Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements

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Human AP-2 is a sequence-specific DNA-binding protein that interacts with inducible viral and cellular enhancer elements to stimulate transcription of selected genes. Here, we report the isolation and characterization of a human cDNA clone containing the entire protein-coding region of AP-2. The deduced primary amino acid sequence of AP-2 does not contain a domain resembling any previously identified DNA binding motif. However, an interesting feature of the AP-2 protein is a clustered arrangement of proline and glutamine residues that have been found recently within the activation domains of other transcription factors. Expression of the AP-2 clone in bacteria yields a protein that binds to DNA and activates transcription in vitro in a comparable manner to native human AP-2. Transfection of cDNA clones into *Drosophila* cells indicates that the AP-2 gene product can also activate gene expression in vivo in a DNA template-dependent manner. Expression of endogenous AP-2 is repressed in a hepatoma cell line and stimulated following retinoic-acid-induced differentiation of a human teratocarcinoma cell line. This indicates that AP-2 may be a transcription factor involved in the control of developmentally regulated gene expression.

[**Key Words:** Enhancer factor; in vitro and in vivo transcription; DNA binding; teratocarcinoma differentiation; *Drosophila* expression system]

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The coordinated control of eukaryotic gene expression requires the combined action of multiple DNA-binding proteins that modulate the frequency of transcriptional initiation by interacting with specific DNA motifs present in promoter and enhancer elements. The expression or activity of these sequence-specific transcription factors may be regulated in a cell-type, tissue-specific, or cell-cycle-dependent manner (Fletcher et al. 1987; Scheidereit et al. 1987; Monaci et al. 1988; Ryseck et al. 1988; for review, see Cereghini et al. 1987; Maniatis et al. 1987; Staudt et al. 1986). In addition, some transcription factors may act as the nuclear targets for signal transduction in response to extracellular messengers (Prywes and Roeder 1986; Sen and Baltimore 1986; Angel et al. 1987; Franza et al. 1988; Hyman et al. 1988; Imagawa et al. 1987; Lee et al. 1987a; Baeuerle and Baltimore 1988; Bohmann et al. 1988; Rauscher et al. 1988). The activity of transcription factors may also be modulated by interactions with other proteins involved in the regulation of gene expression (Olesen et al. 1985; Mitchell et al. 1987; Bell et al. 1988; Chodosh et al. 1988; Comb et al. 1988; Mermod et al. 1988). Therefore, alterations in the combination of transcription factors associated with a particular gene can elicit many different patterns of gene expression. In recent years, several sequence-specific DNA-binding transcription factors have been purified from eukaryotic cells and

have been characterized biochemically. One major challenge has been to determine the mechanisms by which these factors can act, either alone or in concert, to control transcriptional activation.

The enhancer-binding protein AP-2 represents an inducible transcription factor that may be involved in the process of signal transduction and cell-specific expression. AP-2 purified from HeLa cells by DNA-affinity chromatography is a 52-kD protein that specifically interacts with the consensus sequence 5'-CCCCAGGC-3' (Mitchell et al. 1987). This *cis*-acting DNA motif is found in a variety of viral and cellular transcriptional regulatory regions, including the enhancers of SV40 and human metallothionein IIa (hMtIIa) (Haslinger and Karin 1985; Lee et al. 1987b; Mitchell et al. 1987). Interestingly, transcriptional activation by AP-2 may be subject to both positive and negative regulatory mechanisms. In the case of positive control, the AP-2-binding site can act independently as both a TPA- and cAMP-inducible element (Imagawa et al. 1987; Comb et al. 1988; Hyman et al. 1988). Therefore, AP-2 may play a fundamental role in altering the pattern of gene expression within a cell in response to these two distinct signal transduction pathways.

One unusual and interesting aspect of AP-2 is its interaction with other sequence-specific regulatory proteins. In particular, it has been shown that the DNA-

binding activity of AP-2 can be inhibited specifically by SV40 large T antigen, a viral gene product involved in regulating gene expression. Biochemical analysis indicates that protein-protein interaction between AP-2 and large T antigen abolishes the ability of AP-2 to bind DNA and activate transcription *in vitro* (Mitchell et al. 1987). This inhibition of AP-2 function is thought to play a part in the negative autoregulation of SV40 early transcription by the early gene product, large T antigen. It is also possible that the global changes in gene expression associated with large T antigen-induced cellular transformation might be mediated by the sequestration of transcription factors such as AP-2 by large T antigen. Interestingly, the activity of AP-2 also appears to be regulated in a cell-type-specific manner. In contrast to HeLa cells, the human liver hepatoma cell line HepG2 does not contain any detectable AP-2-binding and transcriptional activity (Imagawa et al. 1987). However, it remains to be determined whether the apparent absence of functional AP-2 in HepG2 cells is due to regulation of the gene by transcriptional, translational, and/or post-translational mechanisms.

A thorough understanding of these regulatory processes is severely hampered by the low abundance of transcription factors such as AP-2 in animal cells. Therefore, the most direct and useful approach to circumvent the intrinsic difficulties of evaluating the biochemical properties of these transcription factors is to isolate the genes encoding the proteins. First, this enables the development of reagents, including anti-peptide antibodies, which can be used to investigate their structure, function, and regulation. Second, expression of the cloned factors helps elucidate the role of these sequence-specific DNA-binding proteins in the activation of transcription (Giguère et al. 1986; Bohmann et al. 1987; Kadonaga et al. 1987; Landschulz et al. 1988; Miesfeld et al. 1986; Rusconi and Yamamoto 1987; Courey and Tjian 1988; Hope et al. 1988; Santoro et al. 1988). In addition, a mutational analysis of the cloned factors facilitates the identification of protein domains important for transcriptional activation, DNA binding, and protein-protein interactions (Keegan et al. 1986; Kadonaga et al. 1987; Miesfeld et al. 1987; Courey and Tjian 1988; Evans and Hollenberg 1988; Kadonaga et al. 1988; Struhl 1988; Webster et al. 1988).

Here, we report the molecular cloning of cDNAs corresponding to human AP-2 and the expression of the full-length protein in both prokaryotic and eukaryotic cells. An analysis of the AP-2 gene should enable us to dissect the various mechanisms controlling AP-2-dependent transcriptional activation.

Results

Isolation of AP-2 cDNAs

A reliable method of isolating cDNA clones encoding DNA-binding transcription factors is to obtain peptide sequence from the purified protein, which can then be used to derive oligonucleotide probes for screening cDNA libraries (Kadonaga et al. 1987; Santoro et al.

1988). This method has the advantage that the identity of any isolated cDNA clones can be confirmed directly by a comparison of the actual peptide sequence and the predicted amino acid sequence. Peptide sequence from affinity-purified 52-kD HeLa AP-2 was obtained using several different techniques (see Materials and methods). First, the amino-terminal amino acid sequence of AP-2 was determined from the full-length protein (peptide 1; Fig. 1B). The sequence of peptides 2–6 was obtained following digestion of AP-2 with trypsin. Finally, treatment of AP-2 with 4-vinylpyridine under reducing conditions to alkylate the cysteine residues, followed by cyanogen bromide (CNBr) cleavage, yielded the sequence of peptide 7.

A 45-nucleotide synthetic oligomer corresponding to the deduced coding sequence of the overlapping AP-2 peptides 6 and 7 was selected as a specific DNA probe for screening a HeLa cDNA library. Three independent cDNA clones were isolated from 10^6 λ gt10 phage plaques (Fig. 1A). The DNA sequence of the longest AP-2 cDNA clone, termed AP2-9, is illustrated in Figure 1B. Analysis of the predicted amino acid sequence of AP2-9 reveals a long open reading frame of 1.3 kb, beginning at nucleotide 63 and ending with a stop codon at nucleotide 1376. This open reading frame begins with the amino-terminal sequence corresponding to peptide 1 of AP-2 and also contains the other six peptides in the same reading frame, confirming the authenticity of the AP-2 cDNA clone. Translation of this 437-amino-acid reading frame would theoretically yield a protein of 48 kD, which agrees well with the estimated size of HeLa AP-2 from SDS-PAGE of 52 kD (Mitchell et al. 1987). Thus, the AP-2 cDNA appears to contain the entire coding region of AP-2.

Restriction enzyme mapping and DNA sequence analysis of other AP-2 cDNA isolates indicates that the 1.6-kb AP2-9 insert does not represent a full-length cDNA but is truncated at both its 5' and 3' ends (Fig. 1; T. Williams and B. Lüscher, unpubl.). Indeed, Northern blot analysis using the AP-2 cDNA reveals a major broad band of mRNA in HeLa cells of ~3.3–3.6 kb (Fig. 2A). This diffuse distribution of AP-2 mRNA has been observed with several different preparations of RNA and is in marked contrast to the tight bands observed when the same Northern blots are reprobbed with sequences corresponding to CTF/NF1 (Santoro et al. 1988; N. Tanese, pers. comm.). The heterogeneous distribution of AP-2 mRNA species suggests that alternatively processed forms of the AP-2 mRNA may exist in HeLa cells. Southern blot analysis of human genomic DNA indicates that these potentially different species of AP-2 mRNA are derived from a single copy of the AP-2 sequence in the human genome (data not shown).

Differential expression of AP-2 in HepG2 and teratocarcinoma cells

We have also assayed several human cell lines by Northern blotting and RNase protection analysis to determine whether the level of AP-2 mRNA was regulated in a cell-type-specific or inducible manner. The data in-

Figure 1. DNA and amino acid sequence of HeLa AP-2. (A) Schematic representation of the three partial AP-2 cDNA clones. The boxed region of AP2-9 denotes the long open reading frame starting from the ATG at nucleotide 63 to a TGA at nucleotide 1376. The location of the proline-rich stretch and the α -helical region of the predicted AP-2 protein are indicated by hatching and gray shading, respectively. The extent of the other two cDNA clones is indicated below. (B) The entire nucleotide sequence and the deduced amino acid sequence of the AP2-9 cDNA. The amino acid sequences enclosed by boxes (designated peptides 1–7) indicate the seven peptides obtained from analysis of the HeLa AP-2 protein. (*) The deduced stop codon for translation.

indicate that although the control Sp1 RNAs are relatively invariant in different cell types before or after induction, the AP-2 mRNA is absent from the human liver hepatoma cell line, HepG2 (Fig. 2). This result indicates that the expression of the AP-2 gene may be controlled in a cell-type-specific fashion. In addition, the absence of AP-2 mRNA in HepG2 cells explains the lack of AP-2 DNA-binding and transcriptional activity in these cells (Imagawa et al. 1987). Interestingly, the expression of AP-2 is also regulated during differentiation. Treatment of the human teratocarcinoma cell line NT2 with retinoic acid, which causes the differentiation of these cells

(Andrews 1984), appears to trigger at least a 5- to 10-fold increase in the levels of AP-2 mRNA (Fig. 2). These findings indicate that the expression of AP-2 is inducible and regulated in a cell-type-specific manner.

Expression of AP-2 in bacteria

It was important to establish that the cDNA clones we obtained encode a functional AP-2 protein. Therefore, we expressed the AP-2 cDNA in bacteria and purified the resulting protein product to determine whether it was capable of binding to DNA and activating transcription in vitro. For this purpose, the entire open reading

Figure 2. Tissue-specific and inducible expression of AP-2 mRNA. (A) A Northern blot of total cellular RNA from HeLa, HepG2, NT2, and retinoic acid-differentiated NT2 cells, as indicated above each track. The blot was probed with an oligo-labeled fragment of AP2-9 extending from the *Eco*RI site at nucleotide 1 to the *Nco*I site at nucleotide 1289. (Left) The positions of the 28S and 18S rRNA species. (B) RNase protection analysis of total RNA from HepG2 and HeLa cells using a mixture of AP-2 and Sp1 probes (341 and 270 bp, respectively). The positions of the protected products are indicated by arrows. The two products seen with the Sp1 probe correspond to two different forms of the Sp1 RNA. (C) RNase protection analysis of cytoplasmic RNA from NT2 cells that had been cultivated for 48 hr in the presence (+ RA) or absence (– RA) of retinoic acid. The RNA probes are the same as in B.

frame (ORF) of AP2-9, beginning at the ATG determined from amino-terminal peptide sequencing to be the translational initiation site, was engineered into the *Escherichia coli* expression vector pKK233-2. The resulting plasmid, termed pAP2L, should express AP-2 as a full-length nonfused protein upon induction of the *trp*–*lac* fusion promoter (Fig. 3). A novel polypeptide corresponding to expression of AP-2 is not visible in crude bacterial lysates. However, following DNA affinity chromatography, a 52-kD protein comigrating with HeLa AP-2 is seen when the pAP2L expression construct is present, whereas this protein is absent from bacteria containing the parental expression vector alone (Fig. 3). This first-pass affinity-purified bacterial AP-2 could be purified further to near homogeneity by a second cycle of AP-2 affinity chromatography (Fig. 3).

The DNA-binding specificity of this *E. coli*-expressed

AP-2 was tested by DNase I protection assays using SV40 and hMtIIa enhancer/promoter templates (Fig. 4). The *E. coli*-expressed protein recognizes the same binding sites in the SV40 intergenic control region and hMtIIa basal level elements (BLEs) as native HeLa AP-2. In addition, *E. coli*-expressed AP-2 and HeLa AP-2 are indistinguishable in their DNase I footprinting patterns and relative affinities for the various enhancer and promoter binding sites. These DNase I protection experiments verify that the AP2-9 cDNA clone corresponds to the HeLa sequence-specific DNA-binding protein AP-2.

Bacterially expressed AP-2 activates transcription in vitro

Previous experiments had indicated that HeLa AP-2 not only bound sequence specifically to DNA but also could

Figure 3. Purification of bacterially expressed AP-2 protein. (Bottom) A schematic representation of the AP-2 bacterial expression vector, pAP2L. The hatching indicates the inducible *trp-lac* fusion promoter (P_{trc}) of the parental pKK233-2 plasmid, with the arrow denoting the direction and start site of transcription. The open box represents the AP-2 open reading frame, beginning with the ATG at nucleotide 63 and ending with the TGA at nucleotide 1376. The solid box indicates the 3'-untranslated region of the AP2-9 cDNA. (R1) The *Eco*RI site marking the 3' terminus of the AP2-9 cDNA; (H3) the *Hind*III site used for cloning. All nucleotide numbers are given with respect to the sequence of AP2-9. (Top) An 8% SDS-polyacrylamide gel, where the proteins have been visualized by silver staining following electrophoresis. Input indicates crude bacterial lysate from *E. coli* containing either the parental expression vector (pKK233-2) or the AP-2 expression vector (pAP2L). First- and second-pass eluate refer to the proteins purified after one or two rounds of sequence-specific DNA affinity chromatography, respectively. AP-2 protein purified from human HeLa cells is also shown for comparison. (Left) Relative molecular mass markers.

activate transcription *in vitro* (Imagawa et al. 1987; Mitchell et al. 1987). Therefore, we examined the ability of the bacterially expressed protein to function as a transcription factor *in vitro*. The stimulatory effect of AP-2 was tested using a HeLa nuclear extract that had been depleted of endogenous AP-2 protein by DNA affinity chromatography (Scheidereit et al. 1987; D. Bohmann, pers. comm). When bacterially expressed AP-2 was added to this depleted HeLa extract, it activated hMtlIIa

transcription *in vitro* in a comparable manner to native HeLa AP-2 (three- to fivefold; Fig. 5). Furthermore, the transcriptional activation obtained with bacterially expressed AP-2 is binding site dependent and is not observed with a hMtlIIa template lacking the AP-2 sites in the distal and proximal BLEs (Fig. 5). These results unambiguously establish that the isolated cDNA encodes a sequence-specific transcription factor with the specificity of AP-2.

Cloned AP-2 activates expression *in vivo*

In addition to examining the transcriptional properties of this bacterially expressed AP-2 *in vitro*, it would also be useful to examine the ability of this protein to activate transcription *in vivo*. *Drosophila* tissue culture cells recently have been very successfully adapted to study the ability of cloned Sp1 and CTF/NF1 to activate expression *in vivo* (Courey and Tjian 1988; Santoro et al. 1988). These *Drosophila* Schneider cells appear to be devoid of the homologs of many mammalian transcription factors, including Sp1, CTF/NF1, and AP-2 (Perkins et al. 1988) and have provided a useful *in vivo* complementation system for analyzing the transcriptional activity of several mammalian transcription factors. Therefore, the *Drosophila* Schneider cell cotransfection procedure was also adopted to study the transcriptional activity of AP-2 *in vivo*.

The expression vector used for these experiments, pPADH, consists of the two tandem promoters of the *Drosophila* alcohol dehydrogenase gene linked to the actin 5C poly(A) addition signal. To adapt the AP-2 cDNA for insertion into pPADH, the ATG codon at nucleotides 63–65, corresponding to the amino-terminal methionine, was converted into an *Nco*I site by site-directed mutagenesis. This facilitated the introduction of the entire AP-2 ORF into pPADH to generate the plasmid pADH-AP2 (Fig. 6A). This construct should express AP-2 as a full-length nonfused protein containing a single conservative amino acid change of leucine to valine at the second position due to the presence of the *Nco*I site.

Next, we constructed reporter plasmids containing the bacterial chloramphenicol acetyltransferase (CAT) gene linked to a wild-type hMtlIIa promoter (CAT-HSI) or a truncated version lacking the AP-2-binding sites in the distal and proximal BLEs (CAT-EAG) (Fig. 6B). When CAT-HSI or CAT-EAG are transfected into *Drosophila* cells not expressing AP-2, a low basal level of CAT activity is observed, which is unaffected by cotransfection of the pPADH expression vector (Fig. 6). In contrast, cotransfection of pPADH-AP2, which directs the synthesis of AP-2 in *Drosophila* cells, along with the reporter plasmid CAT-HSI, produces a significant induction (fourfold) of CAT activity. Expression of AP-2 in transfected cells does not affect the levels of CAT activity obtained with the mutant reporter gene lacking AP-2-binding sites. The binding-site-dependent enhancement of CAT activity caused by pPADH-AP2 also could be observed using either a triplicated AP-2-binding site de-

Figure 4. DNase footprint analysis of bacterially expressed AP-2. The DNA-binding ability of the second-pass affinity-purified bacterially expressed AP-2 [*E. coli*; ~2 ng/ μ l], compared to AP-2 protein isolated from HeLa cells (HeLa; 3 ng/ μ l). Amounts of AP-2 protein are noted above in microliters. Control DNase reactions lacking AP-2 are also presented (–). For the hMtIIa template, DNase I footprinting reactions were performed using a ^{32} P 5'-end-labeled *Bam*HI–*Hind*III fragment from pHSI (Karin et al. 1987). Numbering is relative to the transcription initiation site at +1, indicated by the arrow. The GC box and distal and proximal BLEs of the promoter are also shown. For the SV40 virus template, a ^{32} P 5'-end-labeled *Eco*RI–*Hind*III fragment derived from pSVGCO was used (Mitchell et al. 1987). Numbering is relative to the *Bgl*II site at the origin. Various components of the SV40 intergenic control region are shown for reference. The positions of AP-2-binding sites within the hMtIIa and SV40 enhancer elements are indicated by dotted ovals.

rived from the hMtIIa distal BLE attached to the herpes simplex virus–thymidine kinase (HSV-TK) promoter (6- to 10-fold induction; Fig. 6C) or the human enkephalin promoter, where only a single AP-2-binding site is present (2.5-fold induction; Fig. 6D). These results demonstrate that AP-2 also acts as a sequence-specific transcription factor in vivo. In addition, the basal levels of CAT activity of the wild-type templates and the AP-2 minus (–) templates are comparable, suggesting that AP-2 is very likely absent from these *Drosophila* cells. Moreover, the ability of AP-2 to activate transcription of the wild-type templates indicates that the *Drosophila* transcriptional machinery is capable of responding to exogenously added AP-2.

Discussion

To understand the mechanisms governing the positive and negative regulation of AP-2-dependent transcriptional activation, we have isolated and begun to analyze

the structure and function of cDNAs corresponding to this cAMP- and TPA-inducible enhancer-binding factor. The AP-2 peptide sequences we determined enable us to confirm the identity of the predicted protein product and to locate the translational initiation site. The longest cDNA clone contains the entire ORF of AP-2, beginning at the initiator methionine corresponding to nucleotides 63–65 (Fig. 1B). Expression of the full-length cDNA clone in both prokaryotic and eukaryotic systems confirms that AP-2 is a sequence-specific DNA-binding transcription factor.

We have attempted to determine whether AP-2 is related to other DNA-binding proteins and transcription factors. The nucleic acid sequence and predicted amino acid sequence of AP-2 do not display any striking homologies to other genes and proteins present in the EMBL and NBRF data bases. Moreover, examination of the AP-2 protein sequence has not revealed any obvious resemblance to well-characterized DNA-binding motifs such as homeo boxes and zinc finger motifs (Laughon

Figure 5. Bacterially expressed AP-2 stimulates transcription in vitro from the hMtlIIa promoter. (Bottom) A schematic representation of the hMtlIIa templates used for the in vitro transcription analyses, indicating the positions of the AP-2 sites (dotted ovals) relative to the transcription initiation sites utilized in vitro (arrows). (Top) A primer extension analysis of products of in vitro transcription reactions using either the wild-type HSI/TK template (wt) or the 5' deletion 5'Δ-105 lacking the AP-2 sites (Δ) (Lee et al. 1987b). Reactions contained either no exogenous AP-2 (-), 10 ng of bacterially expressed second-pass DNA affinity-purified AP-2 (+), or 20 ng of HeLa AP-2 protein (HeLa). The positions of the hMtlIIa start sites and the internal control MSV/TK start sites are indicated by arrows.

and Scott 1984; McGinnis et al. 1984; Berg 1986; Kadonaga et al. 1987; Bürglin 1988; Evans and Hollenberg 1988; Hoey and Levine 1988; Clerc et al. 1988; Sturm et al. 1988, both this issue), or to elements present in the DNA-binding domains of the transcription factors CTF/NF1 or c-jun (Bohmann et al. 1987; Santoro et al. 1988). Thus, it is possible that AP-2 utilizes a novel type of sequence-specific DNA-binding structural domain. Alternatively, as the predicted secondary structure for the carboxy-terminal half of AP-2 is quite α -helical (Fig. 1A), it may be distantly related to the helix-turn-helix DNA-binding motifs present in the λ repressor and Cro proteins (for review, see Pabo and Sauer 1984).

In the context of transcription, studies on the yeast transcription factors GCN4 and GAL4 and the viral *trans*-acting protein VP16 have indicated that acidic regions are important components of transcriptional activation domains (Hope and Struhl 1986; Gill and Ptashne 1987; Ma and Ptashne 1987; Treizenberg et al. 1988). However, examination of the primary amino acid sequence of AP-2 does not reveal any obvious acidic stretches, although it is possible that an acidic domain could be created by protein folding or post-translational modification. In fact, acidic domains are unlikely to represent the only structural motif to mediate transcriptional activation. For example, the mammalian transcription factor Sp1 has two glutamine-rich regions (29% glutamine) which are largely responsible for its transcriptional activity (Kadonaga et al. 1988; Courey and Tjian 1988). A 22% glutamine-rich region is also

found in the AP-2 protein between amino acids 63 and 103 and could potentially activate transcription by a similar mechanism to Sp1. Strikingly, this glutamine-rich stretch of the AP-2 protein also overlaps a region between amino acids 38 and 97, which contains 30% proline residues (Fig. 1A,B). Notably, such proline-rich regions have been observed in a number of other factors implicated in transcriptional regulation, including CTF/NF1, C/EBP, c-Jun, c-Fos, and Fra-1 (Bohmann et al. 1987; Cohen and Curran 1988; Landschulz et al. 1988; Santoro et al. 1988). Most importantly, in the case of CTF/NF1, this proline-rich stretch maps to a region of the protein determined to be the transcriptional activation domain (N. Mermod and R. Tjian, unpubl.). In addition, although the sequences involved in transcriptional activation by c-Jun are less well defined, they also contain the proline-rich stretch of amino acids (D. Bohmann and R. Tjian, unpubl.). The sequences responsible for transcriptional activation have not yet been identified for C/EBP, c-Fos, and Fra-1. It will be interesting to determine whether these concentrated arrays of proline and glutamine residues perform some specific function in transcriptional activation of AP-2.

In any case, it seems likely that many different types of protein domains will be involved in sequence-specific DNA binding and transcriptional activation. Presently, we are generating mutations in the AP-2-coding region to identify definitively the various functional domains of this protein, which appears to have some novel features. The in vivo and in vitro assays that we have es-

Figure 6. (See facing page for legend.)

Figure 6. AP-2 expressed *in vivo* activates transcription in a binding-site-dependent manner. (A) Schematic representation of the AP-2 *Drosophila* expression vector, pPADH-AP2. The hatching indicates the tandem *Drosophila* alcohol dehydrogenase promoters (P_D and P_P) of the parental pPADH plasmid, where the arrows denote the direction and start sites of transcription. The open box represents the AP-2 open reading frame, beginning with the ATG at nucleotide 63 and ending with the TGA at nucleotide 1376. The solid box indicates the 3'-untranslated region of AP2-9 cDNA. (N) The *Nco*I site, engineered at the 5' end of the AP-2 open reading frame; (R1) the *Eco*RI site, marking the 3' terminus of the AP2-9 cDNA; (X) the *Xho*I site, used for cloning. All nucleotide numbers are given with respect to the sequence of AP2-9. (B–D) Schematic diagrams of the CAT reporter templates used in the cotransfection assays (bottom). (B) CAT-HSI contains the upstream regulatory sequences of hMtIIa, and CAT-EAG is a 5' deletion mutant of CAT-HSI lacking the AP-2-binding sites. (C) The plasmid pBLCAT19 contains the HSV-TK promoter with three copies of a 19-bp AP-2-binding oligonucleotide derived from the hMtIIa distal BLE. The plasmid pBLCAT2Δ contains only the HSV-TK sequences. (D) ENK wt contains the wild-type human enkephalin promoter, whereas ENK LS is a linker-scanning mutant of this promoter lacking the AP-2 site. The start sites and direction of transcription are shown by arrows, and the AP-2-binding sites are represented by dotted ovals. (Top) The results of the CAT assays are shown at the tops of the panels. The presence of reporter plasmid, alone in the transfection (–) or in conjunction with the expression vectors pPADH (v) or pPADH-AP2 (AP-2), is indicated above each lane.

established to study the properties of the full-length protein should also enable us to gain a comprehensive understanding of the domains of AP-2 important for its interactions with DNA, with SV40 large T antigen, and with other factors involved in transcriptional activation.

It is highly probable that certain transcription factors play a fundamental role in regulating gene expression in response to various developmental signals. Interestingly, the level of AP-2 mRNA is elevated substantially following the retinoic-acid-induced differentiation of the human teratocarcinoma cell line NT2. The induction of AP-2 RNA following differentiation of human NT2 cells correlates with the observation that enhancer and promoter elements to which AP-2 binds are less active in undifferentiated mouse teratocarcinoma cells than in their differentiated derivatives (Gorman et al. 1985; Sleight and Locket 1985). It now appears that specific DNA-binding proteins, which are likely to act as transcription factors, are induced upon differentiation of human and mouse teratocarcinoma cells (Hauser et al. 1985; Kryszke et al. 1987; Speck and Baltimore 1987). Therefore, it is likely that the increased levels of these factors are involved in altering the patterns of gene ex-

pression in response to differentiation. Moreover, the finding that AP-2 is not expressed in the human hepatoma cell line HepG2 also suggests that it may be involved in tissue-specific gene expression. Presently, we are testing to see whether the absence of AP-2 in HepG2 cells is a unique property of this particular cell line or whether it represents a more widespread feature of other transformed or normal liver cells, which could be partially responsible for liver-specific gene expression.

The mechanisms by which AP-2-binding sites can act as TPA- and cAMP-inducible elements is an interesting problem that remains unsolved (Imagawa et al. 1987; Comb et al. 1988; Hyman et al. 1988). However, now that the gene encoding AP-2 has been isolated, it will be possible to design various experiments to determine whether the TPA and cAMP responsiveness of the AP-2-binding site might be due to transcriptional, translational, and/or post-translational modulation of AP-2 expression. The availability of expression systems for wild-type and mutant AP-2 in animal cells should also facilitate our studies of the negative regulatory interaction between T antigen and AP-2, which is thought to be mediated via direct protein–protein contact.

In summary, we have isolated cDNA clones corresponding to the human transcription factor AP-2. The availability of this cloned factor should now allow us to dissect the many levels of transcriptional control within a cell, including signal transduction and developmentally regulated gene expression.

Materials and methods

AP-2 purification and peptide sequencing

AP-2 protein was purified from HeLa cell nuclear extracts by sequence-specific DNA affinity chromatography, essentially as described by Mitchell et al. (1987). DNA affinity columns prepared with a sequence from the hMtIIa distal BLE, 5'-GAACT-GACCG CCCGC GGCCG GTGTG CAGAG-3' (hMtIIa nucleotides -188 to -159), were used for the first round of purification, and an SV40 AP-2 column was used for the second round (generous gift of D. Bohmann; Mitchell et al. 1987). Buffers used for second passage and elution from the SV40 columns contained 0.1% lauryldimethylamine oxide (LDAO, supplied by Calbiochem) as detergent instead of 0.1% NP-40. For amino-terminal sequencing of AP-2 protein, 20 µg was subjected to electrophoresis on a 12–18% SDS–polyacrylamide gradient gel before transfer to a PVDF membrane (Millipore). The 52-kD band corresponding to AP-2 was visualized by Coomassie blue staining, excised, and sequenced using an Applied Biosystems 477A sequencer (Matsudaira 1987).

To generate tryptic peptides of AP-2 protein, 20 µg was precipitated using 25% (wt/vol) TCA and resuspended in 8% triethylamine (TEA), 0.1% trifluoroacetic acid (TFA). After adjusting the pH to 8.4 using 1 M HEPES, the protein was digested for 16 hr at 37°C using 50 ng trypsin (Worthington) in 100 mM ammonium bicarbonate.

To generate CNBr peptides of AP-2 protein, 20 µg was TCA-precipitated and resuspended in 8% TEA, 0.1% TFA. The protein was then treated with 4-vinylpyridine under reducing conditions to alkylate the cysteine residues, as described by Enfield et al. (1980), TCA-precipitated, acetone-washed, and resuspended in 200 µl of 70% formic acid. CNBr (1 mg) was added, and the mixture incubated for 36 hr at room temperature in the dark under nitrogen. Peptides generated by tryptic digestion or CNBr cleavage were resolved on a C18, 300 Å, 2.1 × 150-mm column (Vydac) using a TFA/acetonitrile gradient. Peptides were sequenced as described above.

Isolation of recombinant clones encoding AP-2

An oligonucleotide 5'-GTCTG TGAAC TCCTT GCAIA TCTGC TTGGT GGCIA GIAGC ATGTT-3' was deduced from the sequence of the presumed overlapping tryptic peptide 6 and CNBr peptide 7, according to Lathe (1985) (I represents inosine). This oligonucleotide was 5'-end-labeled with [γ -³²P]ATP and polynucleotide kinase and used to screen a HeLa cell cDNA library in λ gt10, kindly provided by Dr. C. Hauser. Lambda plaques transferred to nitrocellulose were screened essentially as described by Kadonaga et al. (1987), except that the two final washes were performed in 2 × SST, 0.5% SDS at 53°C. Three independent clones were isolated and the cDNA inserts subcloned into the *Eco*RI site of pBluescript SK⁺ (Stratagene) for further restriction enzyme analysis. Clone AP2-9 was completely sequenced on both strands using subclones generated by restriction enzyme or exonuclease III digestion, and the two other clones were sequenced at their 5' and 3' termini. Se-

quencing was performed by the dideoxy method using Sequenase reagents (U.S. Biochemical Corp.).

Northern blot analysis

Northern blot analysis of human RNA was performed essentially as described by Hauser et al. (1985), except that the AP-2 probe was prepared by the random hexamer priming method (Feinberg and Vogelstein 1983). Each track contained 40 µg of total RNA (kindly supplied by N. Tanese). Differentiation of NT2 cells was induced by 2–5 days treatment with retinoic acid, as described by Hauser et al. (1985).

RNase protection and preparation of RNA probes

AP-2: The 261-bp *Pvu*II fragment of the AP2-9 cDNA was subcloned into the *Sma*I site of pBluescript SK⁺ such that T7 RNA polymerase would transcribe the noncoding strand. Runoff transcription was performed using DNA template that was linearized in the polylinker with *Bam*HI. Sp1: A genomic fragment of the Sp1 gene (B. Lüscher and R. Tjian, unpubl.) was subcloned into the polylinker of pBluescript SK⁺, and the resulting template was used to generate antisense RNA after transcription with T7 RNA polymerase. Transcriptions by T7 RNA polymerase were according to the recommendations of the manufacturer (Stratagene) in the presence of [α -³²P]rUTP (400 Ci/mmol).

RNase protection was performed as follows: 40 µg of total (HeLa and HepG2 cells) or 30 µg of cytoplasmic RNA (NT2 cells) was coprecipitated with the RNA probes specific for AP-2 and Sp1 (1.5 × 10⁵ cpm each) and hybridized overnight in 30 µl hybridization buffer [80% formamide, 400 mM NaCl, 40 mM PIPES (pH 6.7), 1 mM EDTA] at 65°C. The hybrids were treated with 350 µl of RNase digestion buffer [300 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4 µg/ml of RNase A, and 8 U/ml of RNase T1] for 1 hr at 37°C. The samples were then digested with proteinase K for 20 min at 37°C by the addition of 10 µl of 20% SDS and 5 µl of proteinase K (20 mg/ml). They were extracted with phenol/chloroform, ethanol-precipitated, and analyzed on a 5% polyacrylamide gel containing 8.3 M urea, in 1 mM EDTA and 100 mM Tris, adjusted to pH 8.3 with boric acid.

Expression of AP-2 in bacteria

The AP-2 bacterial vector, termed pAP2L, was constructed by fusing an oligonucleotide 5'-CATGC TTTGG AAGTT AACGG ATAAT ATCAA GT-3' (corresponding to nucleotides 62–93 of AP2-9) to a fragment of AP2-9 spanning from the *Rsa*I site at nucleotide 94 to the *Hind*III site in the polylinker of pBluescript SK⁺ just downstream of the *Eco*RI site at nucleotide 1636. This fusion was ligated into the expression vector pKK233-2 (Pharmacia) cleaved with *Nco*I and *Hind*III. *E. coli* TG-1, transformed with control pKK233-2 vector pAP2L, was grown at 37°C to OD₆₀₀ ~0.3 and then induced by the addition of IPTG to a final concentration of 1 mM. The cells were grown for an additional 90 min at 37°C, and the AP-2 protein was extracted according to Kadonaga et al. (1987). The protein was purified further by sequence-specific DNA affinity chromatography, as described above. This purified AP-2 was used either directly for DNase I footprinting analysis or dialyzed against buffer T [20 mM HEPES–KOH (pH 7.9), 20% (vol/vol) glycerol, 0.2 mM EDTA, 100 mM KCl, 12 mM MgCl₂, and 1 mM dithiothreitol (DTT)] in preparation for in vitro transcription assays. DNase I footprinting reactions were performed using an SV40 template

derived from the plasmid pSVGCO or a hMtIIa template derived from the plasmid pHSI, as described by Mitchell et al. (1987).

In vitro transcription reactions

A HeLa nuclear extract was prepared according to Dignam et al. (1983), as modified by Wildeman et al. (1984). Aliquots of extract in buffer D* [20 mM HEPES–KOH (pH 7.9), 20% (vol/vol) glycerol, 20 mM KCl, 2 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT] were frozen in liquid nitrogen and stored at –70°C. To a thawed aliquot of nuclear extract, 2 M KCl was added to yield a final concentration of 100 mM just prior to use. To deplete endogenous AP-2 from the extract, AP-2 hMtIIa resin (100 µl packed volume) was pre-equilibrated in buffer D* containing 100 mM KCl and then added to 250 µl of nuclear extract. The extract/resin suspension was rotated for 30 min at 4°C and then centrifuged for 1 min at 15,000g. The supernatant was either used directly or stored at –70°C. Each *in vitro* transcription reaction (20 µl) contained AP-2-depleted extract (4 µl) in 10 mM HEPES–KOH (pH 7.9), 10% (vol/vol) glycerol, 0.1 mM EDTA, 0.25 mM DTT, 50 mM KCl, 0.5 mM each rNTP, 4 mM spermidine, and 4 mM MgCl₂ final concentration. Each reaction also contained a control murine sarcoma virus (MSV)-TK promoter fusion template (40 ng) and either pHSI/TK or 5'Δ-105 test template (100 ng), as indicated (Lee et al. 1987b). Transcription reactions were carried out at 30°C for 60 min and analyzed by primer extension analysis, as described by Mitchell et al. (1987).

AP-2 cotransfection assays

The AP-2 *Drosophila* expression vector pPADH-AP2 was constructed by fusing an oligonucleotide 5'-CATGG TTTG-G AAATT GACGG ATAAT ATCAA GT-3' (corresponding to nucleotides 62–93 of AP2-9) to a fragment of AP2-9 spanning from the *Rsa*I site at nucleotide 94 to the *Xho*I site in the poly-linker of pBluescript SK+ just downstream of the *Eco*RI site at nucleotide 1636. This fusion was ligated into the expression vector pPADH cleaved with *Nco*I and *Xho*I, resulting in the transcriptional fusion of the AP-2 open reading frame to the *Drosophila* alcohol dehydrogenase promoter leader sequence. This construct creates an *Nco*I site at the translational start of AP-2 and causes a conservative change of the second amino acid from leucine to valine. The construction of pPADH is described elsewhere (Courey and Tjian 1988).

CAT-HSI was constructed by inserting the 830-bp *Hind*III–*Bam*HI fragment of pHSI (corresponding to hMtIIa nucleotides –764 to +69; Haslinger and Karin 1985) into pBLCAT3 (Luckow and Schütz 1987), and CAT-EAG is a 5' deletion of CAT-HSI to nucleotide –108. The wild-type pENKAT-Δ114 and linker scanning mutant pENKAT-Δ114M (Hyman et al. 1988) were generous gifts of Dr. S. Hyman. The plasmid pBLCAT2Δ was constructed by deletion of the 164-bp *Hind*III to *Nar*I fragment of pBLCAT2 (Luckow and Schütz 1987), followed by Klenow repair and religation (generous gift of N. Mermod). pBLCAT19 was constructed by ligating three copies of the sequence 5'-CTGAC CGCCC GCGGC CCGT-3', corresponding to nucleotides –185 to –167 of the hMtIIa distal BLE, into pBLCAT2Δ restricted with *Sal*III and *Bam*HI.

Drosophila Schneider line-2 cell culture and transfection by the calcium phosphate coprecipitation method were performed by standard procedures (Santoro et al. 1988). Cotransfections contained 2–5 µg of expression vector and 5 µg of reporter plasmid in the presence of pUC118 carrier to a total of 15 µg DNA. Cell lysis and CAT assays were performed as described previously (Santoro et al. 1988).

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