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Functional Characterization of the Bovine Conglutinin Promoter: Presence of a Novel Element for Transcriptional Regulation of a C-Type Mammalian Lectin Containing a Collagen-Like Domain[1]

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Bovine conglutinin is a Ca^{2+}-dependent serum lectin that is specific for *N*-acetylglucosamine and a member of the collectin (collagen-like lectin) family. Here we report the identification of the *cis*-acting elements involved in regulating expression of the conglutinin gene. The 5′-flanking region of the conglutinin gene was cloned and sequenced by gene walking using vector (cassette)-ligation mediated PCR. A genomic fragment encompassing −741 to +50 bp had significant promoter activity when linked to the luciferase reporter gene and transfected into the human hepatoma cell line HepG2. Transfection analysis using a series of luciferase vector/5′-stepwise deletion mutants of the promoter constructs indicated that the sequence of 7 base pairs at around −180 bp from the transcription initiation site was necessary for the full expression of the conglutinin gene. The site-directed mutagenesis in the AP-1 (Activator Protein-1) sequence, immediately downstream of the positively controlling *cis*-element at around −180 bp, resulted in a marked loss of the promoter activity. The novel positively controlling *cis*-element and the AP-1 sequence regulated synergistically the expression of the conglutinin gene. Gel retardation assay and DNase I footprint analysis demonstrated the presence of the nuclear proteins that bind to these two *cis*-elements.

Key words: animal lectin, collectin, conglutinin, gene expression, luciferase.

Conglutinin is a unique bovine plasma protein that mediates the agglutination (conglutination) of erythrocytes coated with activated complement components in the presence of Ca^{2+} (*1, 2*). Conglutinin binds to the carbohydrate moiety of iC3b in a Ca^{2+}-dependent manner (*3*), and conglutination is specifically inhibited by *N*-acetylglucosamine (*4*). About a decade ago, the protein was isolated and identified as a Ca^{2+}-dependent mammalian lectin specific for *N*-acetylglucosamine (*5, 6*). Studies on the primary structure of conglutinin (*7-11*) revealed that it shares unusual structural features with other animal C-type (Ca^{2+}-dependent) lectins (*12*) containing a collagen-like sequence (Gly-Xaa-Yaa). Namely, the subunit polypeptide of conglutinin (45 kDa, 351 amino acids) consists of a short N-terminal cysteine-rich region followed by a collagen-like sequence and a short linker or neck region, and a globular C-terminal carbohydrate recognition domain (CRD) (*12*). Thus conglutinin is now classified as a collectin (collagen-like lectin) (*13*). The collectin family includes two other serum lectins, mannan (mannose)-binding protein (MBP), and bovine collectin-43 (CL-43), and also pulmonary surfactant protein-D (SP-D) and -A (SP-A) (*14*). Conglutinin shows the same characteristic features of gene organization (*10, 15*) as the other collectins (*14*): the signal peptide, the N-terminal segment, and the first 6 Gly-Xaa-Yaa triplets are encoded by a single exon, the rest of the collagen-like sequence is divided among four separate exons of almost equal size, and the neck and CRD are each encoded by a single exon.

The biological significance of conglutinin has been poorly understood for almost a century. Conglutinin levels in dairy cattle decrease after calving, abortion, and infections (*16, 17*), and conglutinin titers of cows were found to be inversely proportional to the severity of experimentally induced infections with *Cowdria ruminantium* (*18*), sug-

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Abbreviations: AP-1, activator protein-1; CL-43, collectin-43; CRD, carbohydrate recognition domain; GRE, glucocorticoid responsive element; HNF-3, hepatocyte-specific nuclear factor-3; MBP, mannan (mannose)-binding protein; SP-A, pulmonary surfactant protein A; SP-D, pulmonary surfactant protein-D; TM, buffer consisting of 50 mM Tris/HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 20% glycerol, and 0.5 mM PMSF.

gesting that conglutinin is a consumable component of the serum. Recent studies demonstrated that conglutinin plays a role in host defense against invading organisms that are rich in surface *N*-acetylglucosamine, mannose, and fucose residues. Conglutinin has been reported to express antibacterial activity against *Escherichia coli* and *Salmonella typhimurium in vitro* (*19*), and to act as an opsonin in phagocytosis (*20*). More recently, conglutinin was reported to inhibit human immunodeficiency virus (HIV) (*21*) and influenza virus infections (*22, 23*). In view of these biological functions of conglutinin, considerable interest has been raised about the mechanisms controlling the conglutinin gene expression. To date, however, the nature of the transcriptional control mechanism of conglutinin has not been elucidated.

Previously we described the structure of bovine conglutinin cDNA, the exon-intron organization and the partial sequence of the 5′-upstream region of the conglutinin gene (*10*). Here we report the functional characterization of the conglutinin gene promoter and its biological significance with respect to the gene expression.

MATERIALS AND METHODS

Materials—Radionucleotides [α-^{32}P]dCTP (111 TBq/mmol) and [γ-^{32}P]ATP (222 TBq/mmol) were purchased from New England Nuclear Life Science Products (Boston, MA). Oligonucleotides used as probes and primers were custom synthesized by Cruachem (Kyoto). *Taq* DNA polymerase (*Thermus aquaticus* YT1), restriction enzymes, and other enzymes for DNA manipulations were purchased from Takara Shuzo (Kyoto), Toyobo (Osaka), Nippon Gene (Tokyo), Amersham Pharmacia Biotech (Bucks, UK), and Gibco BRL Life Technologies (Rockville, MD). Bovine liver was obtained from a slaughterhouse.

Polymerase Chain Reaction (PCR) Cloning of the 5′-Flanking Region of the Conglutinin Gene—The structure of the upstream region of the conglutinin gene was determined using a vector (cassette)-ligation mediated PCR technique as described previously (*10*). Briefly, the DNA fragments flanking the noncoding region of Exon I of the conglutinin gene were amplified by the nested PCR using bovine liver genomic DNA/pUC18 DNA ligates, which were prepared by digestion with restriction enzymes (*Eco*RI, *Xba*I, and *Hinc*II) followed by ligation, as a template, and with a combination of nonspecific forward primers having pUC18 plasmid vector sequence and specific reverse primers having sequences complementary to the noncoding region sequence of the 5′ region of the conglutinin cDNA (*10*). In the case of genomic DNA digested with *Sau*3AI, the restriction fragments were ligated with a synthetic oligonucleotide cassette possessing a 5′-cohesive overhang of the *Sau*3AI recognition sequence without phosphate on its 5′-end (Takara Shuzo), and the 5′-upstream fragment was amplified by nested PCR using the ligate as a template and a combination of cassette primer, C1 or C2 (Takara Shuzo), and the specific reverse primers. The PCR was carried out using a programmed incubator (Atto Zymoreactor II, Model AB-1820, Atto, Tokyo) with conditions as described previously (*10*). The amplified DNA fragments obtained by the third time nested PCR were purified by 2% agarose gel electrophoresis, extracted using the Geneclean II kit (Bio-101, CA) or the Quiex gel extraction kit (Qiagen GmbH, Germany), and subjected to direct sequencing on both strands using specific amplifying primers and pUC general primers with a DNA sequencer (Model 373A, Perkin Elmer, Applied Biosystem Division, CA) and the *Taq* DyeDeoxy Terminator Cycle Sequencing kit.

Luciferase Reporter Gene Constructs—To assay promoter activity, a series of reporter gene constructs comprising the 5′-flanking regions of the conglutinin gene, its 5′-stepwise deletions, and site-directed mutations were prepared and inserted into the polylinker region of the PicaGene basic vector (PGV-B, abbreviated BV) (Toyo Ink MFG, Tokyo), which was a promoterless and enhancerless vector containing the firefly luciferase gene. Plasmid constructions were initiated by amplifying a DNA fragment of the conglutinin gene containing 50 bp of Exon I and −741 bp of 5′-flanking sequences with PCR using genomic DNA of bovine liver as a template and a pair of primers: FP [−741], a forward primer corresponding to the nucleotide sequence of −741 to −722 bp with a *Kpn*I site on its 5′-end; and RP [50], a reverse primer complementary to the nucleotide sequences from +31 to +50 bp with a *Hin*dIII recognition sequence on its 5′-end (see Fig. 1). PCR conditions were as follows: extension at 72°C for 1 min, 30 cycles of three consecutive incubations, denaturation at 94°C for 48 s, annealing at 58°C for 36 s, extension at 72°C for 2 min 42 s, and final extension at 72°C for 7 min. The PCR product was gel purified and digested by *Kpn*I and *Hin*dIII, ligated into the BV vector at the *Kpn*I and *Hin*dIII site, and cloned by transforming competent *E. coli* JM109 (Nippon Gene). Plasmid DNA was prepared using Miniprep Kit Plus (Amersham Pharmacia Biotech) from randomly selected carbenicillin-resistant colonies, followed by selection of the colonies containing insert-positive plasmid DNA using PCR with a combination of GL-1 and GL-2 luciferase primers (Promega, WI). The construct was verified by double-stranded DNA sequencing and designated as BV/−741. The 5′-deletion mutations of the upstream region were produced by PCR with the cloned conglutinin gene/luciferase plasmid construct BV/−741 DNA as a template. Forward primers targeted to specific regions of the promoter were synthesized: FP [−629], FP [−492], FP [−355], FP [−193], FP [−173], FP [−166], FP [−159], FP [−138], FP [−114], FP [−83], and FP [−49]. All were 20mer oligonucleotides corresponding to the nucleotide sequences of the conglutinin promoter regions −629 to −610 bp, −492 to −473 bp, −355 to −326 bp, −193 to −174 bp, −173 to −154 bp, −166 to −147 bp, −159 to −140 bp, −138 to −119 bp, −114 to −95 bp, −83 to −64 bp, and −49 to −30 bp, respectively, and had the *Kpn*I recognition sequence on their 5′-ends (see Fig. 1). The reverse primer RP [50], as described above, was commonly used for the amplification of a series of 5′-deletion mutants. The gel-purified DNA fragments were double-digested with *Kpn*I and *Hin*dIII, and ligated into the BV vector at the *Kpn*I and *Hin*dIII site, generating subclones BV/−629, BV/−492, BV/−355, BV/−193, BV/−173, BV/−166, BV/−159, BV/−138, BV/−114, BV/−83, and BV/−49. BV/−180 was prepared as follows. The DNA fragment containing the promoter region between −193 and +50 was digested with *Bsp*1286I, 3′-end blunted with T4 DNA polymerase (DNA Blunting Kit, Takara Shuzo), and ligated into the BV vector, which

had been digested with *Sma*I and dephosphorylated with bacterial alkaline phosphatase. The DNA sequences of these cloned constructs were verified as above. The mutations of T to C substitution at positions −158, −154, and −161 bp and the deletion at −164 to −163 bp (−GT) in the promoter region from −355 to +50 bp were prepared using the Transformer Site-Directed Mutagenesis kit (Clontech Laboratories, CA) according to the manufacturer's instructions. Briefly, the mutagenic oligonucleotide primers containing substituted or deleted nucleotides (see Fig. 4) and *Nco*I selection primer, both of which had been phosphorylated with T4 polynucleotide kinase (Amersham Pharmacia Biotech), were annealed to the heat-denatured single-stranded target DNA, then the mutated strands synthesized. The target DNA used was the pUC19 plasmid DNA containing the promoter region sequences from −355 to +50 bp at the *Kpn*I and *Hin*dIII site. Positive mutants were selected by resistance to the *Nde*I digestion and transfected into BMH 71-18 *mut* S competent cells. The mutated plasmids were prepared, selected again by resistance to the *Nde*I digestion, and transfected into *E. coli* JM109 competent cells. The mutated promoters were verified by sequencing and cloned into the BV as described. The constructs containing a mutated site at positions −158, −154, and −161 bp, and deleted sites at −164 to −163 bp in the region from −355 to +50 bp are designated BV/−355 (mut A), BV/−355 (mut B), BV/−355 (mut C), and BV/−355 (mut D), respectively (see Fig. 4). The mutated DNA fragment at position −158 bp in the promoter region from −166 to +50 bp was amplified by PCR using BV/−741 DNA as a template, a mutagenic forward primer corresponding to −166 to −147 bp sequences, in which nucleotide T at position −158 bp was substituted by C, and the reverse primer RP [50]. The mutated fragments were ligated into the BV vector and the sequences of the cloned constructs were verified by DNA sequencing and designated as BV/−166 (mut A-2) (see Fig. 4).

Transfection and Enzyme Assay—Human hepatoma cell line HepG2 and human lung carcinoma cell line A-549 were purchased from American Type Culture Collection. COS-1 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal calf serum (Gibco BRL Life Technologies), 4 mM L-glutamine, and 60 μg/ml kanamycin at 37°C under a 5% CO_2 atmosphere. For DNA transfections, the plasmid DNAs of the conglutinin promoter/luciferase constructs and of pCH 110 (β-galactosidase eukaryotic assay vector, Amersham Pharmacia Biotech), which was used as an internal standard of transfection efficiency, were prepared using Quiagen tip-500 (Quiagen GmbH). Five micrograms each of the purified conglutinin/luciferase and pCH 110 plasmid DNAs were co-precipitated with calcium phosphate (*24*) and transfected into cells that had been seeded at 3×10^5 cells/35-mm tissue culture well. The transfection experiments were carried out using two different clones of each conglutinin/luciferase construct with triplicate plates for each clone. After 4 h, the plates were treated with 20% glycerol for 2 min and returned to 5% CO_2. After 16 h the culture medium was changed, and incubation was continued for 24 h before assay of cellular luciferase and β-galactosidase activities. The cells were washed twice with Dulbecco's phosphate-buffered saline (metal minus) (Nissui Pharmaceutical) and lysed in 150 μl of PicaGene LCβ cell lysis buffer (Toyo Ink MFG). After freeze-thawing (−80°C, 30 min/room temperature, 10 min), the lysed cells were collected and centrifuged in a microfuge to obtain the cell extracts. Luciferase activity was measured using a PicaGene luciferase assay kit (Toyo Ink MFG) and a Lumat LB9501 luminometer (EG&G Berthold, Germany). The β-galactosidase activity was determined using a substrate comprising 2.3 mM *o*-nitrophenyl-β-D-galactoside, 86 mM phosphate buffer (pH 7.15), 1.0 mM $MgCl_2$, and 110 mM 2-mercaptoethanol, and an EIA reader (Model 2550, Bio-Rad Laboratories, CA). The enzyme activities were measured in duplicate for each cell extract. Average luciferase activity divided by the average β-galactosidase activity of the same cell extracts was presented as a normalized relative light unit (NRLU).

Nuclear Extracts—Nuclear extracts of HepG2 cells and bovine liver tissues were prepared essentially according to the procedure of Dignam *et al.* (*25*) and Lichtsteiner *et al.* (*26*), respectively. The nuclear extracts were dialyzed against 0.1 M TM buffer, consisting of 50 mM Tris/HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 20% glycerol, 0.5 mM PMSF, and 0.1 M KCl. The dialyzed extracts were either stored at −80°C or partially purified by loading onto Heparin-Sepharose CL-6B (Amersham Pharmacia Biotech) which was equilibrated with 0.1 M TM buffer. Washing and elution were carried out as described by Dynan and Tjian (*27*). The fraction eluted in TM buffer containing 0.4 M KCl was used for DNase I footprint analysis. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories) with bovine serum albumin as a standard.

Gel Mobility Shift Assay—The gel retardation assay was performed essentially as described by Ausubel *et al.* (*28*). The oligonucleotide probes used in this study were prepared as follows. The upper and lower strands of the conglutinin promoter regions, which contain an unknown positively controlling *cis*-element (probe K) and the AP-1 consensus sequence (probe AP-1), each with the *Xba*I recognition site added to their 5′-end, were synthesized. The sequence of probe K corresponded to upper and lower strands of −191 to −167 bp, and that of probe AP-1 to upper and lower strands of −165 to −145 bp, which contains the AP-1 consensus sequence present at −158 to −152 bp. Sequences of oligonucleotides were as follows.

Probe K: 5′CTAGCTGAAGGTGCTCTTTGAACTCTAAT 3′
3′GACTTCCACGAGAAACTTGAGATTAGATC 5′

Probe AP-1: 5′CTAGTGTGTTTTGAGTCATGAACAT 3′
3′ACACAAAACTCAGTACTTGTAGATC 5′

*Xba*I sites are marked by double underlines, and the AP-1 consensus sequence by single underlines. Annealed oligonucleotides were labeled by filling in 5′ protruding ends with the Klenow enzyme, using [α-^{32}P]dCTP. The binding reaction was carried out in 12.5 μl of reaction mixture containing 10 mM Tris/HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 5% glycerol, 10,000 cpm (equivalent to about 1-5 fmol) of probe, 2-2.5 μg of poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech), and 3-10 μg protein of nuclear extracts (the crude preparation from HepG2 cells, or partially purified extracts from bovine liver tissue) at room temperature for 30 min. In competition experiments,

cold annealed oligonucleotides were added at 50-350-fold excess to hot probes with the nonspecific competitor poly(dI-dC)·poly(dI-dC). The DNA-protein complexes formed were separated by electrophoresis on 6% polyacrylamide gels (acrylamide/bisacrylamide, 29:1) containing 6.7 mM Tris/acetate (pH 7.5) and 1 mM EDTA at room temperature with buffer circulation. The gels were then dried, autoradiographed, and analyzed with BAS 2000 image analyzer (Fuji Film, Tokyo).

DNase I Footprinting Analysis—A DNA fragment extending from −315 to +50 bp of the conglutinin promoter was released by digesting one of the 5′-deletion mutants cloned into pUC19 plasmid DNA containing the promoter region sequences from −355 to +50 bp with *Xba*I, the recognition site of which was located between −316 and −311 bp, and *Hin*dIII, followed by phosphorylation with [γ-^{32}P]ATP and T4 polynucleotide kinase. After recleavage with *Hin*cII, the recognition site of which was located between −101 and −96 bp, the fragment (217 bp) containing singly end-labeled promoter region of −315 to −99 bp was purified by acrylamide gel electrophoresis. DNase I footprinting analysis was carried out essentially as described by Angel *et al.* (*29*). Binding reactions were carried out at 0°C for 15 min in a final volume of 50 μl of reaction mixture containing 50 mM Tris/HCl, pH 7.9, 0.1 M KCl, 1 mM EDTA, 12.5 mM $MgCl_2$, 1 mM DTT, 20% glycerol, 2% polyvinylalcohol, 1 μg of poly(dI-dC)·poly(dI-dC), 57 μg of protein of partially purified nuclear extract from Hep G2 cells, and 3-4×10^3 cpm of end-labeled DNA fragment (equivalent to about 10 fmol). Following the binding reaction, 1 μl of freshly diluted precalibrated DNase I (Takara Shuzo) solution (40 ng, 4 units/μg) in 2.5 mM $CaCl_2$-$MgCl_2$ was added, and digestion was carried out at room temperature for 2 min. The reactions were terminated by adding 100 μl of stop solution containing 1% SDS, 20 mM EDTA, and 200 mM NaCl, extracting with phenol and chloroform, and then adding 1 μl of Ethachinmate (Nippon Gene), followed by precipitation with ethanol. The samples and the products of G and G+A sequencing reaction (*30*) which were prepared using the same labeled fragment were coelectrophoresed on 6% sequencing gel. The gels were dried, autoradiographed, and analyzed by BAS 2000 image analyzer.

RESULTS

Cell-Specific Transcription of Luciferase Reporter Gene Driven by the 5′-Flanking Region of the Conglutinin Gene—The 5′-flanking region of the conglutinin gene was sequenced by gene walking with a vector (cassette)-ligation mediated PCR technique (*10*). The DNA fragments obtained by digestion with four restriction enzymes (*Eco*RI, *Xba*I, *Sau*3AI, and *Hin*cII) showed the completely identical nucleotide sequences in their overlapping regions. Figure 1 shows the 741 bp nucleotide sequence thus determined. The adenine nucleotide residue numbered 1 was assigned as the putative transcription initiation site based on the 5′-end sequence of conglutinin cDNA determined by the 5′-RACE method (*10*). To assess whether or not the 741 bp 5′-flanking region contains *cis*-acting promoter elements necessary for the controlled expression of the conglutinin gene in a panel of cell lines, we constructed a plasmid vector containing this region upstream of the promoterless luciferase gene in the expression vector BV. This construct was transfected by the calcium phosphate method into HepG2, COS-1, and A-549 cells, and transient expression of luciferase was measured (Fig. 2A). Plasmid pCH 110 containing the β-galactosidase reporter gene was used as a control to normalize for transfection efficiency between cell lines. Upon transfection into the human hepatoma cell line HepG2, a potent luciferase activity was observed with the full-length conglutinin promoter, which was approximately 50 to 100 times higher than the basal activity with vector alone. In contrast, only marginal luciferase activity could be detected in COS-1 (kidney fibroblast-like) and A-549 (human lung carcinoma) cells. Thus the 741 bp 5′-flanking

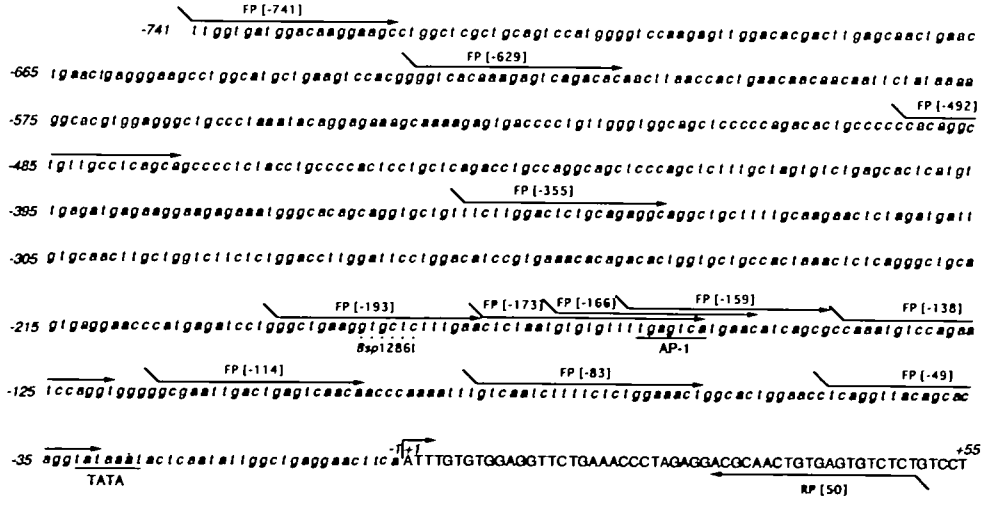

Fig. 1. **Conglutinin promoter sequence and locations of primers used to generate 5′-deletion mutants.** The genomic sequence extending −741 bp upstream and +55 bp downstream relative to the transcription initiation site +1 mapped by 5′-RACE (*10*) (indicated with the hooked arrow) is shown. The 5′-flanking region is shown in italic lower case and denoted by negative numbers, and a portion of Exon I is shown in roman upper case and denoted by positive numbers. The ranges of the sequences of primers used to generate the stepwise 5′-deletion constructs by PCR are indicated by arrows above and below the sequence. Forward primers (FP) are overlined and a reverse primer (RP) is underlined. Tails represent additional sequences containing restriction sites: forward and reverse primers contained *Kpn*I (5′ ATGGTACC 3′) and *Hin*dIII (5′ GCAAGCTT 3′) recognition sequences at their 5′-ends, respectively (see "MATERIALS AND METHODS"). The numbers in parentheses shown above and below the sequence indicate the 5′-ends of primers relative to +1. The recognition site for *Bsp*1286I is shown by the dotted underline. The TATA box and the AP-1 binding consensus sequences are underlined and marked.

sequence is transcriptionally competent in liver cells, thereby conferring cell-specific transcription. The average luciferase activity of the conglutinin gene observed in the HepG2 cell line was comparable to that obtained under the control of SV-40 early promoter (PV), and approximately 30% of that under the control of a SV-40 early promoter as well as an enhancer (CV) (Fig. 2B).

Identification of cis-Acting Elements on the Conglutinin Promoter—To identify the sequences responsible for controlling the conglutinin gene expression, we generated a series of 5′-deletion mutants of various lengths extending from −741 bp of the 5′-flanking region to +50 bp of the 5′-noncoding region of exon I by PCR. These fragments were placed upstream of the promoterless luciferase gene, and the constructs were transfected into the human hepatoma cell line HepG2. The luciferase activity expressed

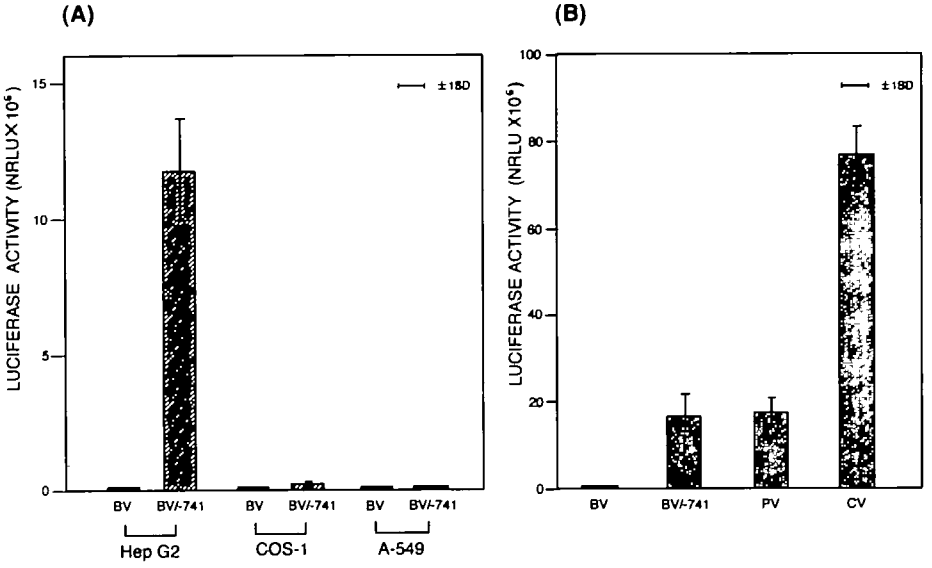

Fig. 2. **Transient expression of luciferase under the control of the 5′-flanking region of the conglutinin gene.** (A) Luciferase activities measured in HepG2, COS-1, and A-549 cell lines transfected with the luciferase reporter plasmid containing the conglutinin promoter. BV, promoterless and enhancerless luciferase reporter plasmid (PicaGene basic vector, PGV-B, Toyo Ink MFG) containing no conglutinin 5′-flanking region; BV/−741, luciferase reporter construct containing the conglutinin promoter ranging from −741 bp to +50 bp. (B) Luciferase activities measured in HepG2 cell line transfected with various luciferase reporter plasmids. BV and BV/−741, as described in (A); PV, luciferase reporter plasmid containing the promoter of the SV40 early gene (PicaGene promoter vector, PGV-P, Toyo Ink MFG); CV, luciferase reporter plasmid containing the enhancer and promoter of the SV40 early gene (PicaGene control vector, PGV-C, Toyo Ink MFG). Differences in transfection efficiencies are corrected by dividing the luciferase activity by the β-galactosidase activity, and the data shown here represent the normalized relative light units (NRLU) (mean±SD, represented by thin lines, of three experiments). Another experiment gave identical results.

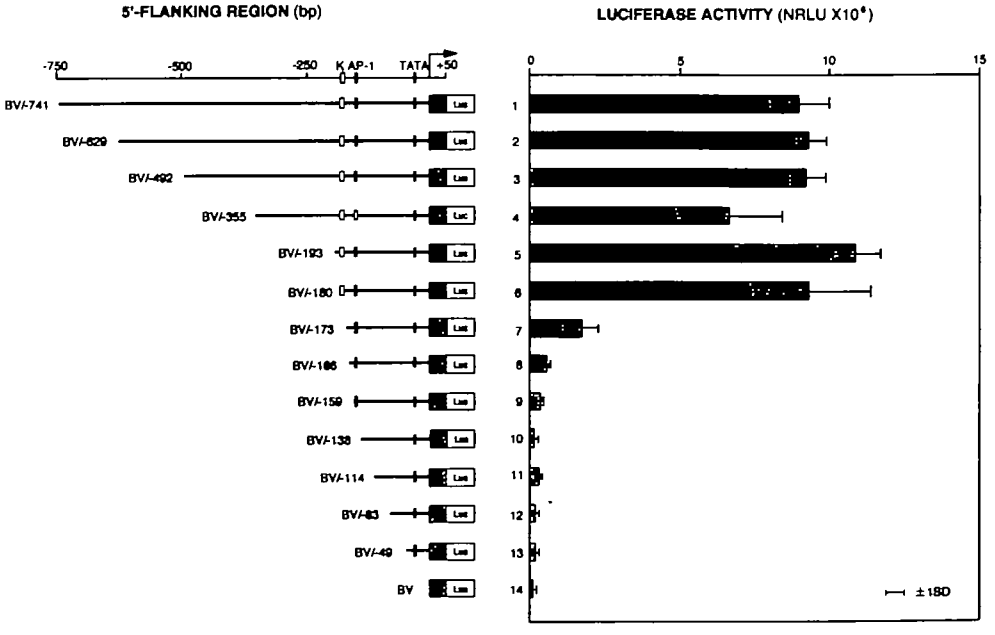

Fig. 3. **5′-Deletion analysis of the conglutinin promoter.** Left: Diagram of conglutinin promoter/luciferase reporter constructs. Stepwise 5′-deletions ranging from various 5′-end points in the 5′-flanking region to +50 bp in the untranslated portion of Exon I were constructed in luciferase reporter plasmid BV (see "MATERIALS AND METHODS" and Fig. 1). Solid lines represent conglutinin 5′-flanking sequence, hatched boxes represent 5′-portion of Exon I, and open boxes represent luciferase vector sequences. The transcription initiation site (+1) is designated by the hooked arrow. Deletion end points relative to the transcription initiation site (+1) are given to the left in base pairs. BV indicates the luciferase plasmid without the conglutinin promoter. K, AP-1, and TATA shown by vertical open boxes on the solid bar indicate the location of an unknown *cis*-element (designated K element, see text), AP-1 binding consensus sequence, and TATA box, respectively (see Fig. 1). Right: Luciferase reporter gene promoter assay. Each conglutinin promoter/luciferase reporter construct was transiently cotransfected into HepG2 cells with a pCH110 β-galactosidase reporter plasmid and standardized to the efficiency of transfection, as described in Fig. 2. Hatched bars represent the luciferase activities of the constructs corresponding to the deletion mutants shown in the left diagram. Values shown are the results of three to five independent experiments, in which individual separate plasmid constructs were used for expression, with CV transfection (see Fig. 2B) as a positive control of expression.

indicated that deletion of sequences to within 193 bp of the transcription start site had little effect on promoter activity (Fig. 3, lanes 1-5). A marked drop in activity, however, was observed with deletion of the region between −193 and −173 bp (Fig. 3, lanes 5 and 7). To focus closely on the most crucial region between −193 and −173 bp, a truncated promoter fragment was generated from the construct BV/−193 by digestion with restriction enzyme *Bsp*1286I, the recognition site of which was located between −185 and −180 bp (see Fig. 1 and "MATERIALS AND METHODS"). The −180 bp deletion mutant produced luciferase activity similar to that observed with −193 bp deletion mutant (Fig. 3, lanes 5 and 6). Thus a region within the sequence from −180 to −174 bp should serve as a regulatory element. Since a computer-assisted analysis of the sequence located between −180 and −174 bp performed using TFSEARCH (ver. 1.3, Parallel Application TRC Laboratory, RWCP, Japan) and the database supplied with Transfac Matrix Table (Release 3.3) (*31*) revealed no consensus binding sequence for known transcription factors, we putatively designate the sequence around −180 bp as conglutinin (abbreviated 'K', see Ref. *2*) element.

During the luciferase-transient expression study using 5′-deletion mutants of the conglutinin promoter, we happened to observe a marked reduction of the promoter activity with a particular clone of one of the BV/−492 constructs. Subsequent study of this particular clone indicated that the nucleotide T at −158 bp is converted to C [Fig. 4, BV/−492 (WT) *versus* BV/−492 (mut A-1)] as a result of a mutation occurring during the PCR amplification of the 5′-upstream fragment and cloning into the BV vector. This artificial point mutation was found only on that particular clone out of the 44 clones of the various deletion mutant constructs examined which contained the AP-1 binding consensus sequence. The position at −158 bp, which is crucial for transcriptional regulation, was found to be the initial base of the AP-1 binding consensus sequence (TGAGTCA), which was located immediately downstream of the above-mentioned K element. The requirement of this AP-1 binding sequence was ascertained by the following mutation experiment. The conglutinin promoter/luciferase construct containing the 5′-flanking region from −355 to +50 bp (BV/−355 in Fig. 3) was mutated at four positions with respect to the AP-1 site by site-directed mutagenesis. Upon mutation of thymine at −158 bp to cytosine or thymine at −154 bp to cytosine, the promoter activity was markedly reduced to 10% of the wild-type level [Fig. 4, BV/−355 (WT) *versus* BV/−355 (mut A) or BV/−355 (mut B)]. In contrast, mutation of thymine at −161 bp, which is in the 5′-flanking region of the AP-1 sequence, to cytosine showed no effect on the promoter activity [Fig. 4, BV/−355 (mut C)]. The functional importance of the initial base of the AP-1 sequence was also observed in the construct containing the −166 5′-flanking fragment [Fig. 4, BV/−166 (mut A-2)]. Interestingly, however, the AP-1 sequence alone was not sufficient for the expression of the full promoter activity, as shown in lanes 7, 8, and 9 of Fig. 3. Thus, constructs containing the AP-1 sequence but not the *cis*-acting K element did not show any significant promoter activity. Therefore, the efficient *in vitro* transcription of the conglutinin gene requires both the unknown *cis*-element at around −180 bp and the AP-1 sequence at −158 to −152 bp. In addition, the deletion of two bases, guanine at −164 bp and thymine at −163 bp, lying between the *cis*-acting K element and the AP-1 sequence resulted in a significant reduction of the promoter activity, to approximately one-third that of the wild-type sequence [Fig. 4, BV/−355 (mut D)]. This suggested that the distance between AP-1 and the unknown K element is very important to their cooperative function.

Detection of DNA/Protein Interaction Using Gel Mobility Shift Assay—Deletion and mutation analyses of the 5′-flanking region indicated that the sequence from −180 to −174 bp and the immediately downstream AP-1 binding sequence served as regulatory sites involved in DNA/protein interactions (see Fig. 3). To examine the ability of these sequences to interact with *trans*-acting factors, ^{32}P-

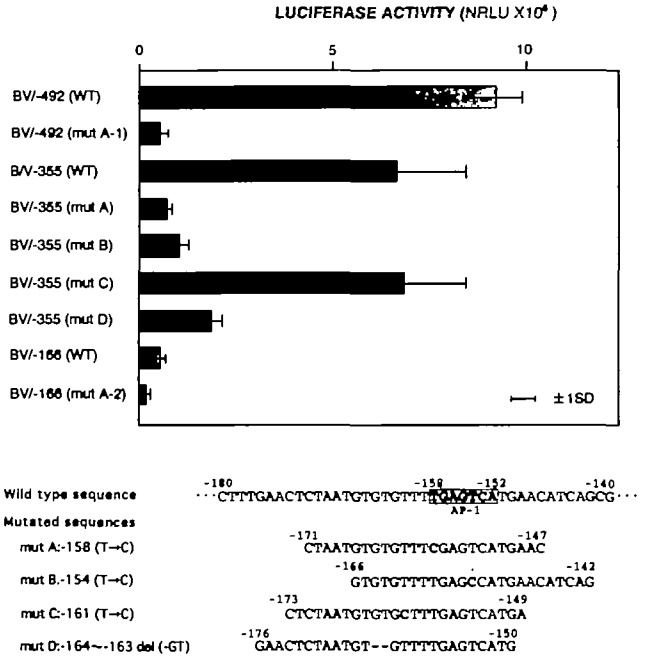

Fig. 4. **Effects of mutations with respect to the AP-1 binding consensus sequence on transcriptional activity of the conglutinin promoter/luciferase reporter constructs.** Conglutinin promoter/luciferase construct BV/−355 in Fig. 3 was mutated at four positions by site-directed mutagenesis using the following mutated oligonucleotide primers (see "MATERIALS AND METHODS"). Mutant A, thymine at −158 bp, the initial base of the AP-1 binding consensus sequence (−158 to −152 bp), which is boxed in the wild-type sequence, is converted to cytosine; mutant B, thymine at −154 bp, within the AP-1 sequence, is converted to cytosine; mutant C, thymine at −161 bp, in the 5′-flanking region of the AP-1 sequence, is converted to cytosine; mutant D, guanine at −164 bp and thymine at −163 bp, which lie between an unknown *cis*-acting 'K' element at around −180 bp (see Fig. 3 and text) and the wild-type AP-1 sequence, are deleted. Dots above the base designate mutation sites and lines between the bases designate deletion sites. The mutated oligonucleotides A, B, C, and D correspond to −171 to −147 bp, −166 to −142 bp, −173 to −149 bp, and −176 to −150 bp, respectively, in the 5′-flanking region of the conglutinin gene. Mutant A was also generated in the constructions BV/−492 and BV/−166, which were designated mut A-1 in BV/−492 and mut A-2 in BV/−166, respectively (see "MATERIALS AND METHODS" and text). Shaded columns represent the luciferase activities obtained with the wild-type constructs, and solid columns represent those obtained with the mutated constructs. Luciferase activities are normalized to β-galactosidase activity resulting from cotransfection with a pCH110 β-galactosidase reporter gene, as described in Fig. 2.

labeled double-stranded deoxyoligonucleotides encompassing each region were synthesized and used for gel mobility shift assays. After incubation with nuclear extracts prepared from HepG2 cells, the reaction products were analyzed by electrophoresis in low ionic strength polyacrylamide gels. The DNA-protein complexes were separated from the labeled probe K (Fig. 5A, lanes 1-5) or probe AP-1 (Fig. 5A, lanes 6-8). The K element bound strongly to nuclear proteins (lane 2), and this binding was reduced by competition with the unlabeled probe K (lanes 3 and 4), but not with the unlabeled AP-1 (lane 5), indicating the high specificity of the interaction between the K element and nuclear proteins. The region containing the AP-1 binding sequence at −158 to −152 bp also bound to nuclear proteins (lane 7), and the binding was reduced by competitive unlabeled AP-1 (lane 8).

In the next experiments, we examined the binding of the K element and AP-1 with nuclear protein from bovine liver tissues. As shown in Fig. 5B, specific DNA-protein complexes were also observed with nuclear extracts from bovine liver cells. The shifted bands formed by the K element (lane 2) were completely abolished by competition with the unlabeled K probe as in the case of HepG2 nuclear extracts, but not with the unlabeled AP-1 probe (lanes 3-5). The labeled AP-1 probe was also shifted with the nuclear proteins from bovine liver cells, and the complex formation was inhibited by the competitor cold AP-1. Thus these results demonstrated the presence in bovine liver cells of nuclear factors that specifically bound to the regulating regions identified by the reporter gene assays. In these shifted bands with either probe K or probe AP-1, the fastest migrating component(s), which was not reduced with 250-fold excess of the unlabeled probe K, seemed to be due to nonspecific binding.

DNase I Footprint Analysis of Factors Binding to the Conglutinin Promoter Region—To identify nuclear factor binding sites involved in transcription regulation, we carried out DNase I footprint analysis of the DNA fragment from −315 to −99 bp of the conglutinin promoter region, with the results shown in Fig. 6. Upon incubation with the nuclear extracts from HepG2 cells, two distinct protected regions (regions A and B) were detected upstream of the transcription start site. Region A at positions −183 to −167 bp overlaps with the *cis*-acting K sequence at −180 to −174 bp as revealed by the reporter gene assay. Thus a factor binding to this relatively limited range of the A region may be positively controlling *trans*-acting transcription factor. A computer search indicated no consensus sequence for previously characterized factors in region A. The factor, that binds to this region, therefore, appears by these criteria to represent a novel DNA-binding protein. Region B at positions −161 to −151 bp overlaps with the known AP-1 consensus binding sequence at −158 to −152 bp. Thus the AP-1 factor appears to be a positive regulatory factor cooperating with the immediate upstream unknown *trans*-acting factor described above. Essentially the same results were obtained by footprint analysis using the nuclear extracts from bovine liver tissues (data not shown). These results are consistent with the results obtained by transient expression studies and gel retardation assays as described. Other partially protected regions were detected downstream of the AP-1 sequence at around −115 to −143 bp, but analysis by transient expression could not find any promoter activity around this area, and the functional significance of this region remains unresolved.

DISCUSSION

In this report, we have characterized the bovine conglutinin gene promoter and identified regulatory *cis*-elements re-

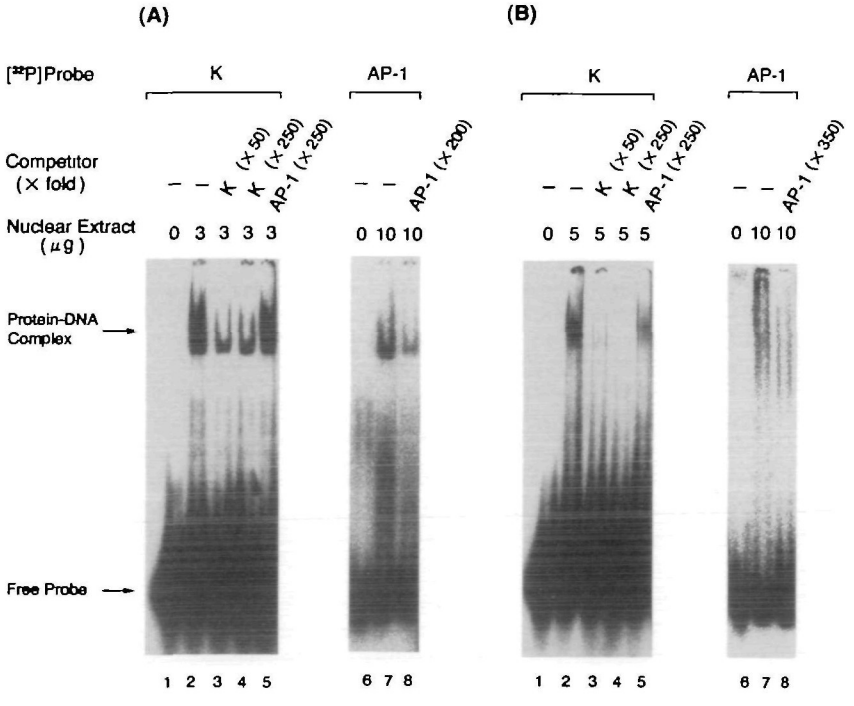

Fig. 5. **Gel mobility shift assay with the positive 'K' *cis*-element and the AP-1 binding sequence oligonucleotides.** (A) Mobility shift assay performed with nuclear extracts from HepG2 cells. The double-stranded deoxyoligonucleotides probe K and probe AP-1, which contained the 'K' *cis*-element at around −180 bp and the AP-1 binding sequence at −158 to −152 bp, respectively (see "MATERIALS AND METHODS"), were 5′-end-labeled and incubated with unfractionated nuclear extracts from HepG2 cells in the absence (−) or presence of unlabeled competitor nucleotides (as indicated at the top of the lanes). The binding-reaction mixtures were loaded on a 6% nondenaturing polyacrylamide gel to allow separation of free (indicated by lower arrow) and retained DNAs (upper arrow). The identities of the labeled probes and unlabeled competitor nucleotides, the amounts of the competitor (relative to labeled probes), and the amounts of nuclear extract (μg protein) are given on the top of the lanes. (B) Mobility shift assay performed with nuclear extracts from bovine liver tissues. The labeled probes, K and AP-1, were incubated with 0.4 M TM heparin-agarose fraction of nuclear extracts from bovine liver tissues in the absence (−) or presence of unlabeled competitor nucleotides (as indicated at the top of the lanes). Other conditions are as described in (A).

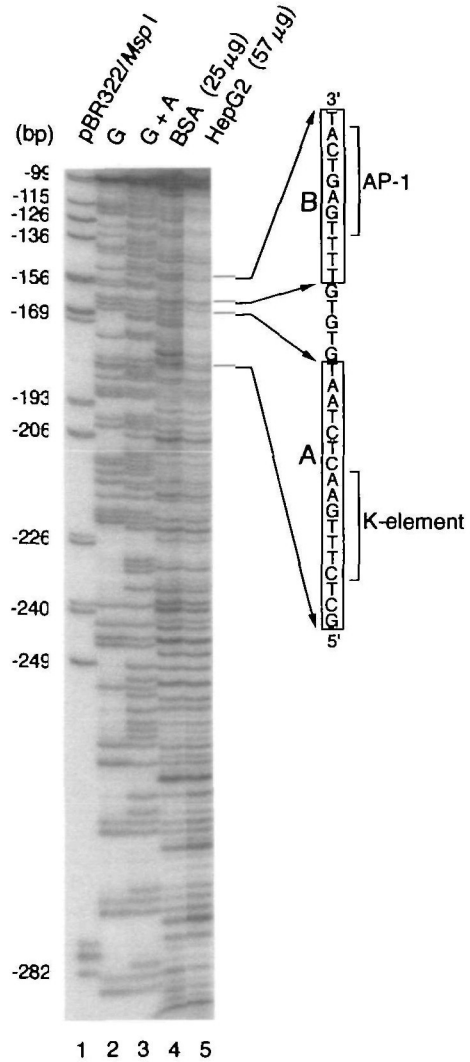

Fig. 6. **DNase I footprint analysis of protein-DNA complexes obtained with nuclear extracts from HepG2 cells on the −315 to −99 bp conglutinin promoter fragment.** The end-labeled DNA fragment was incubated at 0°C with 25 μg of BSA or 57 μg of 0.4 M TM heparin-agarose fraction of nuclear extract from HepG2 cells in 50 μl of reaction mixture. G and G+A indicate chemical sequence ladders prepared with the same labeled fragment, and pBR322/MspI were used as size markers. The numbers on the left represent the nucleotide numbers (in bp) of the conglutinin gene 5′-upstream region. The nucleotide sequences protected from DNase I digestion are indicated on the right panel. The two protected regions shown in the figure, labeled A and B (indicated by open boxes), contain the K element and the AP-1 consensus sequences (indicated by brackets), respectively.

sponsible for expression of the conglutinin gene. Functional analysis of the conglutinin promoter region (741 bp) by transient expression of a conglutinin promoter/luciferase reporter plasmid construct in HepG2 cells revealed the presence of a positively controlling *cis*-element, which may be most important for the conglutinin gene expression. The sequence of this element (CTTTGAA) localized at −180 to −174 bp overlapped with the region identified by DNase I footprint analysis as position −183 to −167 bp (Fig. 6). Since this nucleotide sequence did not contain consensus sequences of known transcription factors, we designate this putative *cis*-element as conglutinin (K) element. Intriguingly, the function of this novel *cis*-element was absolutely dependent upon the presence of the AP-1 *cis*-element from −158 to −152 bp (TGAGTCA) immediately (9 bp) downstream of the K element. In addition, the deletion of two bases located between the element K and the AP-1 sequence resulted in significant diminishment of the promoter activity, suggesting that the distance between the K and the AP-1 elements is important in their synergism. The synergism of two or more factors in the transcription is commonly found especially in tissue-specific expression (*32*). It might be possible that these two elements are bound by a single nuclear factor which has two distinct binding sites. However, this possibility is unlikely because gel mobility shift assay with a longer oligonucleotide probe, which contained both the K element and AP-1 sequences (−191 to −145 bp), using HepG2 nuclear extracts showed a significantly larger mobility shift of the DNA-protein complex than that with the K element or the AP-1 probe (data not shown). In addition a non-protected area (5 bp) was found between the K element and the AP-1 sequence by DNase I footprint analysis, as shown in Fig. 6.

Conglutinin has been reported to be synthesized only in bovine liver (*8, 33*), and the presence of its homologue in human serum is still controversial, although there have been several reports of a protein homologous to bovine conglutinin in human plasma (*34-37*). Because of the practical difficulties encountered in the primary culture of hepatic cells from bovine liver tissues, we performed the promoter analysis of conglutinin gene using the human hepatoma cell line HepG2, where the important *trans*-factors involved in the regulation of the liver proteins are likely to be conserved. In this respect, it is important to point out that the electrophoretic mobility shift assays using the nuclear extracts from HepG2 cells and the bovine liver cells demonstrated exactly the same results: the presence of the DNA-binding proteins bound to the *cis*-elements and AP-1 (Fig. 5). These results complemented the reporter gene assays using the HepG2 cells and suggested strongly that a similar type of regulation mechanism operates in the bovine liver cells.

To date, four other collectins distinct from conglutinin have been described in bovidae: mannan-binding protein (*6, 38*) and collectin-43 (*39*) in serum, lung surfactant protein D (SP-D) (*11, 40*), and lung surfactant protein A (SP-A) (*41*). The promoter analysis of the collectins including conglutinin has been little understood. Arai *et al.* (*42*) reported that human MBP gene was regulated by interleukins, dexamethasone, and heat shock, when studied by MBP mRNA expression and gel shift assay using the human hepatoma cell line HuH-7, suggesting that serum MBP transcription is induced by acute phase response. A study by our group on human MBP gene expression using HepG2 cells revealed that hepatocyte-specific nuclear factor-3 (HNF-3), which is shown to control the expression of numerous hepatocyte-specific genes, may activate the transcription of the lectin (Naito, H. *et al.*, unpublished observations). Lung cell-specific expression of the SP-A gene is mediated by thyroid transcription factor-1 (*43, 44*). These findings are quite different from the mode of gene expression of conglutinin described in this study.

The conglutinin gene shares many structural features with other collectin genes, particularly that of the lung homologue lung surfactant apoprotein D (SP-D), with

respect to the gene organization and cDNA structure, suggesting a common ancestral gene (*10, 11, 15, 33, 40*). Despite the very high level of homology in gene structure between these two collectins, conglutinin is synthesized in liver and secreted into the circulation, whereas SP-D is synthesized in the lung type II alveolar cells and Clara cells and secreted into the alveolar space.

This study indicated that the unknown positive regulatory element which functioned synergistically with the AP-1 binding site was involved in the transcription regulation of the bovine conglutinin. Characterization of the putative nuclear protein that binds to the novel regulatory K element is underway.

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