOX* genes and AP-2

Homeobox genes and the mechanisms by which they are regulated are important contributors to the complex system of cellular differentiation and pattern formation during embryonal development. Homeoproteins of the Dfd subfamily in *Drosophila* (which is the paralogous gene to the human *HOX A4*) have a proline-rich region in the N-terminal half of the molecule. AP-2 expression overlaps with that of many *Hox* genes both spatially and temporally in development. Overlapping expression and similar proline-rich transcriptional activation domains may suggest cooperation between Hox proteins and AP-2 in embryonic patterning.

Transcriptional mechanisms in *ras* signal transduction

Activation of transcription factors by *ras* has been documented. For example, c-Jun is activated by increased phosphorylation of the c-Jun activation domain when oncogenic *ras* is expressed (27,28). Tandem Ets binding sites were shown to be necessary for the rat stromelysin promoter to maximally respond to *ras* (29). Furthermore, tandem binding sites for AP-1, NF-κB and SP-1 families of transcription factors were found to be sufficient to function as oncogene-responsive elements and to mediate transactivation by *ras* (49–51). As transcription factors represent key targets of oncogenic *ras*, several lines of evidence from our work have led us to believe that AP-2 may be involved in the mechanism of *ras*-induced inhibition of the *HOX A4* gene promoter. We found a direct link between N-*ras*-induced transformation and AP-2-mediated transcriptional activity (28). We also found that AP-2 is overexpressed but less transcriptionally active in N-*ras*-transformed PA-1 cells. In addition, there is a delay in the induction of *HOX A4* mRNA expression in N-*ras*-transformed PA-1 cells (43). Using computer analysis of the upstream 4 kb sequence of the *HOX A4* gene promoter, we identified seven potential AP-2-binding sites in our promoter sequence data. Transactivation of the *HOX A4* promoter is inhibited in N-*ras*-transformed PA-1 cells as is the mRNA expression of *HOX A4* in northern blot analysis. Consistent with AP-2 regulation by *ras*, transient overexpression of AP-2 inhibited the transactivation of the *HOX A4* promoter. Therefore, AP-2 appeared to be involved in the mechanism of *ras*-induced inhibition of the promoter.

Role of AP-2 in *HOX A4* gene promoter activity

In this study, we have analyzed the role of AP-2 in the inhibition of differentiation-specific gene expression using the *HOX A4* gene promoter as a model. Constructs were made that contained different regions of the 4 kb *HOX A4* gene promoter with a subset of AP-2-binding sites. Transient transcription assays were carried out to identify a region of the promoter from –2950 to –3315 that responded to RA and was inhibited by *ras*.

Figure 5. *HOX A4*, *HOX D4*, and *Hox d-4* homology. Dashes indicate identical bases. AP-2 sites are underlined and in bold-face type, the conserved RARE is in bold-face type, and the other highly conserved region is underlined.

It has been demonstrated that the activation domain of AP-2 causes auto-interference (28). We therefore used AP-2 deletion mutants to determine the mechanism by which AP-2 inhibits the activation of the *HOX A4* promoter, but the results of these experiments did not conclusively demonstrate any effect consistent with squelching of *HOX A4* activity through the activation domain of AP-2 or through the AP-2-binding site. These results may reflect the requirement for the whole AP-2 protein to be functional as a repressor.

Transcription assays using seven *HOX A4* constructs in N-*ras*-transformed PA-1 cells revealed that the region contained in the 0.365 kb *HOX A4* construct was highly RA-inducible in non-*ras*-transformed cells, and its activity was 5- to 8-fold lower in *ras*-transformed cells. In addition, transcription assays determining the effect of AP-2 overexpression on the *HOX A4* constructs in non-*ras*-transformed cells showed that the transcriptional activity of the 0.365 kb construct was also inhibited with AP-2 overexpression, mimicking the effect seen previously in N-*ras*-transformed cells. Therefore, we focused on the AP-2 site contained in the 0.365 kb *HOX A4* construct as an important site in the regulation of the *HOX A4* promoter.

The disregulation of the *HOX A4* gene may result from a general effect of *ras* on a subset of transcription factors in the cell, including AP-2. AP-2 may be involved in the disregulation of the *HOX A4* promoter directly by binding to the promoter at the AP-2 site and interacting with the general transcription machinery through its activation domain to produce activated transcription. Alternatively, AP-2 may be involved indirectly by interacting with a coactivator that in turn interacts with the basal transcription machinery. Because the results from the AP-2 deletion mutant experiments were inconclusive, we employed site-directed mutagenesis to investigate the mechanism by which AP-2 regulates the *HOX A4* promoter.

We eliminated the only AP-2-binding site in the 0.365 kb *HOX A4* construct and made two plasmids, 167-wt and 167-mt. The data from the wild-type and mutant AP-2 constructs revealed that the AP-2-binding site contained in the 167-wt construct is involved in the mechanism by which RA induces the *HOX A4* promoter during differentiation in non-*ras*-transformed cells. Lower AP-2 transcriptional activity and the failure of RA to induce AP-2 activity in *ras*-transformed cells results in a decreased transcriptional activation of the *HOX A4* promoter. Furthermore, elimination of the AP-2 site did not affect the activity of the *HOX A4* gene promoter in *ras*-transformed PA-1 cells. This AP-2 site affects the magnitude of the induction, which

is regulated at the RARE. In *ras*-transformed cells there is little effect of elimination of the nearby AP-2 binding site.

RAREs have been found in several *Hox* genes. For example, there are RAREs in the region 3′ of the *Hoxa-1* and *Hoxb-1* (52,53) genes. In addition, a RARE was also found in the 5′ region of the *Hoxd-4*, *HOX D4* and *HOX A4* (54,55; Bhattacharya *et al.*, manuscript in preparation) gene. The RAREs in *Hoxa-1* and *Hoxd-4* can mediate an up-regulation in response to RA treatment in cultured cells, and the *Hoxb-1* RARE can mediate early response of *Hoxb-1* to RA in the mouse. There is 100% identity between *HOX A4* and *Hoxd-4* in the RARE. The conserved sequence contains the RARE. The conservation of RAREs in multiple *Hox* genes indicates its importance in regulating the process of differentiation.

RARs have been shown to regulate gene expression by direct interaction with transcription factors. Both RARs and retinoid-x-receptors (RXR) down-regulate the transforming growth factor-β_1 promoter by direct protein–protein interaction with AP-1 (56). AP-2 and a RAR may work synergistically to induce activity of the *HOX A4* gene promoter in non-*ras*-transformed PA-1 cells. A positive regulation may occur between the RAR and AP-2 in the *HOX A4* promoter. Data presented here indicate that activation of the *HOX A4* promoter requires both the AP-2 site and the RARE. The mechanism of interaction of factors at such distant sites is unknown. However, constructs containing either the AP-2 site or RARE alone demonstrate decreased activity from a construct containing both sites together. Interestingly, AP-2 sites exist 110, 264 and 189 bp upstream of the conserved sequence in the *HOX A4*, *HOX D4* and *Hoxd-4* promoters, respectively, implying that AP-2 may be important for the regulation of other *Hox* genes (Fig. 5).

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