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An Alternatively Spliced mRNA from the AP-2 Gene Encodes a Negative Regulator of Transcriptional Activation by AP-2

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AP-2 is a retinoic acid-inducible and developmentally regulated activator of transcription. We have cloned an alternative AP-2 transcript (AP-2B) from the human teratocarcinoma cell line PA-1, which encodes a protein differing in the C terminus from the previously isolated AP-2 protein (AP-2A). This protein contains the activation domain of AP-2 and part of the DNA binding domain but lacks the dimerization domain which is necessary for DNA binding. Analysis of overlapping genomic clones spanning the entire AP-2 gene proves that AP-2A and AP-2B transcripts are alternatively spliced from the same gene. Both transient and stable transfection experiments show that AP-2B inhibits AP-2 transactivator function, as measured by an AP-2-responsive chloramphenicol acetyltransferase reporter plasmid. Furthermore, constitutive AP-2B expression in PA-1 cells causes a retinoic acid-resistant phenotype, anchorage-independent growth in soft agar, and tumorigenicity in nude mice, in a fashion similar to transformation of these cells by oncogenes. To determine the mechanism by which AP-2B exerts its inhibitory function, we purified bacterially expressed AP-2A and AP-2B proteins. While bacterial AP-2B does not bind an AP-2 consensus site, it strongly inhibits binding of the endogenous AP-2 present in PA-1 cell nuclear extracts. However, DNA sequence-specific binding of bacterially expressed AP-2A cannot be inhibited by bacterially expressed AP-2B. Therefore, inhibition of AP-2 activity by the protein AP-2B may require an additional factor or modification supplied by nuclear extracts.

Cellular differentiation and growth regulation require specific alterations in programs of gene expression. These alterations require the interaction of environmental signal molecules, transduction to the nucleus, and a cascade of regulated gene expression. Retinoic acid (RA) is a key morphogen in vertebrate development and a potent regulator of cell differentiation (1, 7, 11, 46, 53). Analysis of a number of gene control regions known to be the target of regulation by RA revealed that the transcription factor AP-2 is an important effector of RA-induced cell differentiation.

AP-2, a 52-kDa protein, was first purified from HeLa cells. Partial peptide sequences led to the isolation of the cDNA from a HeLa cell library (54), and the gene was mapped to a region on chromosome 6 near the HLA locus (15, 32). Functional AP-2 binding sites have been identified in the enhancer regions of viral and cellular genes such as simian virus 40 (SV40), human T-cell leukemia virus type I, human metallothionein-II_A (huMTII_A), murine major histocompatibility complex (*H-2K*^{b}), human proenkephalin, and human keratin K14 genes (20, 23, 26, 27, 33, 36, 54), with a consensus palindromic core recognition element, 5′-GCC NNNGGC-3′ (54). The DNA binding domain is located within the C-terminal half of the 52-kDa protein and consists of two putative amphipathic alpha helices separated by a large intervening span region. This hypothetical helix-span-helix-motif was shown to mediate homodimer formation (56).

AP-2 exerts its crucial function in mediating regulation of gene expression in response to a number of different signal transduction pathways. Phorbol esters along with cyclic AMP induce AP-2 activity independent of protein synthesis (24). In contrast, RA induces AP-2 activity by increasing AP-2 mRNA levels in human teratocarcinoma cells in vitro (30) and is associated with programmed gene expression during retinoid-controlled murine embryogenesis (32). Restricted spatial and temporal expression pattern has been detected in several embryonic tissues, in particular in neural crest-derived cell lineages and in limb bud mesenchyme, at times when they are known to be developmentally retinoid sensitive. Furthermore, analyses of AP-2 expression in embryonic and adult *Xenopus* tissues implicate a causal role for AP-2 in regulation of keratin gene expression during skin differentiation (45). Keratin gene expression is known to be highly retinoid sensitive.

Teratocarcinoma cell lines provide an in vitro system with which to study early embryonic development, including RA-induced cell differentiation and growth regulation (1, 31, 42). We have analyzed the function of AP-2 during these processes. Here, we demonstrate that two differentially spliced mRNAs are transcribed from the AP-2 gene, encoding two different proteins, AP-2A and AP-2B. The AP-2A protein is identical to the 52-kDa AP-2 protein purified from HeLa cells. The AP-2B protein has the activation domain of AP-2 and part of the DNA binding domain but lacks the dimerization domain which is necessary for DNA binding. Transfection experiments indicate that AP-2B is a potent inhibitor of transactivation by AP-2A by inhibiting the interaction of AP-2A with DNA. AP-2B can suppress RA-induced growth regulation and cell differentiation. Our results point to an additional regulatory mechanism involved in *trans* regulation of gene expression by AP-2 and suggest a

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crucial function of AP-2 transactivation during RA-induced cell differentiation.

MATERIALS AND METHODS

Isolation of recombinant cDNA and genomic DNA clones. Twice-poly(A)$^+$-selected RNA (2.5 μg) from 6928 cells treated for 24 h with 10 μM RA was reverse transcribed into cDNA, using an oligo(dT)-primed cDNA synthesis kit (Pharmacia LKB, Piscataway, N.J.). Then 50 ng of cDNA was ligated into *Eco*RI-cut dephosphorylated lambda ZAPII and packaged with Gigapack Gold extracts (Stratagene, La Jolla, Calif.), resulting in 2×10^6 plaques. Genomic AP-2 clones were isolated from a commercially available human placental DNA library (Stratagene). All hybridizations were performed under stringent conditions at 65°C with final washes in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.01% sodium dodecyl sulfate (SDS) according to standard protocols (41).

Northern (RNA) and Southern blot analyses and probes. Total cellular RNA was isolated by guanidine isothiocyanate lysis and centrifugation through a cesium chloride gradient (6) and selected twice by oligo(dT)-cellulose chromatography; 2.5 μg of poly(A)$^+$ RNA was loaded per lane on 1.2% agarose–formaldehyde gels and blotted onto nylon membranes (Schleicher & Schuell, Keene, N.H.). Equal loading was verified by hybridization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For Southern blot analysis, 5 μg of phage DNA was digested with the appropriate restriction enzymes and transferred to nylon membranes. Random-primed double-stranded DNA probes were labeled to specific activities of between 1×10^9 and 3×10^9 cpm/μg (12).

Probe N is an N-terminal *Eco*RI-*Bam*HI cDNA fragment spanning nucleotides −45 to +363; probe C contains an N-terminal *Pvu*II cDNA fragment from nucleotides +90 to +351; probe cA/B is an *Eco*RI-*Spe*I fragment spanning the entire cDNA region common to AP-2A and AP-2B from nucleotides −45 to +874. Probe A is the C-terminal *Nco*I-*Eco*RI cDNA fragment specific for AP-2A (nucleotides 1223 to 1780), and probe B is the C-terminal *Spe*I-*Eco*RI cDNA fragment specific for AP-2B (nucleotides 874 to 1560 in AP-2B). Probes A and B do not cross-hybridize. Probe R is a 1,250-bp *Pst*I-*Xba*I fragment from the 3′ end of the genomic phage 8 (see Fig. 3A) containing the first exon from −45 to +45 within the cDNA and most of the first intron.

RT-PCRs for RNA detection. Total RNA was isolated as described by Chomczynski and Sacchi (7). Reverse transcription (RT)-polymerase chain reactions (PCRs) were carried out in a total volume of 100 μl. Fifty nanograms of RNA was mixed with 35 U of RNase inhibitor (Pharmacia), and then 50 mM KCl, 10 mM Tris-HCl (pH 8.8 at 25°C), 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.1 mM deoxynucleoside triphosphates were added. Two AP-2B cDNA-specific primers (5′-GCGAATCCACTTGCTAACTAGAAGG-3′ and 5′-ATAACCCTGAGCTTTCAGGATGGTC-3′) and two p53 cDNA-specific primers (5′-CGTCCCAAGCAATGGATG AT-3′ and 5′-TGGGAAGGGACAGAAGAT-3′) (50 pmol of each) were added, and the mixture was heated at 95°C for 2 min. Then 100 U of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, Wis.) was added, and the mixture was incubated at 42°C for 60 min. After the completion of first-strand cDNA synthesis, 5 U of *Taq* DNA polymerase (Promega) was added, and the mixture was subjected to 35 rounds of temperature cycling (94°C for 1 min, 60°C for 2 min, and 72°C for 3 min per cycle). AP-2B cDNA-specific primers are expected to yield a 430-bp fragment, and p53 primers are expected to yield a 193-bp fragment. p53 cDNA-specific primers were used in these assays to monitor the presence of RNA because our earlier experiments showed that the expression of p53 was equal in various PA-1 sublines and not significantly altered after treatment with RA (5).

Transfections and CAT assays. To measure AP-2 transactivator activity, a reporter plasmid was constructed by trimerization of the AP-2 binding site from the distal basal-level enhancer element of the human huMTII$_A$ gene, 5′-AGGAACT GACCG CCCGC GGCCC GTGTG CAGAG-3′, and ligation into the *Sal*I- and *Bam*HI-cut plasmid pBLCAT2 (29). Plasmid 5xTRE$_{col}$TK-CAT (2) was used as a reporter for AP-1 activity. For transient expression of AP-2 in Schneider cells, expression vectors were constructed by cloning the full-length AP-2A and AP-2B cDNAs downstream of the *Drosophila* actin 5C promoter (37a). Transient transfections were performed by using standard calcium phosphate precipitation (18). Chloramphenicol acetyltransferase (CAT) activity was measured by the conversion of [^{14}C]chloramphenicol into acetyl- and diacetylchloramphenicol (17). Equal amounts of protein extracts were used for CAT assays, and reproducibility was ensured by transfection in triplicate. For transient expression of AP-2B, the full-length cDNA was cloned into the vector pSG5 (19). Titrations of the AP-2B expression vector were performed by using a constant total amount of empty vector plus AP-2B expression vector. To obtain stably transfected cell clones, the whole expression cassette containing the SV40 promoter, the AP-2B insert, and the SV40 polyadenylation site was excised as a *Sal*I fragment and ligated into the *Eco*RI site of plasmid pSV$_2$neo. Stable transfections were performed by electroporation at 280 V and 500 μF (apparatus from Bio-Rad, Richmond, Calif.), and selection was started 48 h after electroporation with 200 μg of G418 (GIBCO, Grand Island, N.Y.) per ml.

In vitro translation and purification of recombinant AP-2 proteins. AP-2 mRNAs were transcribed by T7 RNA polymerase from *Sal*I-linearized pSG5-AP-2A and pSG5-AP-2B expression vectors. After DNase I digestion, 1 μg of mRNA was translated for 1 h at 30°C, using nuclease-treated reticulocyte lysates (Promega).

For ion metal affinity chromatography (IMAC), we introduced an N-terminal cassette of six histidines 3′ of the first coding methionine by PCR into both AP-2A and AP-2B. The primers were modified by *Xba*I or *Hin*dIII restriction sites at their respective 5′ ends. The N-terminal oligonucleotide used to modify both AP-2A and AP-2B was a 77-mer (5′-AGGGGCATA TCTAGATAA CGAGGGCAA AAAAT GCAC CATCACCAT CACCATATG CTTTGGAAA TTGA CGGAT AATAT-3′ (the first methionine and the six histidines are underlined). Amplification products were cut with *Xba*I and *Hin*dIII, ligated into the bacterial expression vector pSK40 (43), and fully sequenced to verify correct constructs. *Escherichia coli* cultures were induced at an optical density at 600 nm of 0.8 with 0.2 mM isopropyl-thiogalactopyranoside (IPTG) and grown overnight. Soluble extracts were passed over zinc-chelate Sepharose (Pharmacia) essentially as described previously (52). Pooled elution fractions were concentrated and changed to the appropriate buffer for gel shifts by centrifugation in ultrafiltration units (Amicon, Witten, Germany).

Gel mobility shift assays. Purified AP-2 proteins or nuclear extracts prepared by a standard protocol (10) were incubated with 2.5×10^4 cpm of labeled double-stranded oligonucleotides in 1× GSA buffer (10 mM *N*-2-hydroxyethylpiperazine-

N'-2-ethanesulfonic acid [HEPES; pH 7.9], 2.5 mM dithiothreitol, 50 mM KCl, 6 mM $MgCl_2$, 100 μg of bovine serum albumin [BSA] per ml, 0.01% Nonidet P-40, 10% glycerol) supplemented with 1 μg of BSA and 1 μg of poly(dI-dC) at 30°C for 30 min. The wild-type sequence 5'-AG GAACT GACCG CCCGC GGCCC GTGTG CAGAG-3' or the mutated sequence 5'-AG GAACT GACCG ACCGC TGCCC GTGTG CAGAG-3', as a control, was used. Native gel electrophoresis was performed on 4% polyacrylamide gels (29:1) at 10 V/cm.

In vitro protein binding assay. For expression and purification of AP-2 fused to glutathione *S*-transferase (GST) in *E. coli* (44), glutathione-Sepharose beads were loaded with 20 μg of GST or the GST/AP-2A fusion protein and then incubated with 10 μl of [^{35}S]methionine-labeled, in vitro-translated AP-2A or AP-2B protein for 2.5 h at 4°C. The beads were washed five times with a buffer containing 25 mM HEPES (pH 7.9), 25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.5% Nonidet P-40. Bound proteins were released from the beads by boiling in SDS loading buffer and electrophoresed on a 10% polyacrylamide gel.

Cell lines and tissue cultures. PA-1 human teratocarcinoma cells were derived from a female ovarian germ cell tumor (59) and are available from the American Type Culture Collection (CRL 1572). Origins and properties of all subclones used in this study have been described previously (4, 48). Briefly, clone 1 and 9117 cells are highly sensitive to RA-induced cell differentiation, whereas clone 6928 is a highly RA-resistant N-*ras*-transformed derivative of clone 1. In contrast to 9117 and 6928 tumor cell lines, clone 1 cells were derived from early-passage PA-1 cells and are nontumorigenic in nude mice. Clone 1 cells are easily transformed by activated oncogenes, such as *ras* and *myc*, to a tumorigenic and highly RA-resistant phenotype. Therefore, PA-1 cells provide a human cell system with which to test the effect of gene expression on cell differentiation and tumorigenicity.

All cells were cultured in modified Eagle's medium supplemented with 7.5% fetal bovine serum. To obtain growth curves, 10^5 cells were seeded into 60-mm-diameter dishes and treated for 10 days with 10 μM RA (all-*trans*; Sigma, St. Louis, Mo.), and cell numbers of duplicate experiments were compared with those of untreated replicas. Anchorage-independent growth was measured by mixing 10^4 cells with 0.35% agarose. The suspension was placed over a layer of 0.7% agarose, and colonies greater than 45 μm in diameter were counted after incubation for 10 days.

Nucleotide sequence accession number. The GenBank accession number of the AP-2B cDNA sequence reported in this paper is M61156.

RESULTS

A differentially spliced AP-2 mRNA is expressed in PA-1 human teratocarcinoma cells. Northern blot analysis of PA-1 human teratocarcinoma cells reveals several distinct mRNAs that hybridize with N-terminal AP-2 cDNA probes. Because many transcription factors have been shown to belong to gene families with members differing in important effector domains (for a recent review, see reference 14), we investigated the possibility that these mRNAs code for multiple AP-2 proteins differing in the ability to activate transcription. To do so, we constructed a cDNA library from an N-*ras* oncogene-transformed cell clone (clone 6928) that was derived from the human teratocarcinoma cell line PA-1 (4, 47, 48, 50). We chose this subclone because N-*ras*-transformed PA-1 cells express approximately sixfold-higher levels of AP-2 mRNA than do PA-1 cells that are not transformed by N-*ras* (data not shown).

A 261-bp *Pvu*II fragment located within the N-terminal cDNA (referred to as probe C) was used to screen 10^6 recombinant cDNA phage. Among eight positive plaques, two independent positive phage were found to contain full-length cDNAs, as judged from N-terminal sequences. One encodes for a 437-amino-acid protein, which we refer to as AP-2A, that perfectly matches the sequence previously determined by Williams et al. (54). The second cDNA, coding for a 365-amino-acid protein, AP-2B, is identical in its first 295 amino acids and respective nucleotides and differs from AP-2A only in the remaining 70 C-terminal amino acids (Fig. 1). Northern blot analyses (Fig. 2A) revealed that both AP-2A and AP-2B mRNAs are transcribed in PA-1 cells. The same common N-terminal cDNA fragment that we had used to screen the cDNA library (probe C) hybridized to five different mRNAs: two that were recognized by a C-terminal fragment specific to AP-2A (probe A) and three that were recognized by a C-terminal fragment specific to AP-2B (probe B). Interestingly, a sixth, approximately 1-kb mRNA was observed only after hybridization with probe A.

Because transcriptional regulation of AP-2 mRNA expression has been described in human teratocarcinoma cells during induction of cell differentiation with RA (30), we analyzed the expression of AP-2A and AP-2B mRNAs in clone 1 and 9117 cells, two PA-1 cell subclones that are sensitive to the effect of RA. Morphological, biochemical, and growth- and gene-regulatory parameters that are regulated by RA have been described previously for these and other PA-1 cell subclones (4, 28, 47, 48, 50). As observed in other cell lines, AP-2 mRNAs were expressed in PA-1 cells at low levels. AP-2A mRNAs were detected by Northern blot hybridization of twice-poly(A)$^+$-selected RNA after longer exposures. We used quantitative RT-PCR to measure AP-2B mRNAs. As shown in Fig. 2B and C, both AP-2A and AP-2B mRNAs were transiently upregulated during RA-induced cell differentiation, with maximum expression occurring between 8 and 24 h, and downregulated approximately to basal levels at 120 h. Results from PCRs of AP-2A mRNA expression were in good agreement (data not shown) with the Northern blot data of Fig. 2B. Therefore, we concluded that there was no significant difference in the temporal pattern of induction by RA between AP-2A and AP-2B mRNA.

AP-2B mRNA is not only found in PA-1 cells. We have also detected AP-2B by RT-PCR in RNA from HeLa cells and at low levels in the human prostate carcinoma cell line LnCAP (Fig. 2D). In developing mouse embryos AP-2B mRNA was detected at 15 to 16 days of gestation by Northern blot analysis using probe B (Fig. 2E).

Because Southern blot analysis of human genomic DNA had indicated the presence of a single AP-2 gene (data not shown) and AP-2A and AP-2B cDNA sequences diverge downstream of the AGG at positions 881 to 883 in AP-2A, compatible with spliced exon borders (Fig. 1), we hypothesized that AP-2A and AP-2B represent differentially spliced transcripts from the same gene. To test this hypothesis, we isolated and analyzed genomic clones spanning the AP-2 gene locus. We screened 5×10^5 recombinant phage of a genomic library from human placenta DNA (Stratagene) with an N-terminal cDNA fragment that contains the first 363 coding nucleotides of both AP-2A and AP-2B cDNAs (probe N; see Fig. 3A for a graphic summary of all probes). Four independent overlapping phage were isolated, and

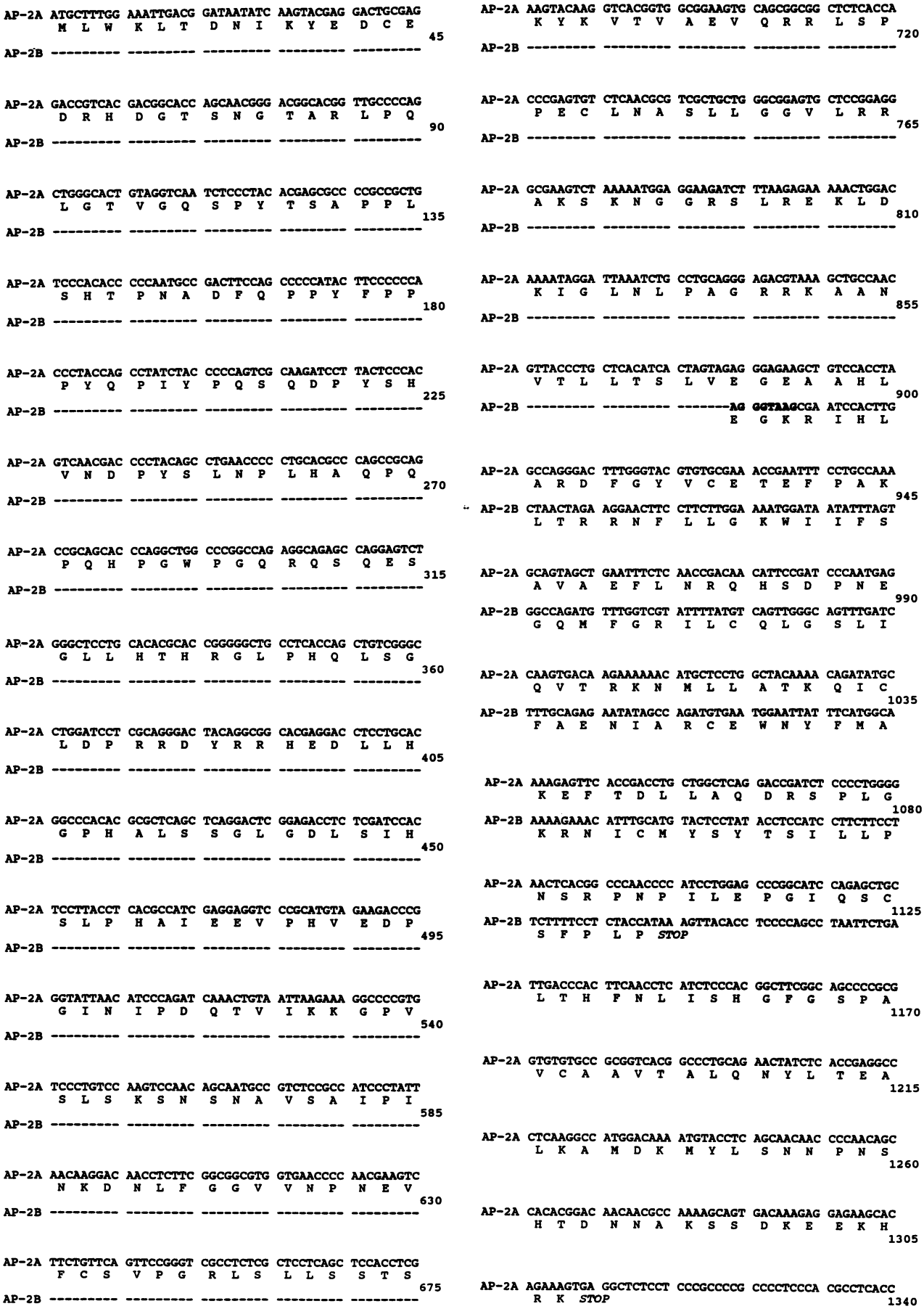
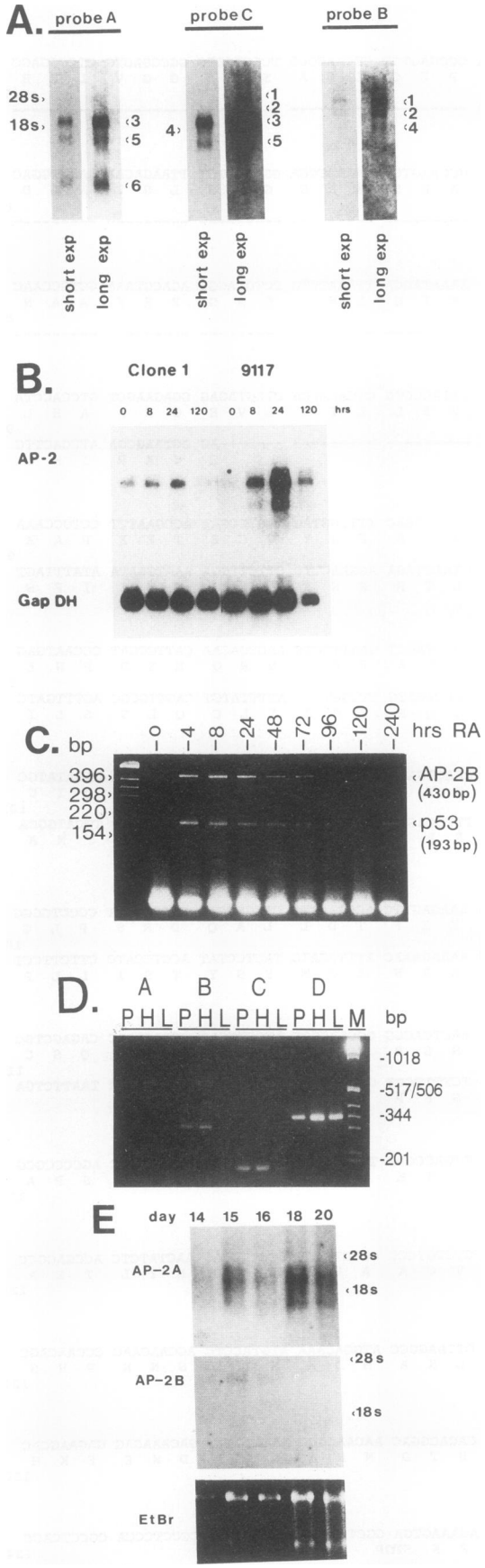


FIG. 1. Nucleotide and amino acid sequences of AP-2A and AP-2B. Dashes in AP-2B indicate identical N-terminal residues. Boldface nucleotides 881 to 888 mark a 5′ splice donor site.



restriction mapping was performed with probes A, B, N, cA/B, which spans the common cDNA sequences, and R, which hybridizes to the first exon as well as to the first intron. As demonstrated in Fig. 3, an exon coding for the C terminus of AP-2B is located next to the last N-terminal exon in common to both AP-2A and AP-2B on the same *Xba*I fragment. The C terminus of AP-2A is encoded by two exons located approximately 5 kb downstream from exon B on two different *Xba*I fragments. Sequencing of the two genomic *Xba*I fragments which contain the C-terminal exons A and B, respectively, revealed that AP-2B is an unspliced transcript, whereas AP-2A mRNA is generated by a splicing event between nucleotides 882 and 883 of the coding sequence. Nucleotides 881 to 888 in the AP-2B cDNA represent a consensus splice site in the genomic sequence (printed in boldface in Fig. 1). RT-PCR of PA-1 cell RNA with a primer common to the AP-2A and AP-2B sequences beginning at nucleotide 746 yielded a product consistent with the predicted AP-2B sequence, thus showing the presence of the AP-2B mRNA by an alternate method (Fig. 2D). The lanes marked A in Fig. 2D show a PCR product formed over a splice junction removing an intron of 1.9 kb. This finding would indicate that the mRNA that codes for the AP-2B protein is not due simply to a failure to splice at the junction at which AP-2A and AP-2B begin to differ but rather that this is a mature, alternative mRNA with substantial processing. Thus, we show by DNA sequence analysis of cDNA clones and genomic DNA clones and by PCR analysis that AP-2B results from an alternative RNA splicing event.

AP-2B is a negative modulator of AP-2 transcription acti-

FIG. 2. Expression of AP-2A and AP-2B mRNAs in PA-1 cells. (A) Northern blots of 6928 cell RNA hybridized to probe C (common to AP-2A and AP-2B cDNAs), probe A (specific for AP-2A cDNA), and probe B (specific for AP-2B cDNA). Each lane contained 2.5 μg of twice-poly(A)$^+$-selected RNA. Blots were exposed for 24 h (short exposure [exp]) and 1 week (long exposure). (B) Northern blots of RNA from clone 1 and 9117 cells treated for 0, 8, 24, and 120 h with 10 μM RA. Twice-poly(A)$^+$-selected RNA (2.5 μg per lane) was hybridized to probe C (common to AP-2A and AP-2B cDNAs) or to GAPDH. (C) Agarose-gel electrophoresis pattern of RT-PCR from 9117 cells. RNA was extracted from cells treated for 0, 4, 8, 24, 48, 72, 96, 120, and 240 h with 10 μM RA, and PCR amplification of AP-2B and p53 cDNA fragments was performed in the same tubes. A smaller 193-bp fragment of p53 and a larger 430-bp fragment of the specific AP-2B C terminus were amplified. (D) Agarose gel electrophoresis pattern of RT-PCR from PA-1 9117 cells treated for 24 h with RA (lanes P), HeLa cells (lanes H), and LnCAP human prostate carcinoma cells (lanes L). The expression by RT-PCR of AP-2B with use of three sets of oligonucleotide primers is shown. In lanes marked A, the 5′ nucleotides of the primer set are at bp 746 and 1318, yielding a 573-bp product. This DNA product spans a 1.9-kb intron from the common region of AP-2A and AP-2B into AP-2B-specific sequences. In lanes marked B, the 5′ nucleotides of the primer set are at bp 766 and 1072, yielding a 307-bp product from region common to AP-2A and AP-2B into AP-2B-specific sequences. In lanes marked C, the 5′ nucleotides of the primer set are at bp 888 and 1072, yielding a 185-bp product. This DNA product is entirely in AP-2B-specific sequences. In lanes marked D is the RT-PCR product of the GAPDH gene; the 5′ nucleotides of the primer set are at bp 29 and 372, yielding a 344-bp product. (E) Northern blots of RNA extracted from total mouse embryos at days 14, 15, 16, 18, and 20. Each lane contained 10 μg of poly(A)$^+$-selected RNA. Top, blot hybridized with AP-2 probe C; middle, blot hybridized with an AP-2B-specific probe, probe B; bottom, ethidium bromide (EtBr) staining of the gel. The blots were exposed for 1 week.

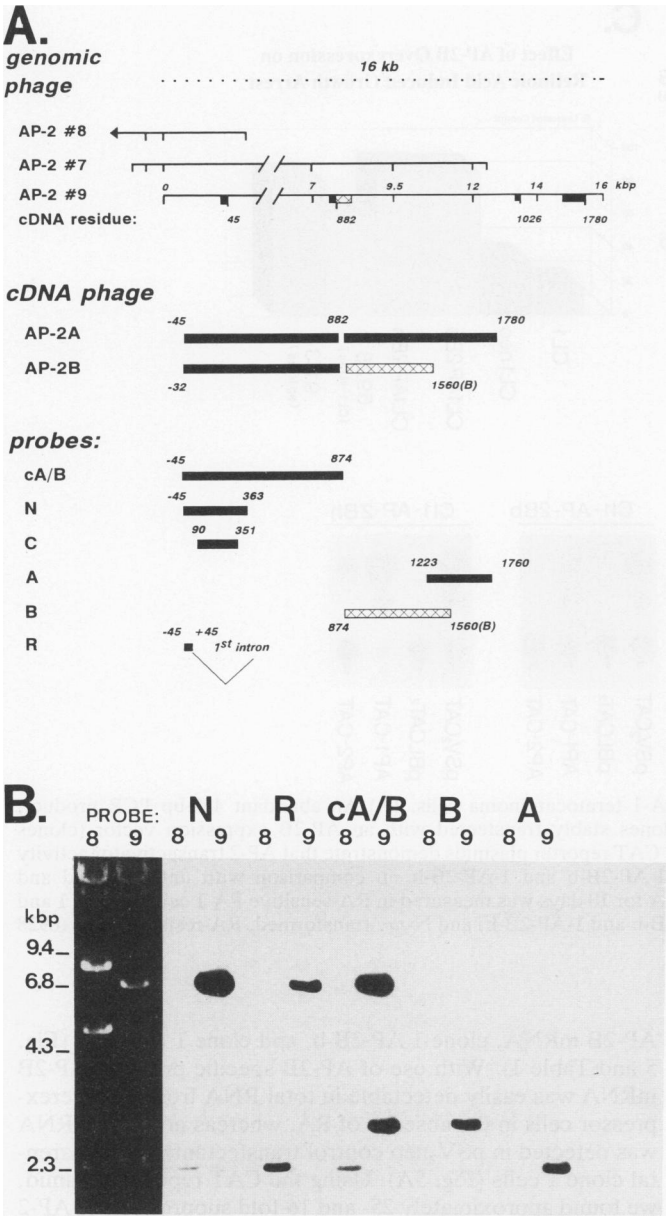


FIG. 3. Genomic organization of the AP-2 gene. (A) Graphic summary of three overlapping genomic DNA phage (AP-2 #8, AP-2 #7, and AP-2 #9) spanning the entire exon sequences of AP-2A and AP-2B cDNAs indicated below. Vertical dashes within the phage inserts indicate *Xba*I sites; the relative distances are indicated above phage AP-2 #9 in kilobase pairs. Dark boxes represent exons specific for AP-2A or in common with AP-2B; the hatched box represents an AP-2B-specific exon. The diagram at the bottom illustrates the locations of all probes relative to the cDNA sequences of AP-2A and AP-2B. (B) Southern blots of *Xba*I-cut DNA from genomic phage AP-2 #8 and AP-2 #9 with various cDNA probes as indicated above the lanes. A C-terminal probe specific to the AP-2B cDNA (probe B) hybridizes to the same *Xba*I fragment as does a probe from the common N-terminal cDNA region (probe c/AB).

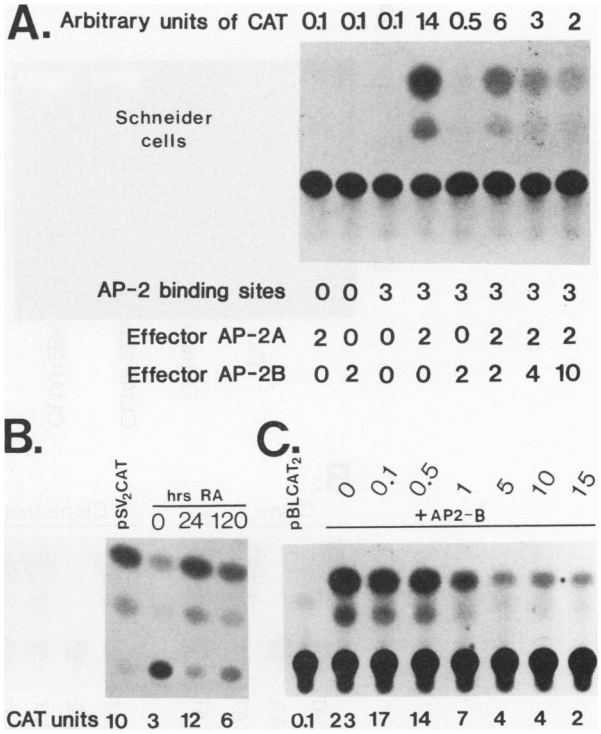


FIG. 4. Effect of AP-2B on transactivation by AP-2. (A) Thin-layer chromatography analyses from CAT assays of Schneider cells transiently transfected with AP-2A and AP-2B expression vectors. An AP-2-sensitive CAT reporter plasmid (see Materials and Methods) was cotransfected to measure the effect on AP-2 transactivation. Micrograms of transfected AP-2A and AP-2B expression vectors are shown at the bottom. (B) Regulation of the endogenous AP-2 activity in 9117 cells by treatment with 10 μM RA. Two micrograms of the AP-2-sensitive CAT reporter was transiently transfected into 9117 cells treated for 0, 24, and 120 h. (C) Inhibition of endogenous AP-2 activity in 9117 cells by transient transfections of an AP-2B expression vector. Amounts of cotransfected AP-2B expression plasmid ranging from 0 to 15 μg are indicated above the lanes.

vator function. To study the biochemical activity of AP-2B, we constructed a CAT reporter plasmid consisting of three oligomerized AP-2 binding sites upstream of the minimal thymidine kinase gene promoter in pBLCAT2 (29). We transfected this reporter together with a constant amount of AP-2A and increasing amounts of AP-2B expression plasmids transiently into Schneider cells, an embryonic *Drosophila* cell line that lacks endogenous AP-2 activity (37). As shown in Fig. 4A, AP-2 binding site-dependent activation of CAT expression by AP-2A was inhibited by AP-2B in a dose-dependent fashion. Expression of AP-2B alone had no effect on the CAT reporter.

The inhibitory activity of AP-2B observed in *Drosophila* cells was also observed upon transient transfection into PA-1 human cells. During RA-induced cell differentiation of 9117 cells, regulation of endogenous AP-2 transactivator function as measured by the CAT reporter (Fig. 4B) corresponded to AP-2A mRNA levels (shown in Fig. 2B). The endogenous AP-2 activity of 9117 cells was suppressed by increasing amounts of an AP-2B expression plasmid (Fig. 4C). Inhibition of CAT expression from the AP-2–CAT vector was not observed when a truncated AP-2 protein corresponding to the region common to AP-2A and AP-2B was transfected (data not shown). From these transfections, we concluded that AP-2B encodes a protein functionally antagonistic to AP-2A.

To further analyze the activity of AP-2B in a mammalian cell system that expresses and regulates endogenous AP-2

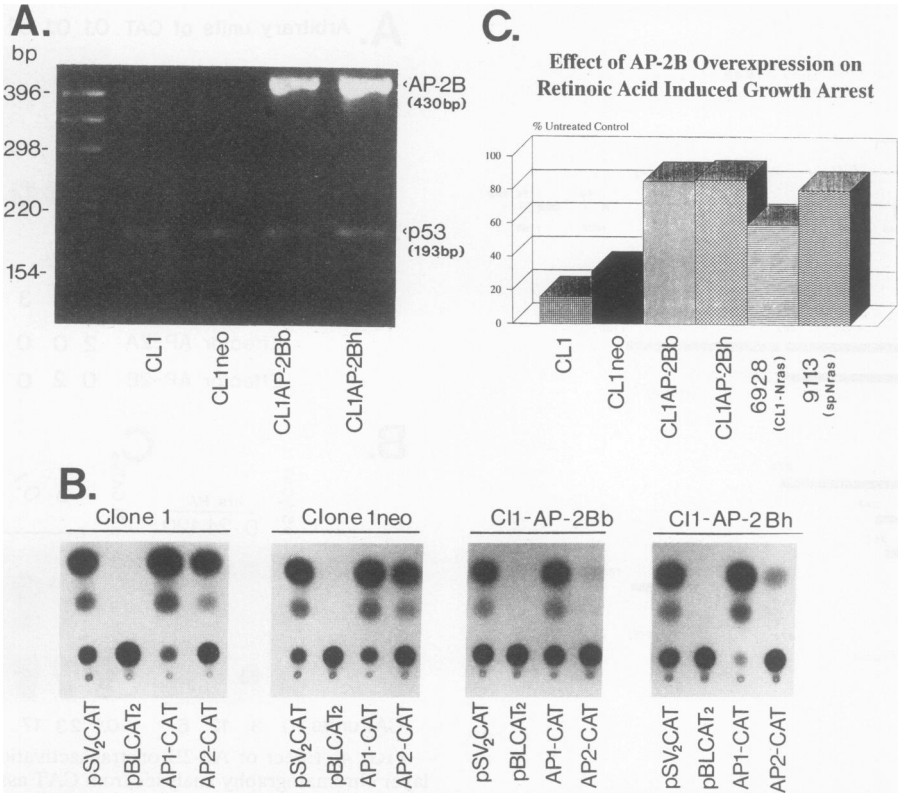


FIG. 5. Effect of constitutive AP-2B expression in RA-sensitive PA-1 teratocarcinoma cells. (A) An abundant 430-bp PCR product, specific for AP-2B mRNA, is amplified in two G418-resistant cell clones stably transfected with an AP-2B expression vector (clones 1-AP-2B-b and 1-AP-2B-h). (B) Transient transfections of AP-1 and AP-2 CAT reporter plasmids demonstrate that AP-2 transactivator activity is severely suppressed in the stably AP-2B-transfected cell clones, 1-AP-2B-b and 1-AP-2B-h, in comparison with untransfected and pSV_2neo-transfected clone 1 cells. (C) Effect of treatment with 10 μM RA for 10 days was measured in RA-sensitive PA-1 cells (clones 1 and 1 neo) in comparison with cells stably expressing AP-2B (clones 1-AP-2B-b and 1-AP-2B-h) and N-*ras*-transformed, RA-resistant cells (6928 and 9113).

during treatment with RA, we generated stable transfectants of clone 1 PA-1 teratocarcinoma cells by using a *neo*-selectable AP-2B expression vector. We chose to transfect clone 1 cells because they are highly responsive to RA-induced cell differentiation and nontumorigenic upon injection into nude mice. In addition, clone 1 cells do not form colonies in soft agar yet are easily transformed by oncogenes into an RA-resistant and anchorage-independent, tumorigenic phenotype (4, 46–49). RT-PCR analysis identified two neomycin-resistant transfectants that stably expressed AP-2B mRNA, clone 1 AP-2B-b, and clone 1 AP-2B-h (Fig. 5 and Table 1). With use of AP-2B-specific primers, AP-2B mRNA was easily detectable in total RNA from the overexpressor cells in the absence of RA, whereas no AP-2 mRNA was detected in pSV_2neo control transfectants or the parental clone 1 cells (Fig. 5A). Using the CAT reporter plasmid, we found approximately 25- and 16-fold suppression of AP-2 transactivation in the AP-2B expressor clones in comparison with untransfected clone 1 PA-1 cells and pSV_2neo-transfected controls (Fig. 5B).

TABLE 1. Effects of constitutive AP-2B expression in clone 1-AP-2B-b and clone 1-AP-2B-h cells on anchorage-independent growth and tumorigenicity

Cell line	Colony formation in soft agar[a]	Tumorigenicity[b]	Latent period (wk)
Clone 1	0	0/3	
Clone 1 neo	0.015	0/3	
6928	4.5	3/3	6
9113	7.5	3/3	6
Clone 1 AP-2B-b	4	1/5	11
Clone 1 AP-2B-h	5	4/5	18, 18, 18, 23

[a] Measured as the percentage of cells forming colonies per 10,000 input cells.
[b] Determined by subcutaneous injection of 3×10^6 cells.

This observation prompted us to determine whether the suppression of endogenous AP-2 activity would affect RA responsiveness of PA-1 teratocarcinoma cells. To address this question, we examined the effect of treatment with 10 μM RA for 10 days on the growth of the two cell clones stably expressing AP-2B. In comparison with untreated controls, 80 and 70% reduction in cell growth was observed in parental clone 1 cells and pSV_2neo transfectants, respectively, equivalent to results for other RA-sensitive PA-1 subclones (4). However, the AP-2B transfectants grew to almost the same cell density with or without RA treatment, similar to N-*ras*-transformed, RA-resistant PA-1 cell subclones 6928 and 9113. The results obtained from growth curves (summarized in Fig. 5C) demonstrate that suppression of endogenous AP-2 transactivator function is associated with cell growth highly resistant to RA.

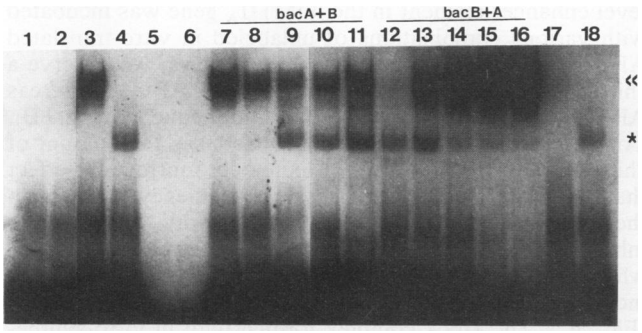


FIG. 6. Gel shift mobilities of recombinant AP-2A and AP-2B proteins. Gel shifts were performed with a wild-type huMTII$_A$ AP-2 binding site (lanes 1 to 4 and 7 to 18) or with a mutated AP-2 site (lanes 5 and 6). Lanes: 1, free oligonucleotide; 2, 10 μg of BSA; 3 and 5, 1 μg of AP-2A; 4 and 6, 1 μg of AP-2B; 7 to 16, mixtures of AP-2A plus AP-2B (in micrograms of protein, 1 plus 0, 1 plus 1, 1 plus 2, 1 plus 3, 1 plus 10, 1 plus 0, 1 plus 1, 1 plus 2, 1 plus 3, and 1 plus 10, respectively); 17, 1 μg of AP-2A plus a 100-fold excess of unlabeled oligonucleotide; 18, 1 μg of AP-2B plus a 100-fold excess of unlabeled oligonucleotide. The asterisk indicates a nonspecific shift of AP-2B that was not competed for by an excess of unlabeled oligonucleotide. « indicates the specific shift of the AP-2–DNA complex.

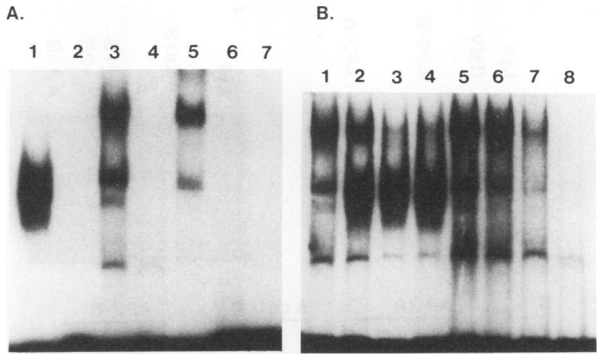


FIG. 7. Effects of recombinant AP-2A and AP-2B proteins on gel-shifted complexes in PA-1 cells. (A) Comparison of gel shift mobilities of recombinant AP-2A (lanes 1 and 2), PA-1 9117 teratocarcinoma cells (lanes 3 and 4), HeLa cells (lanes 5 and 6), and F9 teratocarcinoma cells (lane 7). A 100-fold excess of cold oligonucleotide was added in lanes 2, 4, and 6. (B) Gel shifts resulting from coincubation of PA-1 9117 nuclear extracts with recombinant proteins purified by IMAC. Lanes: 1 to 4, 0, 2, 5, or 10 μg of bacAP-2A added to 9117 nuclear extract; 5 to 8, 0, 0.1, 0.5, or 5 μg of bacAP-2B added to 9117 nuclear extract.

Because all RA-insensitive PA-1 subclones, in particular those that were derived by transformation with oncogenes, exhibit anchorage-independent growth (47, 49), we examined whether the expression of AP-2B in clone 1 cells caused colony formation in soft agar. Table 1 summarizes the results from duplicate experiments and shows that clone 1 cells, consistent with previous results (5), did not exhibit anchorage-independent growth in soft agar. In contrast, the two AP-2B transfectants formed colonies with an efficiency comparable to that of RA-resistant PA-1 subclones that are transformed by an N-*ras* oncogene. In addition, AP-2B expression was able to induce tumorigenicity in clone 1 cells (Table 1). The latent periods for tumor formation, while longer than those of the established N-*ras*-transformed cell lines 6928 and 9113, are similar to that observed when the N-*ras* oncogene was initially transfected in clone 1 cells to derive 6928 cells (48). These results indicate that the failure to respond to differentiation-inducing signals such as RA may be an important factor in the acquisition of tumorigenicity.

AP-2B inhibits AP-2 transactivator function by blocking of sequence-specific DNA binding. To determine the mechanism by which AP-2B negatively modulates AP-2 transactivation, we purified bacterially expressed AP-2A and AP-2B proteins (referred to as bacAP-2A and bacAP-2B) and studied their ability to interact with an AP-2 consensus binding site. AP-2A and AP-2B were modified by an insertion of six histidines after the first methionine to allow purification by IMAC (52) and obtained at approximately 95% purity.

We performed gel shift analysis with bacAP-2A, using oligonucleotides spanning the distal AP-2 binding site in the huMTII$_A$ promoter. We observed a specific shifted complex that could be competed for by an excess of unlabeled oligonucleotides (Fig. 6, lanes 5, 6, 17, and 18), while no shift was observed when the C and G residues at positions 13 and 18 were changed to A and T (lanes 5 and 6). These residues have been shown to be critical for sequence-specific DNA binding by AP-2 (55). A similar mobility shift was obtained with in vitro-translated AP-2A (see Fig. 8B and C) and an unmodified, commercially available AP-2A protein purified from *E. coli* (Promega) (data not shown). Only a nonspecific, shifted complex was observed with bacAP-2B (Fig. 6, lane 4). Incubation of the synthetic AP-2 binding site with bacAP-2A and a 10-fold excess of bacAP-2B did not alter the intensity of the shifted complex compared with incubation with bacAP-2A alone (lanes 7 to 11). These results indicate that AP-2B does not inhibit AP-2 transactivation by occupying the huMTII$_A$ AP-2 site or by a direct protein-protein interaction with the AP-2A protein.

Next we examined a potential interaction of AP-2B with endogenous AP-2 from PA-1 cells. A significant difference in the gel shift pattern enabled us to distinguish between the AP-2 present in nuclear extracts and bacAP-2A (Fig. 7A). Three specific bands with different electrophoretic mobilities were shifted from PA-1 nuclear extracts. A very similar pattern was observed in HeLa cell nuclear extracts (Fig. 7A) except for the absence of the slowest-migrating band. All three bands found in PA-1 cells were specifically competed for by an excess of unlabeled oligonucleotides, as was the band shift from bacAP-2A.

Coincubation of purified bacAP-2A and nuclear extracts from PA-1 cells resulted in competition for binding to the synthetic oligonucleotides. Increasing concentrations of bacAP-2A resulted in increasing amounts of the recombinant-specific band (Fig. 7B, lanes 1 to 4). In contrast, addition of bacAP-2B to nuclear extracts increasingly inhibited DNA binding of the endogenous AP-2 in PA-1 cells, even though AP-2B is incapable by itself of binding the huMTII$_A$ AP-2 site (lanes 5 to 8). To exclude the possibility that this inhibition of DNA binding was an nonspecific effect due to minor protein contamination present in the bacAP-2B, we used IMAC-purified protein fractions from bacteria transformed with an empty pASK40 vector. These fractions, devoid of any AP-2A or AP-2B protein, did not alter the gel shift pattern of PA-1 nuclear extracts (data not shown). Similar experiments were performed to examine the binding of proteins in PA-1 cell extracts to an SP-1 site, and no inhibition of SP-1 sequence-specific binding was observed (data not shown). Therefore, the inhibition activity of AP-2B is specific for AP-2.

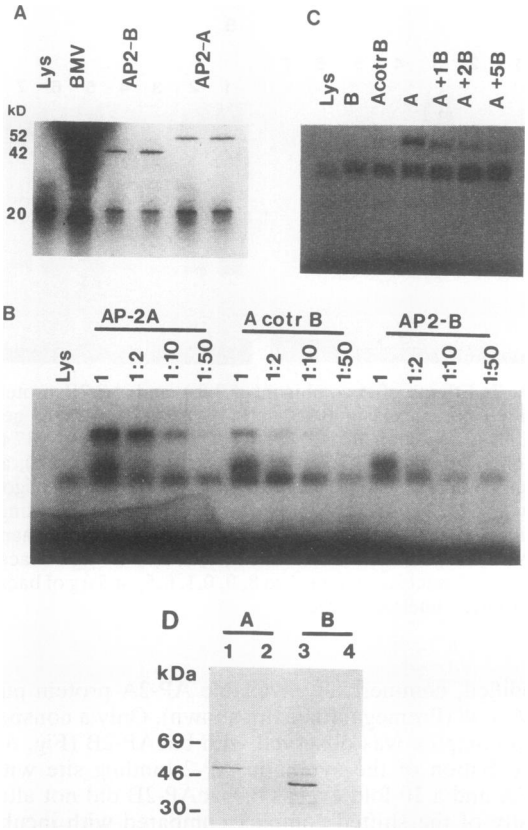


FIG. 8. Evidence that sequence-specific DNA binding by AP-2A is inhibited by AP-2B, using in vitro-translated proteins. (A) In vitro translation. A 52-kDa protein for AP-2A and a 42-kDa protein for AP-2B were translated. Both cDNAs were cloned into the expression vector pSG5 (Stratagene), and 0.5 or 1 μg of T7 transcripts was translated with nuclease-treated rabbit reticulocyte cell lysate (Promega) in the presence of [^{35}S]methionine (A) or unlabeled methionine (B and C). In lane Lys, no RNA was added; in lane BMV, 0.5 μg of RNA from brome mosaic virus was added. Size markers are indicated on the left. (B) Gel shift analysis of in vitro-translated AP-2A and AP-2B and of cotranslated AP-2A and AP-2B (AcotrB). Specific DNA binding was verified by incubation with extract alone and by competition with increasing amounts of unlabeled specific oligonucleotides as indicated above the lanes and absence of competition with nonspecific oligonucleotides (data not shown). (C) Gel retardation analysis of individually translated AP-2A and AP-2B coincubated with specific oligonucleotides. The following amounts of in vitro translation lysate were used: 2 μl of lysate with added RNA, 2 μl of AP-2B, 2 μl of cotranslated AP-2A and -B, 2 μl AP-2A, 2 μl AP-2A plus 2 μl of AP-2B, 2 μl of AP-2A plus 4 μl of AP-2B, and 2 μl of AP-2A plus 10 μl of AP2-B. (D) Specific interaction of in vitro-translated AP-2A and AP-2B with a GST/AP-2A fusion protein. Lanes 1 and 3 were loaded with the GST/AP-2A fusion protein; lanes 2 and 4 were loaded with the unmodified GST protein. Lanes 1 and 2 shows the bound and eluted material after loading of in vitro-translated AP-2A; lanes 3 and 4 shows the bound and eluted material after loading of in vitro-translated AP-2B.

That the mechanism of inhibition of AP-2A by AP-2B occurs by interfering with DNA binding was confirmed with in vitro translation experiments. Figure 8A shows that in vitro translation of AP-2A and AP-2B by in vitro-transcribed RNA produces [^{35}S]methionine-labeled proteins of the correct size by polyacrylamide gel electrophoresis analysis. The ^{32}P-labeled synthetic AP-2 site spanning the distal basal-level enhancer element in the huMTII$_A$ gene was incubated with various combinations of unlabeled in vitro-translated AP-2A and AP-2B. In gel retardation assays, we observe a shifted complex with in vitro-translated AP-2A, whereas AP-2B does not bind this oligonucleotide sequence (Fig. 8B). Cotranslation of AP-2A and AP-2B reduces the amount of shifted oligonucleotide by approximately fourfold, an effect that is not due to a reduced translation efficiency of AP-2A in the presence of AP-2B (data not shown). Slightly less inhibition of DNA binding was observed by gel shift analysis when AP-2A and AP-2B were translated separately and then incubated with the oligonucleotide (ca. twofold inhibition) (Fig. 8C). The DNA complex formed with in vitro-synthesized AP-2 can be effectively competed for with an unlabeled binding-site oligonucleotide. In addition, AP-2A expressed as a bacterial fusion protein with GST and bound to glutathione-Sepharose beads is able to bind to in vitro-synthesized, [^{35}S]methionine-labeled AP-2A and AP-2B (Fig. 8D). The interaction with AP-2A is similar to the homodimerization previously described (56), and the interaction with AP-2B is heterodimerization which we presume leads to inhibition of DNA binding. This heterodimerization probably occurs through a different mechanism than does the AP-2A homodimerization because the dimerization domain identified by Williams and Tjian (56) is not present in AP-2B. Consistent with these experiments, we have noted a decrease in one of the DNA-protein complexes formed with a labeled AP-2 site in gel shifts of clone 1 cells stably overexpressing AP-2B (data not shown).

In summary, these data demonstrate that AP-2B inhibits AP-2 transactivation via inhibition of sequence-specific DNA binding, even though no direct interaction between bacAP-2A and bacAP-2B was observed in vitro. It appears that inhibition of AP-2 by AP-2B requires an additional factor or modification that can be provided by nuclear extracts and rabbit reticulocyte extracts.

DISCUSSION

Differential splicing within the AP-2 gene results in proteins that differ in transactivational properties. Transcriptional regulation of gene expression by AP-2 plays an important role in a variety of embryonic and adult cell differentiation processes, such as neural tube formation, limb bud formation (32), and skin differentiation (26, 45). Thus, AP-2 is the target of regulation through a number of diverse signal transduction pathways. Activation of AP-2 on transcriptional and posttranslational levels can be elicited by signal molecules such as RA, cyclic AMP, and phorbol esters (30). However, little is known about factors that downregulate AP-2 function. Here, we describe for the first time a negative modulator of AP-2 transactivator function. This protein, AP-2B, is generated by differential splicing from the same gene as is the transcription activator AP-2A. AP-2B contains the activation domain of AP-2 and part of the DNA binding domain but lacks the dimerization domain necessary for DNA binding.

Several lines of evidence establish the repressor function of AP-2B. In Schneider cells, which are devoid of endogenous AP-2, expression of AP-2B directly antagonizes the activation of transcription by AP-2A (Fig. 4A). In addition, both transient and stable expression of AP-2B in PA-1 human teratocarcinoma cells suppress the endogenous AP-2 activity (Fig. 4B and 5B).

To prove that AP-2A and AP-2B are derived by differential splicing, we have cloned the entire genomic AP-2 locus (Fig.

3). Five exons code for the N-terminal protein domains common to AP-2A and AP-2B. One exon codes for the end of the region common to AP-2A and AP-2B and the C terminus of AP-2B, whereas the C terminus of AP-2A is located on two exons at least 5 kbp further downstream. Thus, AP-2A and AP-2B are generated by alternative usage of a splice donor site. Differential splicing of transcription factors resulting in proteins with antagonistic functions has been found as a gene-regulatory mechanism in other systems as well (3, 13, 21, 34, 35, 39, 40, 51, 57, 58).

AP-2 activity is a necessary effector of RA-induced cell differentiation and growth control. Several lines of evidence, such as the analysis of enhancer elements and tissue-specific expression during development, point to a key regulatory role for AP-2 in RA-induced cell differentiation. Our results from the analysis of AP-2 expression during differentiation of PA-1 cells further strengthen this evidence. We observed a transient induction of AP-2 mRNAs and transcriptional activity during treatment with RA, which agrees with experiments using N-TERA-2 cells (30).

Because the endogenous AP-2 activity was significantly suppressed in two independent cell clones stably expressing AP-2B mRNA, we were able to study the consequences of repression of AP-2 transactivator function within an RA-sensitive cell line. As demonstrated in Fig. 5 and Table 1, suppression of AP-2 activity in these cell clones was associated with an RA-resistant phenotype and conferred anchorage-independent growth in soft agar and tumorigenicity. This effect is not due to unspecific squelching of the transcriptional machinery because transactivation of an AP-1 CAT reporter used as an internal control remained unchanged (Fig. 5B).

During the past few years, considerable progress has been made in understanding tumorigenesis as a multistep process. The malignant phenotype of cancer cells is associated with changes in many biological properties. Among the features commonly lost in cancer cells is the ability to differentiate and regulate growth appropriately in response to environmental signals. Since we show that the constitutive suppression of endogenous AP-2 transactivator function may abolish the ability of cells to respond to a growth- and differentiation-regulatory signal, it will be interesting to analyze human tumors with activated *ras* oncogenes and investigate the possibility that a loss of AP-2 regulation has a function in tumorigenesis.

In vitro, such a connection between malignant transformation and AP-2 has been established by the observation that the SV40 large T oncoprotein physically interacts with and prevents DNA binding of AP-2 (33). Inhibition of AP-2A binding to DNA by enhanced activity of AP-2B in human tumors might have the same effect as does inhibition by SV40 T antigen. In addition, we have found that N-*ras* transformation of PA-1 cells also results in an inhibition of AP-2 activity, albeit by a different mechanism (24a).

The mechanism of inhibition by AP-2B. The generation of alternative, antagonistic transcription regulators from the same gene seems to be a characteristic of a number of genes. Alternatively spliced *erb*A proteins differ in their ligand binding domains (25), and alternatively spliced *fos*B and CREM proteins differ in their transactivation domains (13, 34, 35). Modification of sequence-specific DNA binding by alternatively spliced DNA recognition domains has been recently identified for different *wt*1 (Wilms' tumor gene) transcripts (3). Variation in the number of zinc fingers due to developmentally regulated alternative splicing alters the binding site specificity of the *Drosophila* transcription factor CF2 (16, 22). An alternative mechanism to vary the primary structure of a transcription factor occurs in an intronless gene, the liver-specific transcription factor LAP (9), whose structure varies as a result of alternative usage of translation initiation sites.

In the case of AP-2B, alternative usage of a splice donor site eliminates the C terminus of AP-2A, an effector domain responsible for sequence-specific DNA binding and homodimerization (55, 56). As expected, both bacAP-2B and in vitro-translated AP-2B proteins which lack this dimerization domain do not bind to a synthetic AP-2 binding site and cannot be cross-linked with AP-2A by glutaraldehyde (data not shown). In addition, IMAC-purified bacAP-2A and bacAP-2B do not interact directly, as assessed by gel mobility shift assays (Fig. 6). The purified bacAP-2B protein, however, inhibited DNA binding of the endogenous AP-2 present in PA-1 cells. In vitro-translated AP-2B can inhibit the DNA binding of in vitro-translated AP-2A, and these in vitro-synthesized proteins interact, as demonstrated by binding of AP-2B to the GST/AP-2A fusion protein (Fig. 8). Thus, we clearly demonstrate that AP-2B functions by inhibiting the endogenous AP-2A from DNA binding and that the interaction of the two proteins occurs via a mechanism conserved in *Drosophila* cells, rabbit reticulocyte extracts, human cells, and human cell extracts.

Currently, we can only speculate on the reason for this difference in interaction with AP-2B between the recombinant AP-2A and the endogenous AP-2 found in cells. The significant difference in the gel shift pattern points to a difference in their physical DNA binding properties. This difference could be the result of a posttranslational modification of the endogenous AP-2 proteins or the formation of a complex with other proteins that may also be required for the interaction with AP-2B. Whatever mechanism mediates their interaction, it appears to be conserved from *Drosophila* to human species. The interaction between AP-2A and AP-2B may involve a protein analogous to the transcriptional activation intermediary proteins known to mediate squelching of transcription (38) or some conserved protein that interacts with the general transcriptional machinery. It will be necessary to characterize thoroughly the endogenous AP-2 transactivator function and its protein-protein interactions to understand fully the mechanism by which multiple signal transduction pathways modulate the transcriptional activity of AP-2.

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