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Regulation of Mouse Serum Amyloid A Gene Expression in Transfected Hepatoma Cells

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Expression of mouse serum amyloid A (SAA1, -2, and -3) mRNAs can be induced up to 1,000-fold in the liver in response to acute inflammation. This large increase is primarily the result of a 200-fold increase in the rates of SAA gene transcription. To analyze the *cis*-acting regulatory element(s) responsible for regulating transcription, we fused 306 base pairs of the mouse SAA3 promoter to a reporter gene, the chloramphenicol acetyltransferase (CAT) gene, and transfected this chimeric DNA into cultured cells. In transient expression assays, this 5′ sequence was sufficient to confer cell-specific expression: CAT activity was readily detectable when the construct was transfected into liver-derived cells but was not detectable in nonliver cells. Furthermore, when liver cells transfected with this construct were treated with conditioned media prepared from activated mixed lymphocyte cultures or with recombinant interleukin-1, a 10- to 15-fold increase in CAT activity was detected. Deletion analyses showed two regions of interest: a proximal region that enhanced CAT expression in a cell-specific manner and a distal region that conferred responsiveness to both conditioned media and recombinant interleukin-1. This distal responsive element had properties of an inducible transcriptional enhancer, and deletion of the proximal cell-specific region rendered the distal element responsive to stimulation by conditioned media in nonliver cells.

Infection, inflammation, or tissue injury initiates physiologic processes in most vertebrates termed the acute-phase response (24, 26). This response includes a change in the circulating plasma protein profile, reflecting synthesis and secretion of proteins involved in immune function and wound repair (13, 25). After tissue injury or infection, monocytes migrate to the site of injury and differentiate into macrophages. These activated macrophages secrete elevated levels of a number of protein factors, including interleukin-1 (IL-1), IL-6, and tumor necrosis factor (13, 36, 40). These circulating factors then transmit their signals to target cells, triggering the acute-phase response. One of the more remarkable phenomena in this response is the accumulation of high levels of plasma proteins present at very low or undetectable levels before induction. Serum amyloid A (SAA) (M_r = 12,000) is one of the major acute-phase proteins in mice and rises in plasma concentration from 0.5 μg/ml to greater than 1,000 μg/ml 24 h after injection of bacterial lipopolysaccharide (19, 32). This large increase in hepatic SAA synthesis is primarily a consequence of increased production of SAA mRNA at the transcriptional level (29, 34). Thus, the induction of SAA mRNA provides an excellent model for studying differential gene expression in response to a specific stimulus.

SAA circulates as an apolipoprotein of high-density lipoprotein (HDL) particles and comprises up to 20% of the total HDL protein during an acute-phase response (2, 12, 19). HDL particles containing SAA have a reduced half-life (10- to 30-fold) in the circulation, suggesting that SAA may facilitate HDL clearance (3). Continuous overproduction of SAA is always associated with chronic inflammation, which often results in secondary amyloidosis, an incurable and frequently fatal disorder (14, 15, 20).

The SAA gene family consists of three genes and a pseudogene (28, 46). Two genes, SAA1 and SAA2, have 96% sequence homology over a 3,215-base-pair (bp) region that includes the exons, introns, and 288 bp of 5′- and 443 bp of 3′-flanking DNA sequences. The third SAA gene, SAA3, diverges substantially from the other two genes; it is up to 70% homologous in the translated exons but less than 25% homologous in the introns and the 5′- and 3′-flanking DNA sequences. When SAA gene-specific probes derived from their unique 3′-terminal exons are used, quantitative measurements of SAA mRNA levels in normal animals and those of animals in the acute phase reveal that SAA mRNA increases approximately 1,000-fold, each gene (SAA1, -2, and -3) contributing an equal proportion (29, 33). The increase in liver SAA mRNA is mediated by a 200-fold increase in the rate of transcription from each of the three SAA genes (29). An analysis of the kinetics of SAA gene transcription and of total SAA mRNA accumulation suggests that SAA mRNA levels are regulated both by transcriptional and posttranscriptional mechanisms. Indeed, in the BNL cell line, a 150-fold rise in SAA3 mRNA was mediated entirely by a posttranscriptional RNA processing event (43).

Studies by Ramadori and colleagues showed a dose-dependent increase in murine SAA hepatic mRNA and SAA plasma protein concentrations after a single injection of purified recombinant murine IL-1 (41). Their studies also showed that primary cultured murine hepatocytes responded to both recombinant mouse IL-1 and purified human IL-1 in a dose- and time-dependent manner that could be inhibited by antibodies to IL-1.

In this study, we sought to identify and characterize the *cis*-acting regulatory sequences responsible for mediating both tissue-specific expression and responsiveness to IL-1.

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We report the identification of a region in the SAA3 promoter necessary for hepatocyte-specific expression and a second DNA region that mediates the IL-1-induced expression of SAA3.

MATERIALS AND METHODS

Cell lines. Three human hepatoma cell lines, Hep3B, HepG2, and PLC/5 (23), were generously provided by B. Knowles (Wistar Institute) and were grown in basal medium consisting of modified Eagle medium and Waymouth MAB (3:1, vol/vol) plus 10% fetal bovine serum (9). HeLa (human cervical carcinoma cell line), NIH 3T3 (mouse fibroblast cell line), and JEG-3 (human placental cell line) were cultured in modified Eagle medium plus 10% fetal bovine serum. All cells were passaged at confluence approximately once a week.

CM and IL-1. Mixed lymphocyte culture conditioned media (CM) were prepared by the method of Maizel et al. (30). These media were obtained by incubating human peripheral blood lymphocytes (10^6/ml) obtained from four healthy donors in RPMI 1640 medium containing 0.25% bovine serum albumin and 0.75% phytohemagglutinin (PHA M form; GIBCO Laboratories, Grand Island, N.Y.). Cells were cultured for 72 h at 37°C, and then the CM was centrifuged and the supernatant was filter sterilized. CM prepared in this manner is known to contain a wide variety of interleukins and monokines that mediate the acute-phase response (9, 13, 27).

Recombinant human IL-1α (specific activity, 2.5×10^9 U/mg) was kindly provided by P. T. Lomedico (Hoffman-La Roche, Inc.).

Plasmid DNAs. pCATβ′ was provided by C. Lowell (Johns Hopkins University School of Medicine). pE8.5 is a *Hin*dIII-*Hin*dIII BALB/c mouse genomic clone containing the SAA3 gene cloned into the *Hin*dIII site of pBR322 (28). For transfections, all plasmid DNAs were prepared by the alkaline lysis procedure (31), with two successive cesium chloride-ethidium bromide gradient centrifugations. Concentrations of DNA were determined by UV spectroscopy.

Plasmid constructions. Plasmids were constructed by standard methods (31). pSAA3/CAT(−306) was constructed by digesting pE8.5, the genomic mouse SAA3 clone, with *Bam*HI and *Sac*I. The 351-bp *Bam*HI-*Sac*I fragment (−306 to +45) was gel purified, and *Bam*HI linkers were ligated to each end. This fragment was then cloned into the *Bam*HI site of pCATβ′. Deletion mutants were generated by BAL 31 exonuclease digestion. The pSAA3/CAT(−306) was first linearized with *Hin*dIII and digested with BAL 31 for various times. The resultant DNA was purified and blunt ended, and *Cla*I linkers were ligated to the blunt ends. After purification, the DNA was digested with *Bam*HI, and the linear forms were gel purified and ligated into a pCATβ′ vector linearized with *Cla*I and *Bam*HI, thus removing the 625 nucleotides between the two sites. The ligation products were used to transform *Escherichia coli* HB101. The extent of BAL 31 digestion of individual clones was determined by sequencing, using the dideoxynucleotide chain termination method with [^{35}S]ATP and a primer to the *Eco*RI region of pBR322 contained in pCATβ′.

Plasmids containing only the cytokine-responsive element (cytokine RE) were generated by digesting pSAA3/CAT(−63) at the unique *Cla*I site and blunt ending with Klenow fragment. A 68-bp fragment (−185 to −118) from the SAA3 5′ *Bam*HI-*Sac*I fragment was generated by *Mbo*II digestion, gel purified, blunt ended with T4 DNA polymerase, and ligated into the pSAA3/CAT(−63) construct. Orientation and insert copy number were determined by DNA sequencing and restriction digests with the unique *Eco*RI and *Bam*HI restriction sites flanking the cloning site.

Transient transfection assay. Sixteen hours before transfection, cells were seeded into 100-mm culture dishes at a density of 5×10^5 to 10×10^5 cells per dish. Cells were transfected with 20 μg of chloramphenicol acetyltransferase (CAT) gene recombinant DNA by the Polybrene procedure (22). Four hours after transfection, cells were washed with Hanks balanced salt solution, subjected to a 25% glycerol shock for 2 min (37), and incubated for 16 to 20 h before treatment with CM or IL-1. To elicit the acute-phase response, cells were treated with 50% CM, which contained equal parts of medium from stimulated mixed lymphocytes and basal medium, or with IL-1-containing medium, which was basal medium with the addition of human recombinant IL-1α. As controls, transfected cells were treated in parallel with 50% control medium, which was prepared by mixing equal volumes of control medium (RPMI 1640 supplemented with 0.25% bovine serum albumin, 0.75% phytohemagglutinin, and antibiotics) and basal medium. Cells were harvested 18 to 24 h after treatment.

CAT assay. Transfected cells were harvested approximately 36 to 40 h after transfection, washed with phosphate-buffered saline, and suspended in 100 μl of CAT assay buffer (0.25 M Tris hydrochloride, pH 7.8). Cell lysates were prepared by four cycles of freezing (−70°C) and thawing (37°C) and by centrifugation at 12,000 rpm for 30 min. Supernatant fluids were collected and tested for protein content and CAT activity. Protein was quantified by the method of Bradford (4), using bovine serum albumin as a standard. The CAT assay was carried out as described previously (16), with modifications described by Cato et al. (6). Our standard assay contained 20 to 100 μg of protein, 0.1 μCi of [^{14}C]chloramphenicol (specific activity, 50 to 57 mCi/mmol; Amersham Corp., Arlington Heights, Ill.), and 1 mM acetyl coenzyme A in a final reaction volume of 120 μl. Reaction samples were incubated overnight at 37°C. The CAT reaction products were extracted with 1 ml of ethyl acetate and chromatographed on silica gel thin-layer chromatography plates, using chloroform-methanol (95:5, vol/vol). Quantitation of the CAT assays was performed by counting the [^{14}C]chloramphenicol spots corresponding to the acetylated and nonacetylated forms in a liquid scintillation counter.

RESULTS

Expression of an mSAA3-CAT hybrid gene. To understand the regulated expression of the mouse SAA3 gene, a restriction fragment of the SAA3 promoter containing 306 bp of proximal 5′-flanking and 45 bp of exon 1 untranslated sequences was inserted 5′ to the bacterial CAT gene (Fig. 1). This construct, pSAA3/CAT(−306), contained the bacterial CAT gene coding sequence, a simian virus 40 small-T-antigen splice site and polyadenylation signal, the ampicillin resistance gene, and the ColE1 origin of replication. pSAA3/CAT(−306) was transfected into three human hepatoma cell lines (Hep3B, HepG2, and PLC/5) and three nonliver cell lines (HeLa, NIH 3T3, and JEG-3) to determine whether transcription was limited to a specific cell type. To control for transfection efficiencies, pSV_2CAT DNA was transfected into duplicate cultures of the six cell lines. This control DNA contains the simian virus 40 enhancer and early promoter in an otherwise identical plasmid and is expressed in many

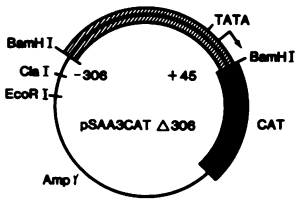

FIG. 1. Structure of the mouse SAA3-CAT hybrid gene construct. A 351-bp fragment (−306 to +45) of the mouse SAA3 gene sequence was excised by *Bam*HI-*Sac*I digestion of the genomic clone pE8.5 (42), *Bam*HI linkers were added, and the construct was ligated into plasmid vector pCATβ′ to produce pSAA3/CAT(−306).

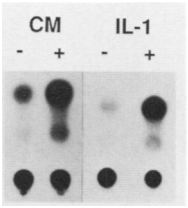

FIG. 3. Induction of the SAA3-CAT gene by IL-1. Hep3B cells (5×10^5) were transfected with 20 μg of pSAA3/CAT(−306) DNA. Approximately 24 h after transfection, cells were treated with (+) or without (−) 50% CM or with culture medium with (+) or without (−) 100 U of recombinant human IL-1 per ml. Cells were harvested 18 h later to determine protein concentration and CAT activity.

different cell types (16). pSV_2CAT was expressed in all six cell lines (Fig. 2). The pSAA3/CAT(−306) construct, however, was efficiently expressed only in Hep3B cells. Lower levels of CAT activity were detected in HepG2 and PLC/5 cells, and none was detected in the three nonliver cell lines. This result demonstrates the presence of regulatory sequences in the SAA3 promoter that enhance expression in hepatocyte-derived cells.

Induction of SAA3-CAT by CM. In response to inflammatory mediators, the liver hepatocyte is the major site of synthesis of many acute-phase proteins, including SAA3. To assay the responsiveness of the pSAA3/CAT(−306) construct to such inflammatory cytokines in cultured cells, transfected cells were subsequently stimulated with CM prepared from activated mixed lymphocyte cultures. This CM has been shown to contain polypeptide inflammatory mediators and can induce acute-phase gene expression when added to hepatocyte-derived cells (27). Transiently transfected cells were harvested after 18 h of CM treatment. In three transfected hepatoma cell lines treated with CM, CAT activity was enhanced 10- to 15-fold, whereas CAT activity was minimally altered in the three nonliver cell lines (Fig. 2). This induction was specific to those hepatoma cells transfected with the pSAA3/CAT(−306) construct, since these cells showed no change in CAT activity when transfected with the control vector pSV_2CAT (Fig. 2). These results indicate that the pSAA3/CAT(−306) construct also contains sequences responsive to inflammatory mediators. In addition, this response was cell specific in that CM-induced CAT expression was observed only in hepatoma cell lines, not in the three nonliver cell lines. Because of the relatively high basal expression and significant induction by CM, Hep3B cells were subsequently used for a more detailed analysis of the cytokines responsible for the induction, as well as of the regulatory elements within the 5′-flanking region of the mouse SAA3 gene.

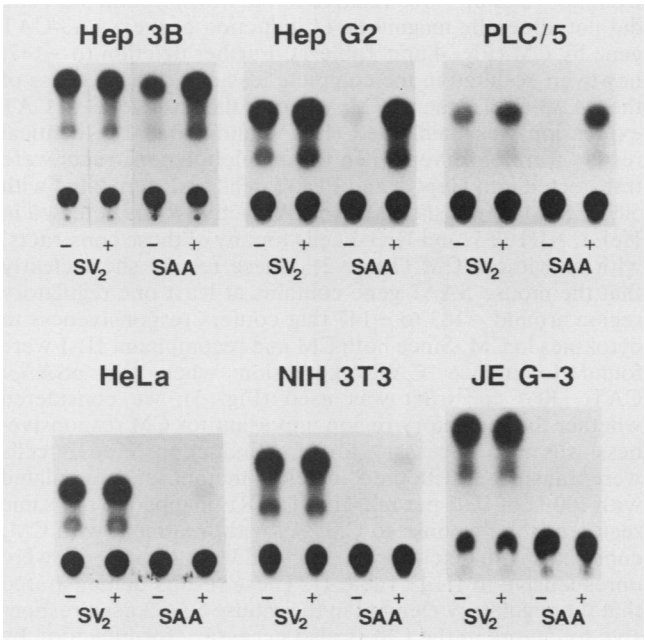

FIG. 2. Cell-specific expression and induction of the SAA3-CAT hybrid gene in cultured cells. Three human liver-derived cell lines (Hep3B, HepG2, and PLC/5) and three non-liver-derived cell lines (HeLa, NIH 3T3, and JEG-3) were transfected with 20 μg of pSAA3/CAT(−306) DNA (SAA) or pSV_2CAT DNA (SV_2) by the Polybrene procedure (22). Approximately 24 h after transfection, cells were treated with control medium (−) or 50% CM (+). After 18 h of treatment, cells were harvested for protein and CAT assays.

Induction of SAA3-CAT by recombinant IL-1. As described above, pSAA3/CAT(−306) was responsive to CM from activated human mixed lymphocyte culture. Because this CM is a mixture of a number of known cytokines with hepatocyte-stimulating activities, we sought to identify specific regulators by using purified recombinant cytokines. IL-1 has been shown to induce endogenous SAA gene expression in mice and in primary hepatocytes (41). We therefore assayed for the ability of IL-1 to regulate CAT expression using the pSAA3/CAT(−306) construct. Addition of human recombinant IL-1 (100 U/ml) to the transfected Hep3B cells resulted in a greater than 10-fold increase in CAT activity (Fig. 3). The magnitude of this induction with IL-1 approximated that with CM (Fig. 3). Treatment with IL-6, however, did not induce CAT expression, and treatment with tumor necrosis factor resulted in only two- to threefold stimulation of CAT activity (data not shown). These results indicate that IL-1 has a significant effect on the transcriptional induction of the CAT gene in Hep3B cells under the control of the mouse SAA3 promoter. Because CM consistently gives a greater induction than IL-1 alone, it is likely that other cytokines such as tumor necrosis factor that are known to be secreted by activated monocytes and macrophages (13) and present in CM play a role in regulating CAT expression in Hep3B cells. These data further suggest that activities other than IL-1 are likely to participate in the several hundred-fold induction of SAA3 gene expression in the liver.

Identification of 5′-flanking sequences that regulate liver-cell-specific expression. As a next step in identifying the DNA sequences in the mouse SAA3 gene necessary for cell-specific expression, portions of the 5′ ends of this region were progressively deleted by BAL 31 nuclease treatment. The structures resulting from the deletions are shown in Fig.

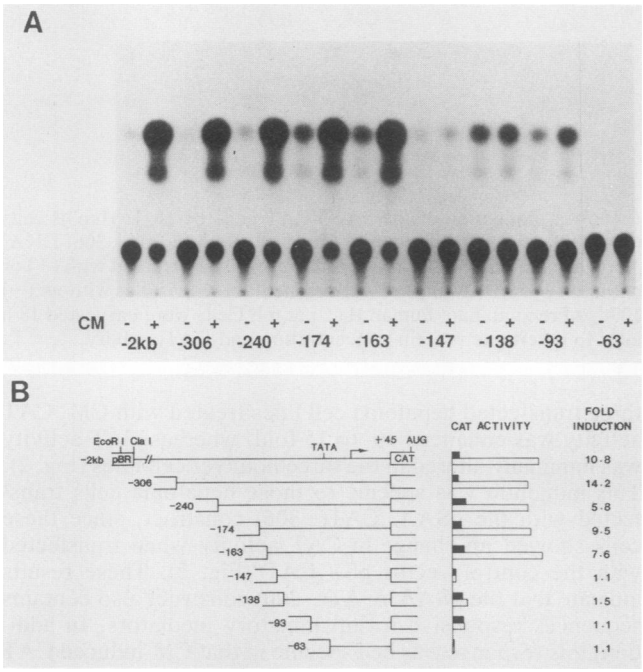

FIG. 4. Deletion analysis of SAA3-CAT fusion genes in Hep3B cells. Hep3B cells (5×10^5) were transfected with 5'-deletion mutants of the mouse SAA3-CAT hybrid gene. Treatment with CM and CAT activity determination were carried out as described in Materials and Methods. (A) Results of the paired CAT activity of each construct treated with (+) or without (−) 50% CM; (B) quantitation of the CAT assay for each mutant. Results are shown as the percentage of conversion of chloramphenicol to both acetylated forms of chloramphenicol in the absence (■) or presence (□) of CM. Fold induction is the ratio of induced (+) over uninduced (−) Hep3B cells.

4, as are their activities when transfected into Hep3B cells. Deletion of the 5'-flanking sequence from kilobase pair (kb) −2 to bp −93 did not have a significant effect on the basal level of CAT expression (Fig. 4 and Table 1). Basal CAT activity, however, was completely abolished with 5' deletions to −63, suggesting that the region around −93 to −63 is essential for basal expression of the SAA3 promoter. Furthermore, this basal activity was specific for liver-derived cells, since only very low levels of CAT activity were detected for any of these constructs when they were transfected into HeLa, NIH 3T3, or JEG-3 cells. Representative results from at least three independent transfections for −306, −93, and −63 mutants are shown in Table 2.

TABLE 1. Induction of SAA3-CAT constructs by CM and IL-1[a]

DNA construct	CAT activity		
	Control	CM	IL-1
pSV_2CAT	13.3	15.4	16.0
pSAA3/CAT(−306)	1.0	14.2	11.4
(−240)	2.5	14.1	17.5
(−174)	1.5	14.7	15.0
(−163)	2.2	17.0	9.2
(−147)	0.6	0.7	2.2
(−138)	1.9	2.8	1.7
(−93)	2.0	2.1	1.0
(−63)	0.1	0.1	0.2
RE(1+)	0.4	3.1	2.5
RE(1−)	0.3	2.5	2.3
RE(2)	5.0	24.9	19.6
RE(4)	5.1	21.8	19.3

[a] Hep3B cells were transfected with 20 μg of DNA of SAA3-CAT constructs and treated with 50% CM (CM) or 100 U of IL-1 per ml (IL-1) for 18 h. CAT activity was quantitated by scintigraphy. Values represent the means of at least three independent transfections with at least two different preparations of each plasmid and are expressed relative to the level of CAT activity in Hep3B cells transfected with pSAA3/CAT(−306), which was assigned a value of 1.0.

TABLE 2. Relative levels of CAT activity in cultured cells transfected with SAA3-CAT mutants and stimulated with CM

DNA construct	CAT activity[a]							
	Hep3B		HeLa		NIH 3T3		JEG-3	
	−	+	−	+	−	+	−	+
pSV_2CAT	13.3	15.4	6.6	5.8	5.1	4.9	16.4	15.8
pSAA3/CAT(−306)	1.0	14.2	0.2	0.3	0.1	0.1	0.1	0.2
(−93)	2.0	2.1	0.1	0.2	0.1	0.2	0.2	0.2
(−63)	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1
RE(1+)	0.4	3.1	0.2	0.3	0.2	0.9	0.2	0.7
RE(1−)	0.3	2.5	0.1	0.7	0.1	0.5	0.1	0.4
RE(2)	5.0	24.9	0.4	4.2	4.5	7.4	3.4	12.7
RE(4)	5.1	21.8	2.7	17.8	2.8	4.1	3.8	10.3

[a] Values represent the means of three separate experiments with at least two different preparations of each plasmid and are expressed relative to the level of CAT activity in Hep3B transfected with pSAA3/CAT(−306), which was assigned a value of 1.0. Transfection and culturing with control (−) or 50% CM (+) was as described in the footnote to Table 1.

Identification of regulatory sequences responsive to cytokines. To identify the specific sequences in the SAA3 promoter that mediate the induction of CAT by IL-1 or CM, the 5'-deletion mutants described above were transfected into Hep3B cells and then stimulated with 50% CM. Deletions from kb −2 to bp −163 relative to the transcription start site did not affect the magnitude of induction of the SAA3-CAT gene by CM (Fig. 4 and Table 1). Further deletion to −147, however, resulted in the complete loss of responsiveness of the SAA3-CAT gene to CM, whereas the basal level of CAT expression was unaffected (Fig. 4 and Table 1). Identical results were observed when these deletion constructs were transfected into HepG2 and PLC/5 cells and stimulated with 50% CM (data not shown). No CAT activity was detected in HeLa, NIH 3T3, and JEG-3 cells for any of these constructs, with or without CM (Table 2). These results show clearly that the mouse SAA3 gene contains at least one regulatory region around −163 to −147 that confers responsiveness to cytokines in CM. Since both CM and recombinant IL-1 were found to induce CAT expression when the pSAA3/CAT(−306) construct was used (Fig. 3), we considered whether the regulatory region important for CM responsiveness was also necessary for IL-1 induction. Hep3B cells were transfected with the 5'-deletion mutants and stimulated with 100 U of IL-1 per ml. The IL-1 RE mapped to the same region as the response to CM. As with treatment with CM, constructs with deletions to −147, −138, −93, and −63 were unresponsive to IL-1 (Table 1). These results demonstrated that the regulatory element in the mouse SAA3 gene responsive to factors in the CM is also necessary for induction by IL-1.

The RE can function independently and in nonliver cells. To determine whether the RE could function as a general transcriptional enhancer, we constructed a series of recombinants by using the pSAA3/CAT(−63) mutant. The pSAA3/CAT(−63) mutant was found to have no detectable CAT activity when transfected into all six cell lines with or

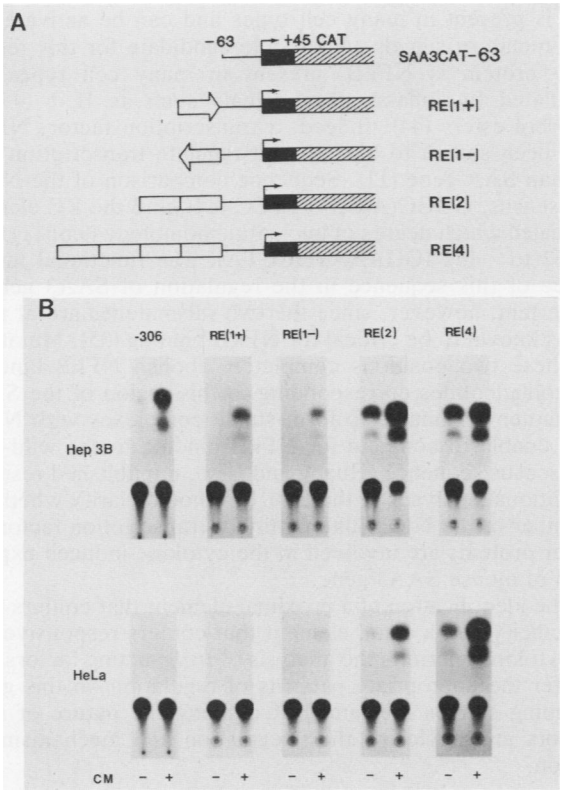

FIG. 5. Effect of RE on induction of the SAA3-CAT gene in cultured cells. (A) Constructs used. A 68-bp *Mbo*II restriction fragment (−185 to −118) was cloned into the unique *Cla*I site of pSAA3/CAT(−63) as described in Materials and Methods. Constructs containing one, two, and four copies of this fragment are shown. Arrows indicate orientations of the inserts with respect to the direction of transcription. Orientations of this fragment in the RE(2) and RE(4) constructs were not determined. (B) CAT activity in Hep3B and HeLa cells transfected with 20 μg of DNA and cultured as described in Materials and Methods.

without CM (Fig. 4 and Table 2). It does, however, contain an intact TATA box and sequences surrounding the SAA3 transcription start site. Because of the extremely low basal CAT activity, this construct provides a convenient assay for sequences affecting promoter strength. A 68-bp *Mbo*II restriction fragment (−185 to −118) containing the RE was isolated from the mouse SAA3 gene and subcloned into the pSAA3/CAT(−63) mutant at the unique *Cla*I site immediately 5′ of the SAA3 promoter. Constructs (Fig. 5A) containing a single copy of this RE in the same orientation [RE(1+)] with respect to the direction of transcription or in the opposite orientation [RE(1−)], two copies [RE(2)], or four copies [RE(4)] were transfected into Hep3B cells. Minimal CAT activity was detected with RE(1+) and RE(1−) in the absence of CM (Fig. 5B). The basal level of CAT expression increased significantly when either two or four copies of RE were present. With induction by CM, CAT activity was significantly enhanced, regardless of the basal CAT expression. Thus, this sequence can function in either orientation and respond to CM in a dose-dependent manner.

The constructs containing the distal RE but not the proximal liver-specific element were transfected into HeLa, NIH 3T3, and JEG-3 cells to test whether expression was permissive in nonliver cells. The cells were treated with 50% CM after transfection. Surprisingly, whereas pSAA3/CAT(−306) showed no CAT activity in these three nonliver cell lines regardless of treatment with CM (Fig. 2 and Table 2), all four constructs [RE(1+), RE(1−), RE(2), and RE(4)] responded in these cells to the CM stimulation (Fig. 5B and Table 2). The magnitude of response correlated with the copy number of the 68-bp RE, with the constructs containing four copies of the RE showing the greatest induction. Although the CAT activities were, in general, lower in these nonliver cells than in Hep3B cells, these results clearly demonstrate that the cytokine-inducible activity of the RE itself is not restricted to liver cells. They also demonstrate that HeLa, NIH 3T3, and JEG-3 cells all have the appropriate receptors for the cytokines and possess intact and functional signal transduction pathways leading to the ultimate transcriptional induction of the hybrid gene.

DISCUSSION

The expression of a number of acute-phase reactants is induced in response to various inflammatory cytokines and hormones produced during inflammation. Increased levels of SAA mRNA have been observed in the mouse after single injections with IL-1 or in hepatocytes after treatment with IL-1 (41). This investigation was therefore aimed at identifying the regulatory sequences in the mouse SAA3 gene important for its responsiveness to inflammatory cytokines in general and to IL-1 in particular.

Using the SAA3 promoter and 5′-flanking regions of the SAA3 gene fused to the CAT reporter gene, we demonstrated that this series of hybrid gene constructs when introduced into liver-derived cells was responsive to stimulation both by the CM derived from activated mixed lymphocyte culture and by purified recombinant IL-1. Furthermore, the cytokine responsiveness as well as the basal expression of this fusion construct were cell specific. This elevated basal CAT expression, particularly in Hep3B cells, was somewhat unexpected in view of the fact that SAA is expressed only at very low levels in the noninduced livers. These results, therefore, suggest that sequences outside of the SAA3 promoter region represented in our constructs may be important in suppressing SAA expression in normal livers. No CAT activity was detected in three non-liver-derived cells, HeLa, NIH 3T3, and JEG-3, either with or without cytokine stimulation, whereas CAT expression was induced by cytokines in the three liver-derived cells tested. By deletion analysis, we have identified in the 5′-flanking region of the SAA3 gene at least two important regulatory elements. The proximal element located around bp −93 and −63 relative to the start of transcription promotes more efficient SAA3 promoter-directed expression in cells of hepatic origin relative to the expression found in nonhepatic cells such as lymphoid and cervical cells. We anticipated that some liver-specific signals might exist in the SAA3 gene, given the very high concentration of SAA3 mRNA after induction of mice with lipopolysaccharide in the liver (34). In fact, at the peak of inflammation, SAA mRNA is one of the major mRNA species in liver, constituting approximately 2% of the total cellular mRNA (34). This short, 30-bp element bears no obvious sequence similarity to protein-binding sites found in several genes expressed at high levels in liver such as those encoding albumin (17), α_1-antitrypsin (18), fibrinogen (8), and transthyrethin (7). Perhaps the sequence that we have identified binds activator proteins such as C/EBP, which does not have a strict binding consensus sequence and is found in a variety of tissues but in high concentrations in liver (5). Alternatively, this sequence may be neutral in

hepatoma cells but suppresses transcription in other cell types. Such a negative regulatory element has been found in the promoter of the human α_1-antitrypsin gene, which is able to drive hepatoma-specific transcription from a heterologous promoter (10). Identifying the specific proteins recognizing this sequence and determining the effects of this sequence on other promoters in various cell lines will help resolve these questions.

We have also identified a sequence further 5′, between −185 and −118 relative to the transcription start site, that conferred responsiveness to activated mixed lymphocyte CM to the SAA3 promoter. Most of the induction by CM could be reproduced by using human recombinant IL-1, suggesting that IL-1 is among the more important inflammatory cytokines in the CM necessary for the induction of the SAA3 gene. The 10- to 20-fold effect of IL-1 on SAA3-driven CAT expression is hardly comparable to the several hundred-fold effect of lipopolysaccharide on hepatic SAA3 mRNA concentrations. However, our recent results showed that whereas IL-6 alone did not induce CAT expression, IL-6 and IL-1 acted synergistically to induce SAA3-CAT expression (J. Huang and W. S. L. Liao, unpublished results). Furthermore, treatment with tumor necrosis factor resulted in two- to threefold stimulation of SAA3-CAT expression. It is apparent from our results that many cytokines are likely to contribute to the regulation of SAA3 gene in the mouse liver. Whereas IL-1 is the major cytokine that regulates SAA3 gene expression, the expression of a number of other liver acute-phase genes, such as those encoding α_2-macroglobulin, hemopexin, and haptoglobin, is regulated primarily by IL-6, not IL-1 (1, 21, 38, 39). Thus, we have identified one cytokine RE in the mouse SAA3 gene, and other cytokine-responsive sequences are likely to be found. The possibility also exists that other sequences, e.g., in the 3′ untranslated region, regulating RNA processing and degradation may affect overall SAA3 expression (43). A surprising aspect of this study was the observation that expression of the intact SAA3-CAT hybrid gene was restricted to liver-derived cells; however, deletion of an internal 56-bp fragment (between −118 and −63) allowed expression in nonliver cells, albeit at significantly lower level than in Hep3B cells. Insertion of multiple copies of the RE resulted in a marked increase in both basal and induced expression in nonliver cells. It is not clear whether increased basal expression and the cytokine responsiveness of nonliver cells are due to the removal of an element that actively suppresses transcription in nonliver cells or to a distance effect from removing sequences between the TATA box and the RE. Recent studies showed that when this 68-bp RE was placed in front of simian virus 40 promoter, it conferred cytokine responsiveness to this heterologous promoter (unpublished data). Thus, this sequence functions as an enhancer.

The DNA element from −185 to −118 has been shown to act as an enhancer in BNL mouse cells but was not significantly regulated by cytokines because of high constitutive expression (42). In this study, we observed that regardless of cell type, liver- or non-liver-derived cells, this sequence was able to confer regulation in response to cytokine stimulation. In cells of hepatic origin this sequence was more active; whereas a single copy of the 68-bp fragment was sufficient to generate significant CAT expression in Hep3B cells, two copies were required for a comparable signal in HeLa cells. These results indicate that the cytokine receptors and the signal transduction pathways through which CM exerts its effect are functional in the cells tested. It further indicates that the transcription factor(s) that interacts with this 68-bp RE is present in many cell types and can be activated by inflammatory signals. A possible candidate for this regulatory protein is NFκB, present in many cell types and regulated by inflammatory signals such as IL-1 (45) or phorbol esters (44). Indeed, a transcription factor, NFκB, has been shown to bind to and regulate transcription of a human SAA gene (11). Sequence comparison of the NFκB consensus, GGGG(A/C)NTTTCC (44), and the RE element revealed a high degree of nucleotide homology (9 of 11) from −162 to −152 (GGAAATGCCTA). The functional importance of this sequence in the regulation of SAA3 gene is uncertain, however, since the two mismatches are at positions known to be critical for NFκB binding (35). Mutations at these two positions completely abolish NFκB binding. Oligonucleotides corresponding to this region of the SAA3 promoter are unable to form stable complexes with NFκB and unable to compete for NFκB binding to the wild-type consensus sequence (Huang and Liao, unpublished results). Additional analyses of the IL-1 RE should clarify whether a member of the NFκB-like family of transcription factors or other proteins are involved in the cytokine-induced expression of mouse SAA3 gene.

The identification of a proximal element that confers liver specificity and a distal element that confers responsiveness to cytokines permits the analysis of *trans*-acting factors that confer the appropriate patterns of regulation on this gene. Ongoing studies are aimed at defining the nature of such factors and exploring their regulation and mechanisms of action.

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