Transcription Factor AP-2 Functions as a Repressor That Contributes to the Liver-specific Expression of Serum Amyloid A1 Gene*

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We previously identified transcription factor AP-2 as the nuclear factor that interacts with the tissue-specific repressor element in the rat serum amyloid A1 (SAA1) promoter. In this report, we provide evidence for a second AP-2-binding site and show that both AP-2 sites participate in mediating the transcription repression of SAA1 promoter. This proximal AP-2 site overlaps with the NFκB-binding site known to be essential for SAA1 promoter activity. Protein binding competition experiments demonstrated that AP-2 and NFκB binding to these overlapping sites were mutually exclusive. Furthermore, the addition of AP-2 easily displaced pre-bound NFκB, whereas NFκB could not displace AP-2. These results thus suggest that one mechanism by which AP-2 negatively regulates SAA1 promoter activity may be by antagonizing the function of NFκB. Consistent with a repression function, transient expression of AP-2 in HepG2 cells inhibited conditioned medium-induced SAA1 promoter activation. This inhibition was dependent on functional AP-2-binding sites, since mutation of AP-2-binding sites abolished inhibitory effects of AP-2 in HepG2 cells as well as resulted in derepression of the SAA1 promoter in HeLa cells. In addition to SAA1, we found that several other liver gene promoters also contain putative AP-2-binding sites. Some of these sequences could specifically inhibit AP-2-DNA complex formation, and for the human complement C3 promoter, overexpression of AP-2 also could repress its cytokine-mediated activation. Finally, stable expression of AP-2 in hepatoma cells significantly reduced the expression of endogenous SAA, albumin, and α-fetoprotein genes. Taken together, our results suggest that AP-2 may function as a transcription repressor to inhibit the expression of not only SAA1 gene but also other liver genes in nonhepatic cells.

Expression of cell type-specific genes is tightly controlled by the combined actions of positive and negative transcription regulators that interact with specific DNA sequences (1–3). In addition, the tissue-specific expression of transcription factors themselves can contribute significantly to their functional role in regulating cell-type determination or differentiation (4, 5). To achieve accurate tissue-specific transcription, a complex array of signals must be integrated through the actions of transcription activators and repressors, culminating at the gene promoter to regulate initiation of RNA synthesis (6–8). For instance, expression of MyoD is restricted to the skeletal muscle cells and contributes to myogenesis by interacting with other positive transcription factors to activate the transcription of muscle-specific genes (9). In contrast, neuron-restrictive silencer factor is expressed in most nonneuronal tissues and in undifferentiated neuronal progenitors (10, 11). Because of its tissue distribution and its repressive effects on neuronal-specific genes, this silencer factor may function as a master negative regulator for neurogenesis (10, 12).

A growing list of transcription factors has been shown to function as either transcription activators or transcription repressors, depending on the promoter and cellular context of their target genes (13, 14). Examples of this group of transcription factors include the Drosophila transcription factor Kruppel, which converts from an activator to a repressor in a concentration-dependent manner (15, 16); human thyroid hormone receptor β, which converts from a repressor to an activator upon ligand binding (17, 18); and YY1, which exerts positive or negative effects on transcription depending on the presence or absence of the E1A protein (19, 20). Transcription factor AP-2 is yet another transcription regulator with dual functions. First identified in HeLa cell nuclear extracts (21, 22), AP-2 was named for its transcription activation function. It acts as an activator in regulating many genes, including those involved in the morphogenesis of peripheral nervous system, face, limbs, skin, and nephric tissues (23–25). Recently, AP-2 has been shown to negatively regulate the transcription of stellate cell Type I collagen, K3 keratin, acetylcholinesterase, protymosin, ornithine decarboxylase, retina fatty acid-binding protein, and CCAAT/enhancer binding protein α (C/EBPα) (26–31). Exactly when a particular transcription factor with dual functions acts as an activator or as a repressor depends not only on its intrinsic features and concentration and on the presence of other transcription factors but perhaps also on the structure, relative position, and orientation of the promoter sequence itself (13, 14).

Serum amyloid A (SAA), one of the major acute-phase pro-

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1 The abbreviations used are: C/EBPα, CCAAT/enhancer binding protein α; SAA1, serum amyloid A1; bp, base pair(s); CRU, cytokine response unit; CM, conditioned medium; RT-PCR, reverse transcription-polymerase chain reaction; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; AFP, α-fetoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; mAP-2, mutant AP-2.
AP-2 as a Repressor for Liver Genes

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teins (32, 33), represents an excellent model system to study cytokine-induced regulation and liver-specific expression. Its serum concentration increases 1,000-fold after acute inflammation, being regulated primarily by the 200–300-fold increase in SAA gene transcription (32, 34, 35). In mice, the SAA gene family consists of four genes, SAA1, SAA2, SAA3, and SAA5, and a pseudogene (36, 37). Whereas expression of SAA1, SAA2, and SAA3 is dramatically induced after inflammation and each contributes equally to the increased SAA mRNA levels in the liver, SAA5 expression is induced to a much lower level and with different induction kinetics (38, 39). In addition to being highly regulated by the inflammatory cytokines, expression of SAA1 and SAA2 are also highly cell type-specific, restricted to the liver hepatocytes (32, 34, 35).

Studies of the rat SAA1 promoter demonstrate that 304 bp of its 5′-flanking sequence are sufficient not only for its liver cell-specific expression but also for its cytokine-induced expression in response to inflammatory mediators (40). Further deletion analyses identified a 66-bp DNA fragment spanning bp −138 to −73 that could confer its cytokine responsiveness. Within this cytokine response unit (CRU) reside binding sites as a repressor in opposing NF-κB-mediated transcription and contributes to the low basal expression of SAA1 and perhaps the transience of its expression after inflammation (41). Subsequent transient transfection analyses revealed a tissue-specific repressor element distal to the CRU that conferred repression on the SAA1 promoter in HeLa cells but had no such inhibitory activity in liver cells (42). This repressor element formed an intense DNA-protein complex with nuclear extracts from HeLa cells but not from liver cells.

We have recently purified this DNA-binding protein from HeLa nuclear extracts and shown by protein sequencing and biochemical and immunological analyses that it is identical to the transcription factor AP-2 (43). Here, we report on the repression effects of AP-2 on the expression of transfected and endogenous liver genes and suggest that it may function as a negative regulator to repress the expression of some liver genes in nonhepatic cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Conditioned Medium—HeLa, HepG2, and Hep3B cells were grown in basal medium that contained minimum essential medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids, 1 mM pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. HepG2 and Hep3B cells were used for our transient transfection and stable cell line studies because they have been demonstrated to confer proper regulation of many endogenous and transfected genes, including SAA (44–47). Conditioned medium (CM) known to contain a mixture of inflammatory mediators capable of inducing acute-phase gene expression was prepared from activated mixed lymphocyte cultures as described (48) and used as a mixture with an equal volume of basal medium.

Oligonucleotides and Plasmids—The wild-type AP-2 consensus sequence (AP-2) (5′-GGAACTGACCCGCCGCGTGTTGCACG-3′) and its mutant (mAP-2) (5′-GGAACTGACCCGCCGCGTGTTGCACG-3′) are from the human metallothionein IIA promoter (49). The wild-type (5′-CTTCACTCTATATTCGCTCCTAGAAAGG-3′) and mutant (5′-CTTCACTCTATATTCGCTCCTAGAAAGG-3′) AP-2d and wild-type (5′-CATTTGTTAAGCCAGCTCCGGA-3′) and mutant (5′-CATTTGTTAAGCCAGCTCCGGA-3′) AP-2 oligonucleotides correspond to the distal (bp −285 to −256) and proximal (bp −97 to −70) AP-2-binding sites, respectively, in the SAA1 promoter. These oligonucleotides were used as primers in polymerase chain reactions (PCR) or as probes or competitors in electrophoretic mobility shift assay (EMSA).

pTK/SAA1 (−285/−70) was constructed by inserting a DNA fragment from the rat SAA1 promoter (bp −285 to −70) into the Smal site of pBluescript vector, which contains the thymidine kinase (TK) minimal promoter and the chloramphenicol acetyltransferase (CAT) reporter gene. This SAA1 promoter fragment was synthesized by PCR using AP-2d and the complement of AP-2p oligonucleotides. The constructs pTK/SAA1 (−285/−70)_AP-2, pTK/SAA1 (−285/−70)_mAP-2, pSAA1_5T/A, pSAA1/CAT, pSAA1/CAT_−120, pSAA1/CAT_−120/−18, pSAA1/CAT_−120/−42, pSAA1/CAT_−120/−42 [mAP-2] are also highly cell type-specific, restricted to the liver hepatocytes (32, 34, 35).

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RESULTS

The Proximal Region of SAA1 Promoter Contains Two AP-2-binding Sites—We had previously identified a repressor element in the rat SAA1 promoter that could inhibit its promoter activity in HeLa cells (42). Furthermore, we showed this repressor element contained a high affinity binding site for AP-2 (43). In this study, we further investigated the mechanisms for the repressive effects of AP-2 by searching for additional AP-2-binding sites in the SAA1 promoter. In addition to the AP-2-
binding site (bp −272 to −265) identified initially, one additional sequence (5′-CCCCAGGG-3′) located at bp −85 to −78 was found to be a potential AP-2-binding site (Fig. 1A). These two sites were designated, relative to their positions in the SAA1 promoter, the distal AP-2-binding site (AP-2d) and the proximal AP-2-binding site (AP-2p) (Fig. 1A). It is interesting to note that the sequences at the AP-2p site are highly conserved between rat, human, and mouse SAA1 genes (40, 52). To determine whether the AP-2p site could indeed interact with AP-2, oligonucleotide competition experiments were carried out using radiolabeled AP-2d sequence and purified AP-2 in EMSA. In the absence of any competitors, a strong AP-2-DNA complex was detected (Fig. 1B). As expected, both the consensus AP-2 binding sequence from the metallothionein IIA promoter and the AP-2d site very effectively competed for AP-2-DNA complex formation, whereas the mutated AP-2d-site oligonucleotides could not compete (Fig. 1B). When the wild-type and mutated AP-2p oligonucleotides were used as competitors, the wild-type AP-2p oligonucleotides showed dose-dependent competition of the complex formation, whereas the mutant AP-2p site did not. This result indicates that the AP-2p site functioned as a binding site for AP-2, although it had lower binding affinity than the AP-2d site.

Interestingly, the position of the AP-2p site overlaps that of NFκB, which is known to be essential for SAA1 promoter function (40, 50). The overlapping nature of these two transcription factor-binding sites raises an intriguing question, that is, whether binding of one factor to the DNA would affect the binding of the other factor. To address this question, gel shift assays were performed using the AP-2p probe, which contains both AP-2- and NFκB-binding sites, and recombinant AP-2 and NFκB. The reactions were carried out under probe-limiting conditions to allow AP-2 and NFκB to compete for limited amounts of target DNA. When added individually to the reactions, both AP-2 and NFκB formed specific DNA-protein complexes (Fig. 2). However, when AP-2 and NFκB were added together, no DNA-protein complexes with slower mobilities were observed, arguing against simultaneous binding of these two transcription factors to the same DNA molecule. To examine their relative binding affinities, radiolabeled AP-2p probe was first incubated with a constant amount of AP-2 or NFκB. After a brief incubation, increasing amounts of NFκB or AP-2 were added to the reaction mixture. As shown in Fig. 2, the addition of AP-2 readily displaced the prebound NFκB from this overlapping region (lanes 8–12). At a 2.3-fold molar excess of AP-2, no NFκB remained bound to the probe. In sharp contrast, NFκB had little effect on the prebound AP-2, even at a 28-fold molar excess of NFκB (lanes 2–6). Taken together, our results indicate that AP-2 and NFκB bind to this overlapping region in a mutually exclusive manner. Furthermore, AP-2 has much higher DNA binding affinity than NFκB. It is therefore tempting to speculate that one mechanism by which AP-2 may inhibit cytokine-induced SAA1 promoter function is by antagonizing the ability of activated NFκB to bind to its cognate binding site.

Binding of AP-2 to the SAA1 Promoter Is Required for Its Inhibitory Effects—Site-specific mutation of NFκB- or C/EBP-binding sites in the CRU completely abolished SAA1 promoter cytokine responsiveness (40, 50), indicating the importance of these two transcription factors in SAA1 gene transcription. Since we showed that the NFκB-binding site overlaps with that of AP-2 and the presence of AP-2 effectively prevents NFκB from binding to its cognate site, we sought to determine whether AP-2 binding to the AP-2p site is required for its repressive effects on the SAA1 promoter. Two reporter constructs, pSAA1/CAT(−120) (wild-type AP-2p site) and pSAA1/CAT(−120)mAP-2p (mutant AP-2p site), were generated. As determined by EMSA, the 2-bp mutation at the AP-2p site completely abolished AP-2 binding but still retained full NFκB binding activities (data not shown). These two reporter constructs were cotransfected into HepG2 cells with the AP-2-expression vector, pSP2, to assess the effects of AP-2 site mutation on the ability of AP-2 to repress the reporter gene expression. As shown in Fig. 3, the CAT activities for both the wild-type and the mutant constructs could be induced 5–6-fold.

![Diagram](https://example.com/diagram.png)
by CM. However, cotransfection with AP-2 expression vector completely inhibited CM-induced expression of the wild-type pSAA1/CAT(−120) reporter. In contrast, expression of AP-2 had no significant effects on the cytokine responsiveness of the mutant pSAA1/CAT(−120) reporter. As controls, cotransfection with the frameshift AP-2 mutant construct had no inhibitory effects on either reporter construct. These results indicate that AP-2-mediated repression on a pSAA1/CAT(−120) reporter requires a functional AP-2 site.

**Mutation of AP-2-binding Sites Results in Derepression of SAA1 Promoter in HeLa Cells**—Because liver and liver-derived cell lines such as HepG2 do not normally express AP-2, a study of the effects of AP-2 in these cells requires cotransfection with AP-2 expression vectors. In contrast, HeLa cells express abundant amounts of AP-2, thus making cotransfection unnecessary as well as providing an alternative approach to examine the specificity of AP-2 repression effects. To investigate whether the lack of SAA1 promoter activities in HeLa cells is, at least in part, due to repression by the endogenous AP-2 through its binding at AP-2d and AP-2p sites, we generated a double AP-2-binding site mutant construct, pTK/SAA1(−285)/FS21, in which both the distal and the proximal AP-2 sites were mutated. As expected, the construct containing the wild-type AP-2 sites, pTK/SAA1(−285)/−70), was nonresponsive to CM stimulation when transfected into HeLa cells, presumably due to inhibition by the endogenous AP-2. Mutation of AP-2-binding sites, however, resulted in derepression of SAA1 promoter activity (Fig. 4). This result indicates that endogenous AP-2 in HeLa cells can indeed inhibit the cytokine-induced SAA1 activation and that binding of AP-2 is required for its repressive effects on the SAA1 promoter.

**Other Liver Gene Promoters Contain Potential AP-2-binding Sequences**—Two AP-2-binding sites were identified in the SAA1 promoter region, a high affinity AP-2d site and a weaker AP-2p site. To determine whether AP-2 may also regulate the transcription of other liver genes, we searched for potential AP-2-binding sites using the AP-2d sequence 5′-ATACCTAC-GACGC-3′ and the AP-2 consensus sequence 5′-GGCN9GCC-3′ (25, 53). As shown in Table I, at least 12 genes that are expressed mainly in the liver were found to contain putative AP-2-binding sites. To determine whether these sequences in the liver gene promoters could interact with AP-2 and therefore compete for its binding, oligonucleotides corresponding to six of the genes, chosen for their acute-phase response or liver-enriched expression, were synthesized and used as competitors in EMSA. HeLa cell nuclear extracts were used as the source of AP-2 binding activity, and radiolabeled AP-2d sequence was used as a probe. As shown in Fig. 5, all six oligonucleotides showed dose-dependent competition for AP-2 binding. Although they contained highly homologous core AP-2 binding sequences, they nevertheless exhibited considerable differences in their abilities to compete for AP-2 binding. For example, although the sequence from the human complement C3 promoter was 20 times more effective in competing for AP-2 binding than the SAA1 AP-2d site, sequences from the α-fibrinogen and apolipoprotein E promoters were 4 and 20 times, respectively, less effective. The fact that these oligonucleotides could inhibit AP-2-DNA complex formation suggests that AP-2 may bind to these sequences and perhaps regulate their expression in vivo.

**AP-2 Inhibits Cytokine-induced Human Complement C3 Promoter Activity in HepG2 Cells**—The fact that human complement C3 promoter contains such a high affinity AP-2-binding site suggests AP-2 may play a functional role in C3 gene regulation as well. To determine whether AP-2 can similarly repress C3 promoter activity, we transfected HepG2 cells with the cytokine-inducible pC3/Luc(−199) reporter with or without the AP-2-expression vector. When cotransfected with the empty vector, the luciferase activity was dramatically induced (25-fold) in response to CM stimulation. However, cotransfection with AP-2 expression vector pSAP2 resulted in more than 90% inhibition of the pC3/Luc(−199) reporter gene activity (Fig. 6A), whereas cotransfection with the frameshift AP-2 mutant pSAP2/FS21 had no inhibitory effects. To further demonstrate that the inhibitory effects were specific for AP-2, HepG2 cells were also transfected with the dominant-negative AP-2 mutant pSAP2Δ166−277 (53). As shown in Fig. 6B, expression of this mutant effectively neutralized the inhibitory effects of AP-2 on human C3 promoter.

**Stable Expression of AP-2 in Hep3B Cells Reduced the Expression of Endogenous SAA, Albumin, and α-Fetoprotein Genes**—Our transient transfection experiments demonstrated that expression of AP-2 in HepG2 cells can inhibit the expression of transiently transfected reporter genes driven by either

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**Fig. 3.** Mutation of AP-2-binding site abolishes the inhibitory effects of AP-2 on the SAA1 promoter. Wild-type pSAA1/CAT(−120) (WT) and mutant pSAA1/CAT(−120)ΔAP-2 (mt) reporter plasmids were cotransfected into HepG2 cells with empty vector (Vector), wild-type AP-2-expression vector pSAP2 (AP-2), or frameshift AP-2 mutant pSAP2/FS21 (fsAP-2). Approximately 20 h after transfection, cells were treated with basal medium (−) or 50% CM (+). Cells were harvested 20 h later for CAT assays.

**Fig. 4.** Mutation of AP-2-binding sites derepresses the SAA1 promoter in HeLa cells. Schematic diagrams of wild-type pTK/SAA1(−285)/−70) construct (WT) and the double AP-2-binding site mutant pTK/SAA1(−285)/−70)ΔmAP-2,Δp (mt) are shown at the top. HeLa cells were transfected with 80 ng of wild-type or mutant CAT reporter plasmids and treated with (+) or without (−) CM. After 20 h of treatment, cells were harvested for CAT assays.
Putative AP-2-binding sequences in liver gene promoters

| Genes* | Sequence          | Region      | Reference No. |
|--------|------------------|-------------|---------------|
| r SAA1 | CTATACCTAGGCAGCT | −157/−151   | 40            |
| r α1-AGP | AACTCCCTAGGGGTTG | −362/−346   | 69            |
| r PEPCK | GGCGCCCTGAGGCCCTC | −213/−229   | 70            |
| r α-FG | CTTTCCCTAGGGTGCC | +67/151     | 71            |
| h C3   | CATGCCCTAGGTACT | −178/−162   | 72            |
| h LCAT | GGCTCCCTAGGCTG  | −138/−122   | 73            |
| h TF   | AAGGCTTGGCCTGTC  | −370/−354   | 74            |
| h TF receptor | ACCTGCCTAGAAGGT | −86/−70     | 75            |
| h apoE | CTAGGCCCTAGGAGGGG | −76/−60     | 76            |
| h apoB | CGGCCGCTAGGGTCTG | −292/−276   | 77            |
| h apoCIII | CCTGCGCTAGGGGCC | −112/−96    | 59            |
| h SAA1 | AATGGCCCTAGGCCG  | −574/−558   | 52            |

*a, human; r, rat; α1-AGP, α1-acid glycoprotein; PEPCK, phosphoenolpyruvate carboxykinase; α-FG, α-fibrinogen; C3, complement component 3; LCAT, lecithin-cholesterol acetyltransferase; TF, transferrin; apoE, apolipoprotein E; apoB, apolipoprotein B; apoCIII, apolipoprotein CIII.

The regions that matched with the core AP-2 binding sequence are underlined.

**FIG. 6.** AP-2 inhibits CM-mediated activation of human C3 promoter in HepG2 cells. A, HepG2 cells were cotransfected with pC3/Luc(−199) reporter construct and 0.5 μg of wild-type AP-2-expression vector pSAP2 (AP-2) or frameshift AP-2 mutant pSAP2/FS21 (fsAP-2). Approximately 20 h after transfection, cells were treated with (+) or without (−) CM. Cells were harvested 20 h after stimulation. Extracts were prepared and assayed for luciferase activities. The fold induction of luciferase activities was calculated relative to the nonstimulated control sample, to which a value of 1.0 was assigned. B, pC3/Luc(−199) DNA was cotransfected into HepG2 cells with 1 μg of AP-2-expressing vector pSAP2 alone or with 1 or 2 μg of dominant-negative AP-2 mutant pSAP2Δ166–277 construct (AP2Δ166–277). Twenty hours after transfection, cells were treated with (+) or without (−) CM. Relative luciferase activities were calculated as above.

SAA1 or C3 promoter. Together with the oligonucleotide competition results that suggest many other liver gene promoters contain AP-2-binding sites, we sought to examine whether AP-2 may have similar effects on the expression of endogenous liver genes in their normal chromosomal context. Stable Hep3B cell lines that constitutively express moderate levels of AP-2 were generated by transfecting AP-2 expression vector, pIND/AP-2, into cells followed by selection with G418. Among the 11 stable cell lines isolated, three clones, H3B.A1, H3B.A2, and H3B.A6, were chosen for further studies. As controls, three Hep3B cell lines, H3B.V1, H3B.V2, and H3B.V3, that had the vector DNA stably integrated were selected in a similar manner. The AP-2 mRNA levels in these cell lines were determined by RT-PCR. As with the parental Hep3B cells, no detectable AP-2 expression was observed in H3B.V1, H3B.V2, and H3B.V3 cells (Fig. 7A). In contrast, AP-2 mRNA was easily detected in H3B.A1, H3B.A2, and H3B.A6 cells. Likewise, AP-2 protein can be detected by Western blot in stably transfected AP-2-expressing cells but not in the cells transfected with vector only (data not shown). To determine AP-2 DNA binding activities in these stable cells, EMSAs were performed using radiolabeled AP-2 probe. Significant AP-2-binding activities were detected in H3B.A1, H3B.A2, and H3B.A6 cells, whereas no detectable AP-2-DNA complexes were observed in H3B.V1, H3B.V2, and H3B.V3 cells (Fig. 7B). For comparison, HeLa cell extracts were also incubated with the AP-2 probe. A strong AP-2-DNA complex with the same electrophoretic mobility as those with the stable cell lines was detected. This protein-DNA

**FIG. 5.** Competition of various oligonucleotides from liver gene promoters for AP-2 binding. The distal AP-2-binding sequence from the SAA1 promoter was radiolabeled and used as probe in EMSA. HeLa cell nuclear extracts were incubated with labeled probe in the presence of increasing amounts of double-stranded oligonucleotide competitors. The competing oligonucleotides were from the promoters of rat SAA1 (AP-2 site), human C3, human apolipoprotein B (ApoB), rat phosphoenolpyruvate carboxykinase (PEPCK), rat α1-acid glycoprotein (α1-AGP), α-fibrinogen (α-FG), and apolipoprotein E (ApoE). The efficiency of competition was calculated and presented as the amount of oligonucleotides required for 50% inhibition of AP-2-DNA complex formation.
The expression levels of these genes in AP-2-expressing cells were calculated relative to those in the vector control cells, to which a value of 100 was assigned.

**DISCUSSION**

Transcription factor AP-2 joins a growing list of transcription regulators that are able to function as either transcription activators or transcription repressors depending on the cellular and promoter context of their target genes (13, 14, 54). Initially characterized as an activating protein in SV40 gene transcription (21, 22), AP-2 has since been shown to participate in the transcription activation of many other genes, including those related to neuronal and epidermal development (23–25, 55, 56). More recently, AP-2 was also shown to function as a transcription repressor in repressing the expression of cell type-specific genes (29, 31).

Expression of SAA1 is highly inducible by the proinflammatory cytokines and is highly tissue-specific, being expressed primarily in the liver. Our earlier studies identified a 66-bp CRU in the SAA1 promoter that is responsible for its cytokine-induced regulation (41, 50). Within the CRU, two cis-regulatory elements, the NFκB- and C/EBP-binding sites, were essential for its promoter responsiveness to cytokine stimulation (40, 50). In addition to the CRU, we identified two DNA regulatory elements that have the characteristics of transcription silencers and can serve as binding sites for the transcription factor AP-2. Interestingly, AP-2 is expressed abundantly in HeLa and many other nonliver tissues but is absent in liver or liver-derived hepatoma cells (57, 58). This inverse correlation between AP-2 and SAA1 expression is consistent with the repressive function of AP-2 on SAA1 gene transcription and may...
contribute to liver-specific expression pattern of SAA1 by diminishing or abrogating its expression in nonliver cells.

This study together with our earlier studies leads us to formulate a model for the regulatory mechanisms that control the inflammation-induced and tissue-specific expression characteristics of SAA1 (Fig. 8). In nonstimulated hepatic cells such as HepG2, YY1 functions as a constitutively expressed transcription repressor that occupies its site in the CRU and accounts for the low basal expression of SAA1. In response to stimulation, inflammatory mediators dramatically increase the activities of the positive transcription factors NFκB and C/EBP in the nucleus. In the absence of AP-2, as in liver cells, two potent transactivators could displace YY1 and bind to their respective binding sites in the SAA1 promoter and function cooperatively to activate SAA1 gene transcription (Fig. 8). In HeLa cells, however, in addition to YY1, AP-2 is also constitutively expressed and could bind to its respective site in the CRU. Because of its higher binding affinity, AP-2 prevents activated NFκB from binding to the overlapping binding site. Consequently, without NFκB binding, the SAA1 promoter cannot respond to cytokine induction despite the presence of activated NFκB and C/EBP in the nucleus. Thus, in this case, the tissue-specific expression of AP-2 itself plays an important role in determining the expression pattern of SAA1. As it is noteworthy that removal of the repressive effects of AP-2 in HeLa cells by mutating the two AP-2 sites did not lead to full activation of the SAA1 promoter. This may reflect the nature of the cellular milieu in HeLa cells, which is not optimum for SAA1 gene transcription. For example, compared with HepG2 cells, HeLa has relatively low levels of C/EBP, a critical transcription factor for SAA1 gene activation. In addition, results from AP-2-expressing cell lines suggest that AP-2 is likely not the only repressor that inhibits SAA1 promoter activity in nonhepatic cells.

Five lines of evidence support the notion that AP-2 plays a central role in repressing SAA1 expression. First, two high affinity AP-2-binding sites were localized to within 300 bp of the SAA1 gene transcription initiation site. Moreover, protein binding studies indicated that the proximal AP-2 binding sequence that overlaps with that of NFκB binds to AP-2 with significantly higher affinity than NFκB at these overlapping sites. The fact that AP-2 binds with higher affinity and the constitutive presence of AP-2 in the nucleus plus the normally inactive state of NFκB in nonstimulated cells gives AP-2 a distinct advantage over NFκB in competing for the overlapping binding sites. Consequently, in cells that express AP-2, i.e. many nonliver cells, this overlapping site would normally be occupied by AP-2 and prevent subsequent NFκB binding. Thus, AP-2 may mediate repression on the SAA1 promoter by antagonizing the function of NFκB. Second, a 20-bp region spanning the NFκB site to the AP-2p site is 100% conserved between rat and human SAA1 genes and 80% with that of mouse SAA1 gene (40, 52). Such high sequence conservation suggests functional importance of this region. Third, forced expression of AP-2 in HepG2 cells efficiently inhibited CM-mediated induction of the SAA1 promoter. This inhibition was highly specific because mutation of the AP-2-binding sites or expression of a dominant-negative mutant of AP-2 relieved AP-2-mediated inhibitory effects. Fourth, whereas the wild-type SAA1 promoter is nonresponsive to cytokine stimulation when transfected into HeLa cells, mutation of AP-2-binding sites led to derepression of the promoter and responsiveness to cytokine induction. Finally, stable expression of AP-2 in Hep3B cells significantly reduced endogenous SAA1 gene expression in response to inflammatory cytokines.

Transcription repression may be achieved by various mechanisms. In principle, negative regulators could inhibit transcription by interfering with any of the several steps in the transcription initiation pathway (60, 61). A repressor might interfere with the activity of a DNA-bound activator by quenching or masking its activation potential (62). Alternatively, it may block nuclear localization of an activator (63). In addition, negative regulators might interfere with the function of general transcription machinery (6, 64, 65). Finally, repressors might block transcription activation by binding to DNA at a region that overlaps that of transcription activators, thus excluding the binding of activators (26, 41). In the rat SAA1 promoter, one possible mechanism by which AP-2 represses the SAA1 promoter in HeLa cells is by blocking the activity of the transactivator NFκB. In this regard, it is also interesting to note that although the 3′ region of NFκB-binding site overlaps with the AP-2p site, its 5′ region overlaps with the binding site of another transcription repressor YY1 (41). However, unlike AP-2, YY1 is expressed in all tissues examined, including the liver, and modulates the cytokine responsiveness of the SAA1 promoter rather than controls its cell-specific expression. Another
distinguishing feature between these two repressors is that whereas NFκB is unable to displace prebound AP-2 at the overlapping region, it can readily displace prebound YY1 (41). Because the binding between AP-2 and NFκB and between YY1 and NFκB at their respective overlapping sites is mutually exclusive, the function of NFκB is therefore opposed by two repressors, with relative binding affinities at these overlapping sites of AP-2 > NFκB > YY1.

Transcription repressors that can function as general negative regulators and contribute to cell differentiation have been reported (10, 12, 66–68). One such negative regulator is the neuron-restrictive silencer factor, which could potentially repress a large number of neuron-specific genes in nonneuronal cells (10, 12). In this regard, the function of AP-2 in repressing liver gene expression in nonliver cells may resemble that of neuron-restrictive silencer factor. In addition to the SAA1 and C3 promoters, we identified potential AP-2-binding sites in at least 10 other liver gene promoters. Several of these sequences could effectively compete for AP-2 binding in EMSA, implying AP-2 may also bind to these promoters and perhaps regulate their expression. Thus, AP-2 may, by restricting its expression in AP-2-expressing nonliver cells, help regulate the expression of many liver genes in a cell-specific manner.

Our results indicate that AP-2, when expressed in a liver cell background, is capable of conferring repression not only on cytokine-inducible genes but also on constitutively expressed liver-specific genes. The reduced expression of SAA, albumin, and AFP was not due to nonspecific effects caused by the manipulation of cells during G418 selection because (a) the comparisons were made with control Hep3B cell lines that were transfected with an empty vector and were selected in a similar manner, (b) consistent repression was observed in three independent AP-2-expressing Hep3B cell lines, and (c) the expression of two housekeeping genes, GAPDH and β-actin, in these cell lines was not altered. Therefore, together with our transient transfection studies, these results further support the notion that AP-2 may have broad functional role as a general negative regulator in repressing the expression of some liver genes in nonhepatic cells. Although AP-2 may possess the negative regulator in repressing the expression of some liver genes, it is not dependent on functional AP-2-binding sites and could be neutralized by a dominant-negative mutant of AP-2. In addition to SAA1 and C3, 10 other liver gene promoters were found to contain potential AP-2-binding sites, some of which are very effective competitors for AP-2 binding. Finally, stable expression of AP-2 in a liver cell background significantly reduced the expression of several endogenous liver genes. Although it is tempting to speculate that the observed inhibition in human liver gene expression in nonliver cells but also as a contributor to the cell-specific expression of other liver genes.

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REFERENCES
1. McKnight, S. L. (1996) Genes Dev. 10, 367–381
2. Latchman, D. S. (1996) Int. J. Biochem. Cell Biol. 28, 965–974
3. Meijer, O. C., Williamson, A., Dallman, M. F., and Pearce, D. (2000) J. Neuroendocrinol. 12, 245–254
4. Chen, L. M., Karlsson, M., Nossal, G. J., Ye, Z. S., Jacks, T., and Baltimore, D. (1993) Genes Dev. 7, 570–582
5. Li, S., Crenshaw, E. B. d., Rawson, E. J., Simmons, D. M., Swanson, L. W., and Rosenfeld, M. G. (1996) Nature 382, 529–533
6. Johnson, F. B., and Krasnow, M. A. (1992) Genes Dev. 6, 2177–2189
7. Pabo, C. O., and Sauer, R. T. (1992) Annu. Rev. Biochem. 61, 1053–1095
8. Goodrich, J. A., Cutler, G., and Tjian, R. (1998) Cell 84, 825–830
9. Weisbrod, H. J., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benerez, R., Blackwell, T. K., Turner, D., Rupp, R., Hellenberg, S., Zhuang, Y., and Lassar, A. (1991) Science 251, 761–766
10. Jiang, J., Paquette, A. J., and Anderson, D. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9881–9886
11. Schoenherr, C. J., Paquette, A. J., and Anderson, D. J. (1996) Science 276, 1360–1363
12. Jiang, J., Rushlow, C. A., Zhou, Q., Small, S., and Levine, M. (1992) EMBO J. 11, 3147–3154
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Steel, D. M., Donoghue, P. C., O’Reilly, R. M., Uhlar, C. M., and Whitehead, A. S. (1996) Scand. J. Immunol. 44, 493–500

Huang, J. H., Rienhoff, Jr., H. Y., and Liao, W. S. (1990) Mol. Cell. Biol. 10, 3619–3625

Kannan, P., Buettner, R., Chiao, P. J., Yim, S. O., Sarkiss, M., and Tainsky, M. A. (1994) Genes Dev. 8, 1258–1269

Li, X., and Liao, W. S. (1992) Nucleic Acids Res. 20, 4765–4772

Wilson, D. R., Juan, T. S., Wilde, M. D., Fey, G. H., and Darlington, G. J. (1990) Mol. Cell. Biol. 10, 6181–6191

Li, X., and Liao, W. S. (1992) Nucleic Acids Res. 20, 4765–4772

Reinke, R., and Feigelson, P. (1985) J. Biol. Chem. 260, 4397–4403

Beale, E. G., Chrapkiewicz, N. B., Scoble, H. A., Metz, R. J., Quick, D. P., Noble, R. L., Donelson, J. E., Biemann, K., and Granner, D. K. (1985) J. Biol. Chem. 260, 10748–10756

Fowlkes, D. M., Mullis, N. T., Comeau, C. M., and Crabtree, G. R. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2313–2316

Fong, K. Y., Botto, M., Walport, M. J., and So, A. K. (1990) Genomics 7, 579–586

McLean, J., Wion, K., Drayna, D., Fielding, C., and Lawn, R. (1986) Nucleic Acids Res. 14, 9097–9406

Lucero, M. A., Schaeffer, E., Cohen, G. N., and Zakin, M. M. (1986) Nucleic Acids Res. 14, 8692

McKinnon, W. K., McClelland, A., Roberts, M. P., and Ruddle, F. H. (1986) J. Cell Biol. 103, 1781–1788

Paik, Y. K., Chang, D. J., Reardon, C. A., Davies, G. E., Mahley, R. W., and Taylor, J. M. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3445–3449

Blackhart, B. D., Ludwig, E. M., Pierotti, V. R., Caiati, L., Onasch, M. A., Wallis, S. C., Powell, L., Rease, R., Knott, T. J., Chu, M. L., Mahley, R. W., Scott, J., McCarthy, B. J., and Levy-Wilson, B. (1986) J. Biol. Chem. 261, 15364–15367