Structure and Regulation of the Senescence Marker Protein 2 Gene Promoter[†,‡]

Chung-Seog Song,[§] Jin Man Kim,[∥] Arun K. Roy,[§,∥] and Bandana Chatterjee*[,§]

Department of Cellular and Structural Biology and Department of Obstetrics and Gynecology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284

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ABSTRACT: The liver-specific expression of the senescence marker protein 2 (SMP-2) in the male rat is markedly reduced during the androgen-sensitive state of young adulthood, whereas it is up-regulated during the androgen-insensitive phases of prepuberty and senescence. Nuclear runoff studies show that the age-dependent changes in SMP-2 expression are due to transcriptional regulation of the gene. In order to explore the mechanism of the regulatory process, we have cloned the upstream flanking regions of two distinct SMP-2 genes (SMP-2A and SMP-2B) and established their nucleotide sequence. These clones contain approximately 2.2 kb of the 5′-flanking sequence, exons 1 and 2, the first intron, and a portion of the second intron. The SMP-2 genes, as well as the upstream sequences, contain the sequence motifs for a number of cis-acting regulatory elements, such as the hepatocyte-specific element (HP1) and the androgen response element (ARE). S1 nuclease and primer extension analyses have established the transcription initiation sites for these genes. For functional analysis of the upstream sequences, we have constructed a hybrid plasmid containing the SMP-2A gene sequence (−1970 to +38 bases) fused to the structural gene for chloramphenicol acetyltransferase (CAT). Upon transfection into rat hepatoma cells (FT02B), this construct was able to drive expression of the CAT gene. The same construct, however, failed to function in fibroblast-derived L cells, indicating tissue-specific regulation of the construct promoter.

Studies on changes in hepatic gene expression during aging have led to the discovery of differential temporal regulation of three major senescence marker proteins in the rat (Chatterjee et al., 1981). The androgen-inducible expression of α_{2u}-globulin (M_r ~19K) and the senescence marker protein 1 (SMP-1, M_r ~34K) in the male rat begins at the onset of puberty (around 40 days of age), is maximal in the young adult, and thereafter declines to an almost nondetectable level during senescence (>750 days of age). Contrarily, the senescence marker protein 2 (SMP-2, M_r ~31K) gene is expressed maximally during both prepuberty and senescence, and mRNA expression drops markedly in the postpubertal adult male. SMP-2 is an androgen-repressible gene, and its high level of expression is maintained in young adult females (Chatterjee et al., 1987a).

Reversible changes in the expression of SMP-2 and α_{2u}-globulin genes during maturation and aging are due to age-dependent alterations in androgen sensitivity of the liver (Roy et al., 1983; Demyan et al., 1989; Chatterjee et al., 1989). In the course of the life span of the rat, the liver goes through triphasic androgen responsiveness: prepubertal androgen insensitivity, androgen responsiveness during adult life, and reversal to an androgen-refractory state during senescence. Accordingly, hepatic androgen insensitivity in both prepubertal and senescent animals favors SMP-2 expression, whereas the gene is repressed in the young adult.

The physiological significance of SMP-2 gene regulation during aging is underscored by the observation that calorie restriction (a well-documented means of life-span extension in rodents and other animal species) delays age-associated reactivation of the SMP-2 gene (Chatterjee et al., 1989). In this regard, it is pertinent to note that restricted calorie intake also retards age-dependent loss of androgen responsiveness of rat liver (Chatterjee et al., 1989). As androgenic hormones seem to repress SMP-2 gene expression, the temporal extension of tissue androgen sensitivity in calorie-restricted rats is likely to protract androgenic repression of the SMP-2 gene.

In order to understand the molecular basis for negative regulation of the SMP-2 gene by androgens, and the resulting changes in its expression during aging, we are studying the structure and relevant cis-regulatory DNA sequences of the SMP-2 gene. In this paper, we describe the isolation, sequence characterization, and tissue-specific regulation of the SMP-2 gene promoter.

Materials and Methods

Isolation of Recombinant Phages and SMP-2 Genomic DNA. A charon 4A based rat genomic library, constructed from *Eco*RI partial digests of the genomic DNA (Sargent et al., 1979), was screened in situ with a previously characterized SMP-2 cDNA clone, pSP4a (Chatterjee et al., 1987a). Of the 6 recombinants that gave positive signals out of 6×10^5 plaques, 2 independent clones, λSMP-A and λSMP-B, were shown to contain SMP-2 sequences. Phages were purified by several rounds of plaque purification, and the recombinant phage DNA was isolated (Maniatis et al., 1982). Southern hybridization of the *Eco*RI-digested recombinant phage DNA with the ^{32}P-labeled SMP-2 cDNA identified 4889 bp (from λSMP-A) and 4439 bp (from λSMP-B) DNA fragments carrying up to approximately 2 kbp of the 5′-flanking sequence and the first two exons of the SMP-2 gene.

Oligonucleotide Synthesis and DNA Sequence Analysis. The entire nucleotide sequence of the 4889 and 4439 bp genomic DNAs isolated as described above was established by Sanger's dideoxy chain termination procedure. The DNA fragments (with *Eco*RI termini) were subcloned at the *Eco*RI site of the M13 mp 19 vector, and the M13 clones with progressive deletions at the 3′ end (achieved through T_4 DNA polymerase mediated 3′ → 5′ exonuclease digestion) were

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* Address correspondence to this author.

[§] Department of Cellular and Structural Biology.

[∥] Department of Obstetrics and Gynecology.

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produced by the "CYCLONE" technique (Dale et al., 1985). Recombinant M13 phage growth, DNA preparation, and DNA chain termination sequencing with [α-^{35}S]dATPαS were performed according to the protocols of Bankier and Barrell (1983). Electrophoresis was carried out on an 8% polyacrylamide/8 M urea gel. The sequences were read from both strands, and each sequence was read an average of 4 times in both directions. Sequence data were analyzed by the Pustell DNA analysis program (IBI, New Haven, CT).

Long stretches of gaps in the sequence data were filled via synthetic oligonucleotide primers that were annealed to internal sequences of the recombinant M13 DNAs for dideoxy chain termination reactions. Oligonucleotides of defined sequences (17–19 bases) were synthesized in the Cyclone DNA synthesizer (Biosearch-Milligen, San Rafael, CA). The oligonucleotide product was purified on a 16% polyacrylamide/urea gel, excised from the gel following its visualization by UV shadowing, and recovered by extraction at 65 °C with water.

S1 Analysis and Primer Extension. The 30-base-long oligonucleotide primer (5′GAAAAGGTATTCCTTCAAACCAAGTATAGT3′) complementary to bases +97 to +68 of the RNA strand of the SMP-2A gene, and located within the first exon, was end-labeled with ^{32}P, using T_4 polynucleotide kinase and [γ-^{32}P]ATP. The primer was annealed to a single-stranded M13 DNA template containing the 4889 bp *Eco*RI fragment of the λSMP-A and extended with *Escherichia coli* DNA polymerase Klenow fragment. After digestion with *Pst*I (that cleaves upstream from the transcription start point with respect to the SMP-2A gene), the DNA strands were separated on an alkaline agarose gel. The 255 bp end-labeled probe was visualized by autoradiography and easily separated from the labeled 30-mer oligo-primer. The resulting probe (255 bp) was annealed to rat hepatic mRNAs and digested with S1 nuclease (Berk & Sharp, 1977). The S1 nuclease resistant DNA fragment was resolved by 8% polyacrylamide gel electrophoresis in 50% urea.

For primer extension, 20 μg of poly(A+) RNA was used; the same primer as in the S1 analysis, described above, was annealed to the total liver mRNA at room temperature and reverse transcriptase mediated extension occurred for 15 min at 37 °C, followed by 30 min at 42 °C.

DNA and RNA Blot Hybridization. DNAs, digested with restriction endonucleases, were fractionated on 0.7% agarose gels, transferred to Nytran membranes (S&S, Keene, NH), and hybridized with SMP-2 cDNAs that were labeled with ^{32}P by random priming (Feinberg & Vogelstein, 1983). The conditions for prehybridization, hybridization, and washing were the same as described by Wahl et al. (1979). For RNA blots, total RNAs were extracted from the liver by the phenol–SDS procedure (Rosenfeld et al., 1972). RNA was size-fractionated on a 1.4% agarose gel containing 2.2 M formaldehyde, transferred to the Nytran membrane, and hybridized with radiolabeled SMP-2 cDNA probes.

Estimation of the Rate of Transcription of the Specific SMP-2 Gene by "Nuclear Runoff" Experiments. Fischer F344 rats (purchased from Charles River Laboratories, Wilmington, MA) were sacrificed under ketamine anesthesia and the livers processed immediately. Isolated liver nuclei (Tata, 1974) were incubated in the presence of [α-^{32}P]UTP (2000 Ci/mmol) and the other three ribonucleoside triphosphates (McKnight & Palmiter, 1979; Chatterjee et al., 1987b). The ^{32}P-labeled RNAs were extracted from the nuclei following the procedure of Chirgwin et al. (1979), and the labeled total RNAs were hybridized to the SMP-2 cDNA containing recombinant plasmid pSP4a (Chatterjee et al., 1987a) immobilized on nitrocellulose filters. The bound radioactivity was counted following its elution with NaOH (McKnight & Palmiter, 1979), and the ratio of bound to total input radioactivity was used as an index of the relative rate of transcription of the SMP-2 gene. [α-^{32}P]UTP incorporation under the assay condition is >90% sensitive to α-amanitin (1 μg/mL).

SMP-2 Promoter–CAT Plasmid Construct. The vector pSVOCAT (Gorman et al., 1982) often shows a cryptic promoter function originating within the pBR322 sequence and produces a background CAT activity in gene transfer experiments. To circumvent this problem, a reverse orientation plasmid, pSVOCAT-reverse (a gift from Dr. Gwen S. Adrian), was used. In this plasmid, the transcriptional direction of the CAT gene is opposite that in pSVOCAT. This reverse orientation vector was constructed by double digestion of the pSVOCAT DNA with *Bam*HI and *Nde*I, creation of blunt ends by fill-in reactions, and religation of two flush-ended DNA fragments. The orientations of the resulting plasmids were determined through restriction mapping.

The hybrid plasmid pSMPA-CAT was derived from pSVOCAT-reverse and the genomic subclone in pBR322 harboring the SMP-2A promoter spanning from −1970 to +38 bp. This promoter was generated from an M13 recombinant clone 15E isolated during T_4 DNA polymerase mediated 3′ → 5′ exonuclease digestion (cyclone reaction) of the entire 4889 bp genomic DNA for SMP-2A. The clone 15E carries the SMP-2 sequence from −1970 to +140 bp and includes 80 bases of the first exon. In order to delete the exon sequences, the 2008 bp DNA (from −1970 to +38 bp) was isolated as follows. The released insert from the clone 15E (with a single *Hin*dIII site) was digested with *Hin*dIII, and the 0.9 and 1.21-kb DNAs were electrophoretically separated. The 1.21-kb DNA fragment (with a single *Ava*II site) was further digested with *Ava*II, and the 1.1-kb DNA thus generated was isolated. The 0.9-kb DNA (with *Eco*RI and *Hin*dIII termini) and 1.1-kb DNA (with *Hin*dIII and *Ava*II termini) were subcloned in mp 19. The recombinant clone carrying the 0.9-kb DNA was digested with *Eco*RI, and the *Eco*RI site was dephosphorylated at the 5′ end by alkaline phosphatase reaction. The insert was then released from the mp 19 vector by *Hin*dIII digestion. The 1.1-kb DNA was similarly treated, so that only the 5′ end of the *Ava*II terminus was dephosphorylated. The 0.9- and 1.1-kb DNA fragments were ligated at *Hin*dIII ends to produce the 2.008-kb DNA which was subsequently treated with the Klenow fragment to create blunt ends, ligated with *Sal*I linkers, and introduced into pBR322 at the *Sal*I site. The SMP-2A promoter was later excised from pBR322 and ligated to pSVOCAT-reverse at an engineered *Sal*I site. The *Hin*dIII site of pSVOCAT-reverse was converted into a *Sal*I site by *Hin*dIII digestion of DNA, creation of blunt ends, ligation to *Sal*I linkers, and subsequent *Sal*I digestion. The plasmid construct pSMPA–CAT (identified through restriction mapping) has the SMP-2A promoter fused to the CAT gene. The polarity of the promoter is same as the transcriptional polarity of the CAT gene. Plasmids were grown and isolated and purified by two rounds of CsCl density gradient centrifugation.

Cell Propagation and DNA Transfection. The rat hepatoma cell line FTO-2B (a gift from Dr. Ann M. Killary) and the mouse fibroblast-derived cell line L aprt− tk− were maintained in a 1:1 Dulbecco's modified Eagle's medium/Ham's F12 medium mixture containing 10% fetal calf serum and penicillin/streptomycin (100 units/mL). Cells were transfected at a confluency of 30–40%.

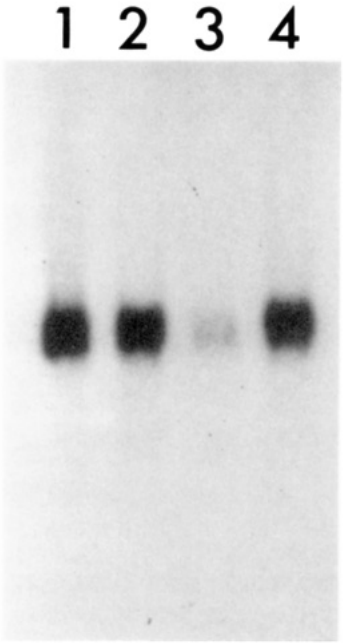


FIGURE 1: Steady-state level of SMP-2 mRNA in rat liver as analyzed by Northern blotting. The lanes are as follows: 1, 100-day-old female; 2, 28-day-old male; 3, 100-day-old male; 4, 800-day-old male. Each lane has equal amounts of RNAs pooled from three individual animals. Hybridization of the same blot with labeled albumin cDNAs did not show any significant changes in albumin mRNA levels.

Cells were plated at 500 000 per 10-cm plate 24 h prior to transfection, and refed with the fresh medium 4 h before transfection. Twenty micrograms of the plasmid DNA was coprecipitated with calcium phosphate and added to the cells. Following 5 h of incubation, the medium was removed, and the cells were treated for 3 min with the same medium, except containing 10% glycerol, washed twice with phosphate-buffered saline, and refed with the medium containing 10% fetal calf serum. At 48 h after transfection, cells were harvested, and the cell extract was prepared. CAT assays were performed according to Gorman et al. (1982).

RESULTS

Changes in SMP-2 mRNA Expression during Maturation and Aging. Age-dependent changes in the expression of SMP-2 were initially observed at the functional mRNA level in an in vitro translation assay of total hepatic poly(A+) RNA and subsequent gel electrophoretic analysis of the polypeptides (Chatterjee et al., 1981). That such temporal changes in the SMP-2 expression stem from altered steady-state levels of the corresponding mRNA is shown by Northern blot analysis (Figure 1). In contrast to the high level of SMP-2 mRNA within total poly(A+) hepatic RNAs from both prepubertal (28-day-old, lane 2) and senescent (800-day-old, lane 4) male livers, the young adult male (100-day-old, lane 3) liver has a conspicuously low level of SMP-2 mRNA. The adult female liver also has a high expression of SMP-2 mRNA (lane 1). Age-dependent variations in the levels of SMP-2 mRNA reflect corresponding changes in relative transcriptional rates of the SMP-2 gene (Table I).

Isolation and Identification of Genomic Clones Carrying the Upstream Flanking Sequence for the SMP-2 Gene. The genomic clones for SMP-2 were isolated by screening of the rat genomic library, constructed as an *Eco*RI partial digest in the Charon 4A vector, with the SMP-2 cDNA probe. This probe was an insert from the previously characterized plasmid recombinant clone pSP4a (Chatterjee et al., 1987a). The plasmid pSP4a contains the coding sequence for SMP-2 mRNA and the entire 3′-noncoding region. This cDNA clone lacks first 47 bases of the 5′-noncoding sequence. From the six isolates that gave positive hybridization signals, two independent clones were identified which contain the 5′ end of the SMP-2 gene. Digestion of the recombinant phage DNAs from these two clones yielded several DNA fragments (Figure 2, lanes 2 and 3) which, upon Southern blot and hybridization with the radiolabeled SMP-2 cDNA insert of pSP4a, showed one hybridizable band from each of the two clones: a 4889 bp DNA fragment from λSMP-A (Figure 2, lane 4) and a 4439 bp DNA fragment from λSMP-B (Figure 2, lane 5).

Nucleotide Sequence of the Upstream Region of the SMP-2 Gene. The complete sequences of the 4889 bp DNA from λSMP-A, designated as fragment A, and the 4439 bp DNA from λSMP-B, designated as fragment B, were determined by dideoxy chain termination of a series of overlapping M13 mp 19 recombinant clones carrying SMP-2 genomic inserts with progressive deletions at the 3′ end. Such nested sets of deleted fragments were created by the "CYCLONE" technique (Dale et al., 1985). Sequence data reveal that the genomic fragments A (4889 bp) and B (4439 bp) originate from two separate genes (designated here as SMP-2A and

Table I: Transcriptional Rates of the SMP-2 Gene during Aging[a]

age of male (M) or female (F) rats (days)	SMP-2 gene transcription (sp hybridizable dpm/6 × 10^6 dpm of total RNA transcripts)	transcriptional act. compared to young adult female (%)
30 M	820 (820, 840, 800)	54.12
150 M	313.3 (350, 300, 290)	20.66
800 M	1050 (1000, 1100, 1050)	69.30
150 F	1516.6 (1500, 1510, 1540)	100

[a] Averages from three experiments were used to compute percent normal female, and individual values are given within parentheses. The background radioactivity (binding of labeled nuclear RNAs to the nonrecombinant pBR322 DNA immobilized on the nitrocellulose paper) was subtracted from the individual dpm values. Inclusion of unlabeled female liver poly(A)-rich RNA (10 μg/mL) in the hybridization reaction abolished more than 90% of specific binding. The transcriptional rates of the constitutive expression of the albumin gene in the liver do not vary during aging (Murty et al., 1988).

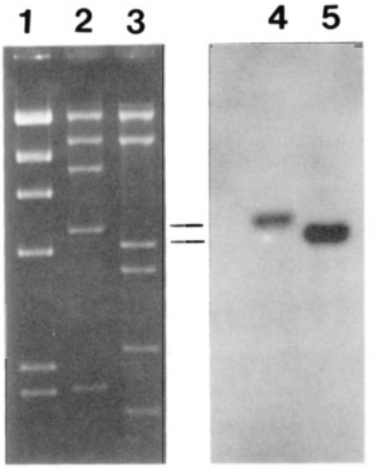


FIGURE 2: Identification of the SMP-2 sequence within the *Eco*RI-digested products of λSMP-2A and λSMP-2B DNAs. The ethidium bromide stained gel of the restriction enzyme cleaved and electrophoretically separated DNAs is shown on the left and the corresponding Southern blot autoradiogram on the right. Lane 1, λDNA cleaved with *Hin*dIII (molecular weight markers); lanes 2 and 4, *Eco*RI-digested λSMP-2A; lanes 3 and 5, *Eco*RI-cleaved λSMP-2B. The radiolabeled SMP-2 cDNA insert of the plasmid pSP4a was used as the hybridizing probe. Only the 4889 bp DNA of λSMP-2A (lane 4) and the 4439 bp DNA of λSMP-2B (lane 5) hybridize with the cDNA probe.

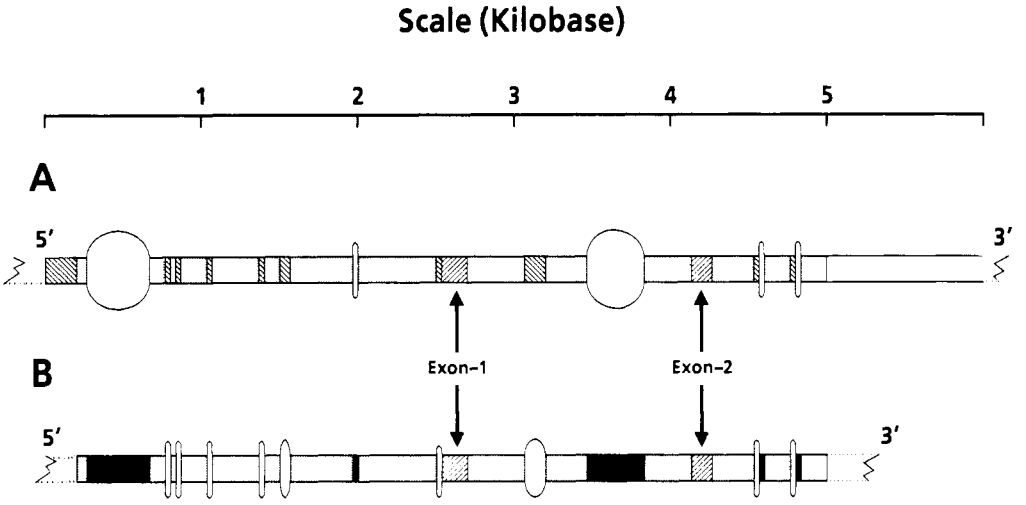


FIGURE 3: Schema showing similarities and dissimilarities in the sequence organizations between the SMP-2A and SMP-2B genes. Gray areas represent 80–90% sequence homology between the two genes. The hatched boxes with downward-slanted stripes from left to right represent sequences unique to the A gene, and the solid blocks represent B gene specific sequences. The deleted sequences in A and B genes are indicated with curved joining lines. The open area in the A gene represents additional sequences at the 3′ end. Exons 1 and 2 are indicated.

Table II: Consensus Sequences of Different Cis-Acting Elements in SMP-2 Genes

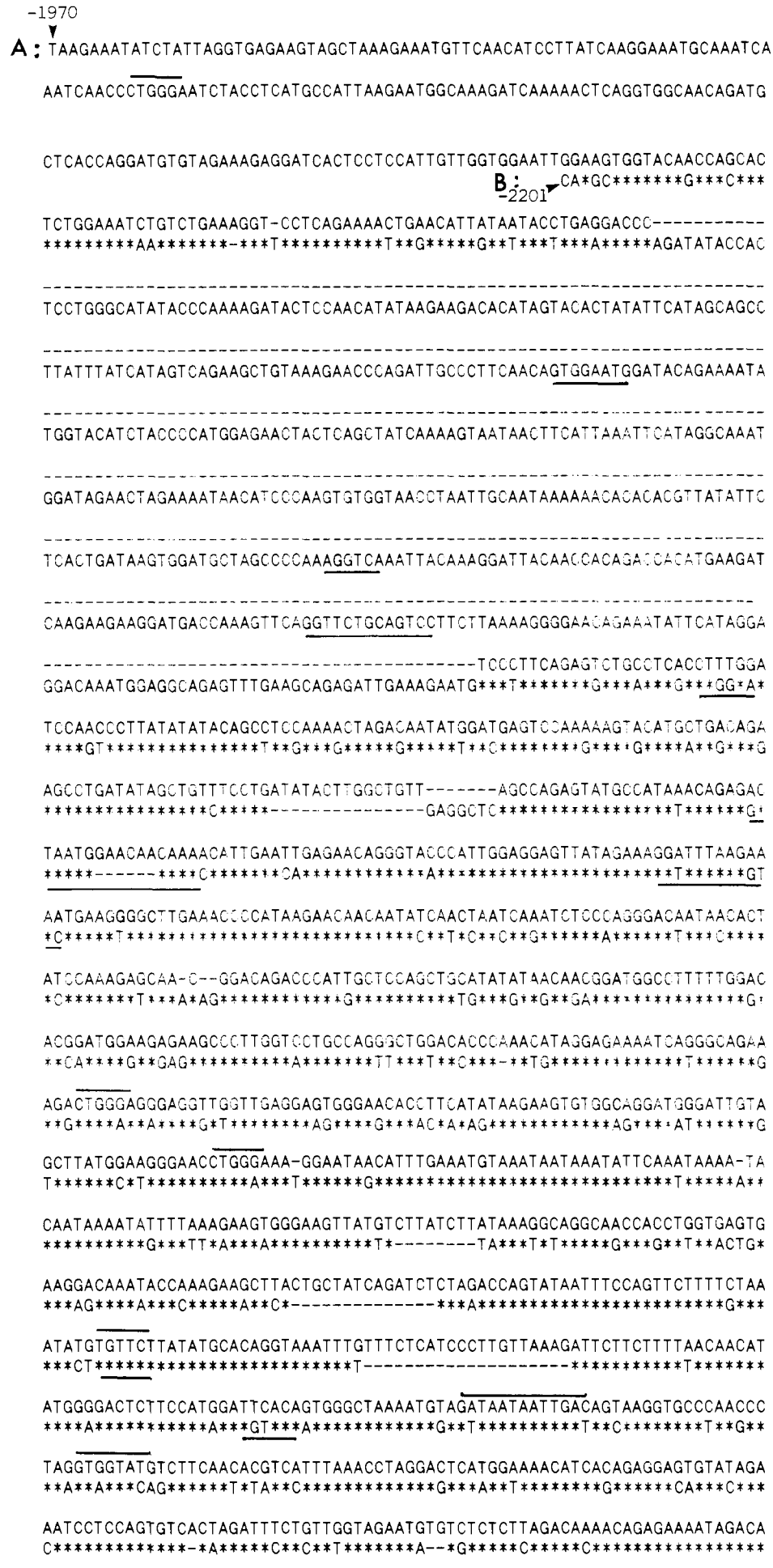
cis-acting element	consensus sequence	location (first base) on SMP-2A gene	location (first base) on SMP-2B gene
hepatocyte-specific promoter (Ryffel et al., 1989)	GNTNNTNNNNNC	−876, −377 +1100, +1559 +1636 +1660, +1663	−1737, −1498 −1443, +907 +1759, +1850 +1859, +2144
GRE (Klock et al., 1987)	TGTACA	+1801, +1946 +2652	−895, +791 +2150
ARE, GRE, half-palindrome (Beato, 1989)	TGTTCT	−980, −154	−959, −151
ERE: half-palindrome (Beato, 1989)	TGACCT	+359, +566	+349, +1680
ERE: half-palindrome (Beato, 1989)	AGGTCA	+1538	−1805, +1737
acute-phase signal (Fowlkes et al., 1984)	CTGGGA	−1892, −1261 −1178, +1407	−1629, +1606
enhancer, antithrombin III (Prochownik, 1985)	$GTGG^{AAA}_{TTT}G$	−843	−1993, +293 +613

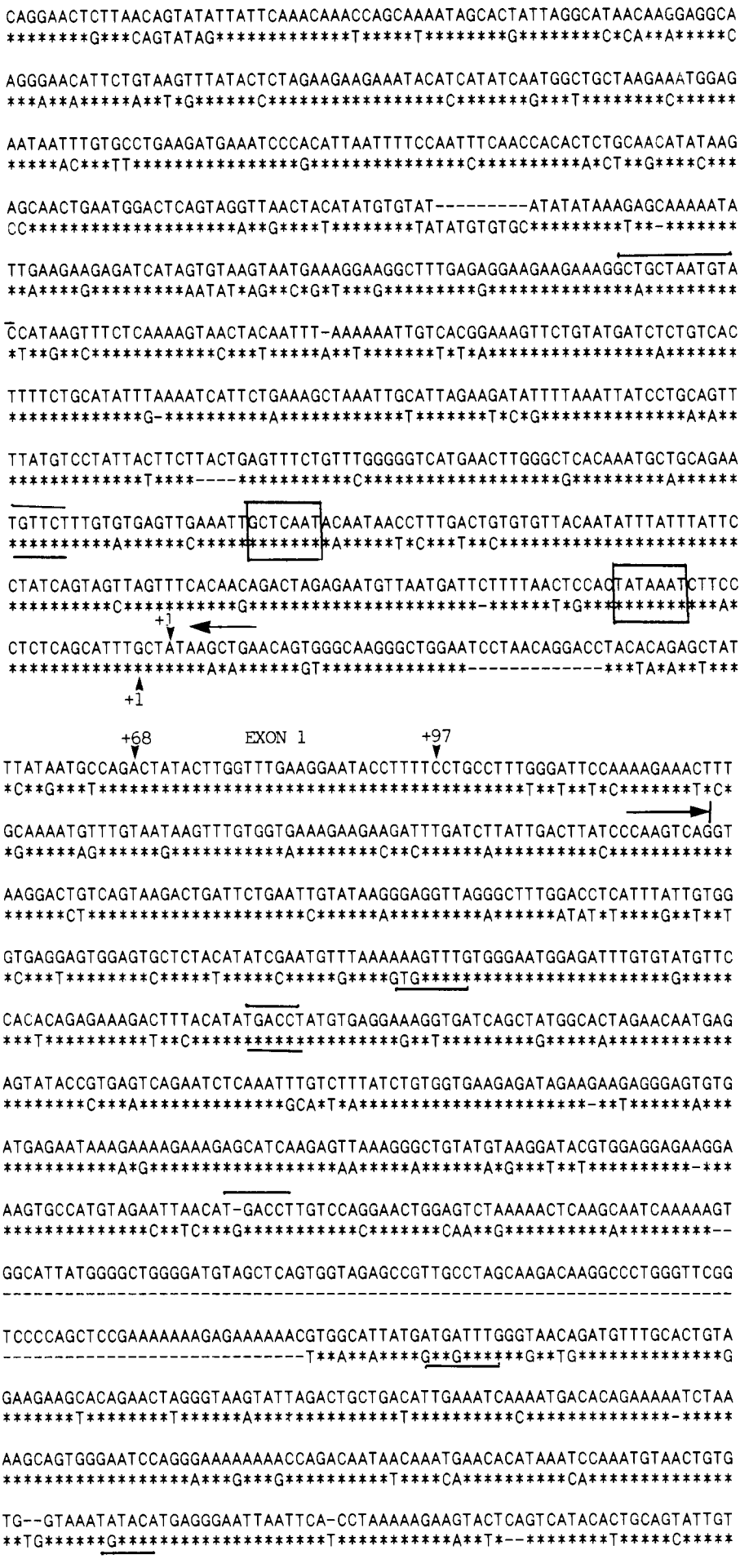
SMP-2B genes) and both of the cloned fragments A and B contain, in addition to the 5′-flanking sequences, the first two exons and a portion of the second intron of the SMP-2 gene. Figure 3 displays a schema of the similarities and dissimilarities in the DNA sequence organizations between SMP-2A and SMP-2B genes. In general, although these two genes have large areas of sequence homologies, marked sequence variations in several regions are also evident. Within the first exon, A and B genes have 87.5% sequence homology (119 out of 136 bases are identical), and in the second exon, the homology is 95.8% (65/68). It is interesting to note that the first and second exons of both genes A and B have slight sequence divergences, not only with themselves but also with the SMP-2 cDNA insert of the previously isolated recombinant plasmid pSP4a (Chatterjee et al., 1987a). Moreover, sequence divergence has been observed in several other SMP-2 cDNA clones that have been isolated in our laboratory. These sequence variations point to the existence of multiple SMP-2 mRNAs, some of which evidently originate from different SMP-2 genes.

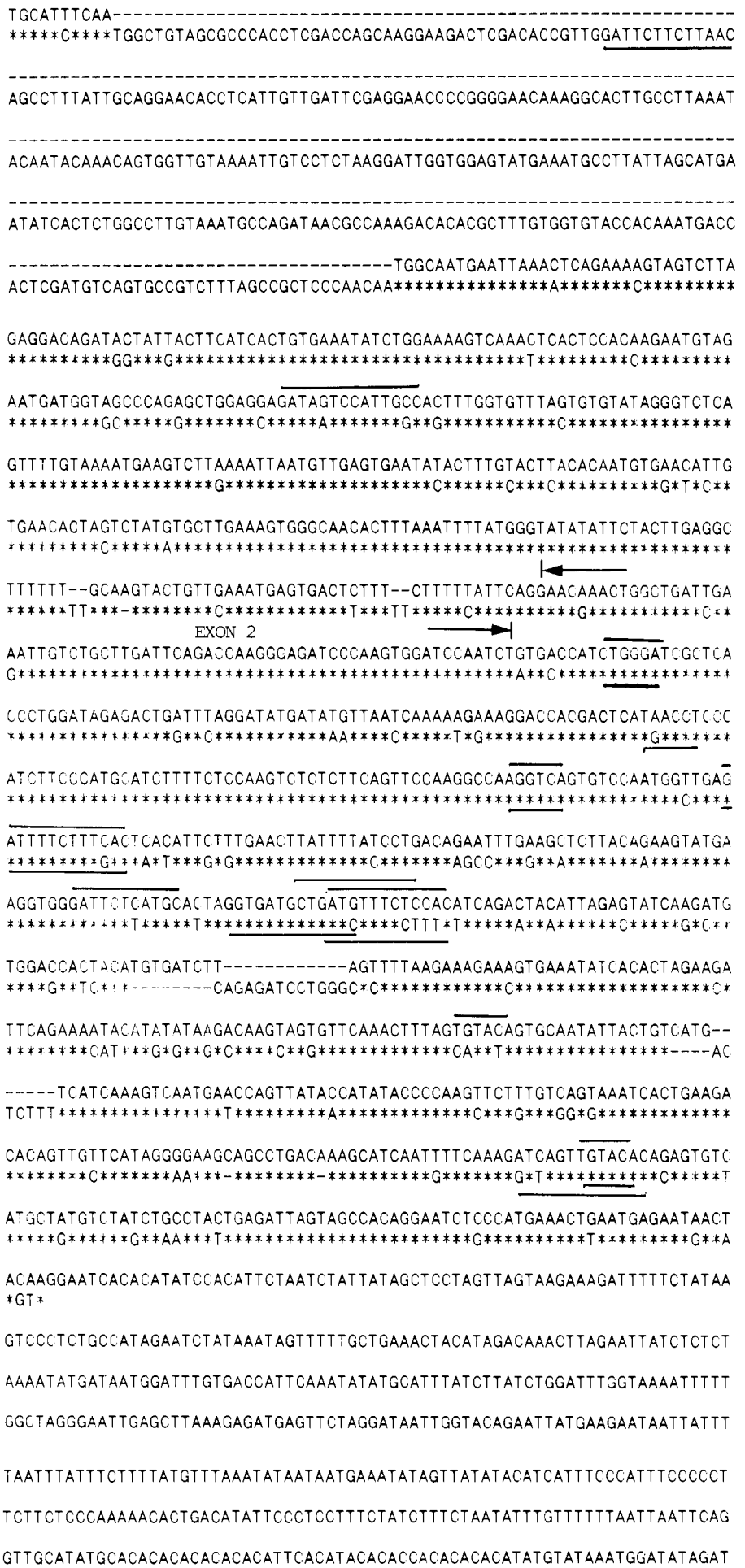
A comparative analysis of the noncoding sequences of the A and B genes for SMP-2 reveals 80–90% homology between these two genes (gray areas of Figure 3). In addition, both these genes have regions of unique sequence represented as hatched boxes (downward-slanted stripes from left to right) for the A gene and as solid blocks for the B gene. Thus, the 101 bp of DNA sequence (from +612 to +712 bases) of the A gene is missing in the B gene. The B gene, however, has 473 bp of DNA sequence (−2023 to −1550 bases) at the 5′ end, and this sequence is nonexistent in the A gene. The 3′ end sequence of the genomic fragment A extends 885 bases further than the 3′ end of the fragment B. Figure 3 also depicts short lengths of unique sequences for the A and B genes.

The nucleotide sequences of the cloned DNA fragments A and B are shown in Figure 4. The two sequences have been aligned for maximum homology. Using computer search, we have scanned the sequences in Figure 4 for motifs that could play regulatory roles. A number of such motifs (overlined for SMP-2A and underlined for SMP-2B) are listed in Table II, and their possible significance is considered under Discussion. The SMP-2A gene has a TATA-like sequence, TATAAAT, 21 bases upstream from the transcription start site. The sequence GCTCAAT, 126 bases upstream from the transcription start site, may fulfill the role of a CAAT box. The SMP-2B gene also shows a typical TATA sequence (18 bases upstream) and a CAAT box (122 bases upstream).

The intron sequence depicted in Figure 4 is in compliance with the general GU/AG rule at the 5′ and 3′ ends of exon–intron junctions. For both A and B genes of SMP-2, the first intron starts with GT at the 5′ terminus and ends with AG at the 3′ terminus. The second intron for the A gene begins


-1970
A:
B:
-2201


+1
+1
+68
EXON 1
+97


EXON 2

AAATACACACACATATATGGATAGAGATAGATACACACACACACACACACACACACACACACACAT

ATATATATATATATATATATATATATATATATATATGGATATAGATAGATTCACACACACATGTACATAA

ACATATACATATGCATATATATTCCTAAATATATTCCTTTATTTATATATTCCTACATTGATATATATCT

TCATTTCTTTTTTTCCCTTTTCTTTTATTGGATATTTTCTTCATTTACATTTCAAATGTTATCCCTTTTG

CTGATTTCTACTCTGGAAAACCCACTCCCATCACCCCTCCCTCTGCCTCTATGATAGTGCTCTTCCTCCC

ACCCATAAGGTCCCTCCTCCCTGTCCTAATATTCCACTATACTGGAGCA

FIGURE 4: Nucleotide sequences of the SMP-2A gene fragment (4889 bp) and the SMP-2B gene fragment (4439 bp) aligned for maximum homology. Upper sequence, A gene; lower sequence, B gene. The likely cap sites for the two genes are marked as +1. The "TATA" and "CAAT" motifs are boxed. The putative cis elements as described in Table II are overlined (A gene) and underlined (B gene). The beginnings and endings of exons 1 and 2 are indicated by arrows. The asterisks (*) indicate identical bases. The hyphens (-) indicate missing bases. The A fragment has 885 bases of additional sequence at the 3′ end.

with GT as well. Furthermore, the long pyrimidine track within the first intron neighboring the 3′-splice site [$(C/T)_{12}$ATTCAG:G for the gene A and $(C/T)_{14}$ATTCAG:G for the gene B] conforms with the consensus $(T/C)_{11}$NCAG:G for the 3′-splice site sequence (Padgett et al., 1986).

Determination of the Transcription Start Site. The transcription start site for the SMP-2 gene was determined by both S1 nuclease analysis and primer extension. A 5′-end-labeled oligonucleotide, complementary to bases +97 and +68, located within exon 1, was extended upstream beyond the transcription start site with Klenow DNA polymerase and the resulting double-stranded DNA cleaved to yield a DNA probe of 255 bases. Hybridization of the probe with rat liver total poly(A+) RNA, followed by S1 nuclease digestion, yielded several protected bands, the longest of which has 97 bases (indicated with an arrow in Figure 5A). A similar analysis of rat preputial gland poly(A+) RNAs showed no protected bands (data not shown). The most predominant band of the S1 gel is, however, 86 bases long. The transcription start site for the SMP-2A gene has been assigned to the adenosine nucleotide which is 67 bases upstream from the 3′ end of the 30-mer oligo-primer (this "A" base has been indicated as +1 in Figure 4) and is 21 bases downstream from the TATA-like sequence TATAAAT. The 86-base-long predominant band in the S1 analysis gel may be the alternate transcription start site for the A gene. In that case, the canonical TATA sequence will still be only about 30 bases upstream of the cap site. The 86-base-long DNA band may also be accounted for by the cap site of the B gene for the following reason. The 30-nucleotide-long oligoprimer chosen for the S1 analysis affords a perfect sequence complementary to both the A and B genes, as they have an absolute sequence match in this region (see Figure 4). Compared to the A gene sequence, the B gene, however, lacks 13 bases upstream of the ATG translation initiation codon. Nevertheless, the B gene has upstream TATAAAT and GCTCAAT sequences that are identical with the TATA and CAAT box motifs of the A gene. On the basis of these considerations, th guanosine nucleotide, which is 57 bases upstream from the 3′ end of the 30-mer oligonucleotide of the B gene, has been assigned as the +1 site. We feel that the multiple bands of the S1-digested DNAs either may be degradation products or may be indicative of alternative start sites.

The same 30-mer oligo-primer as in the S1 analysis was used in the primer extension analysis which yielded consistent results. As shown in Figure 5B, extension of rat liver poly(A+) RNA produced major bands of 97–95 bases and secondary bands of around 86 bases. No band was seen in the absence of RNA.

Functional Analysis of the SMP-2 Gene Promoter. The in vivo promoter activity of the upstream sequence of the SMP-2A gene was tested by its ability to promote transcription of the receptor CAT gene. In a plasmid CAT construct, the SMP-2A promoter was fused to the structural gene for CAT to produce pSMPA–CAT. As shown in Figure 6, the construct pSMPA–CAT contains the 4.5 kb of the vector and an insert of 2008 bp that includes the putative promoter region of the SMP-2A gene. Digestion of pSMPA–CAT with *Pst*I yielded the DNA fragments of expected size (Figure 6, inset). When assayed for chloramphenicol acetyltransferase activity, the pSMPA–CAT-transfected FT02B hepatoma cell extract (Figure 7, lane 6) catalyzed formation of acetylated chloramphenicols, whereas the enzyme activity was nondetectable in the extracts of cells transfected either with the parent vector pSVOCAT-reverse (without promoter) or with a plasmid where the SMP-2A promoter is fused in reverse orientation with respect to the CAT gene (lanes 4 and 5, respectively). As a positive control, pSV2CAT DNA containing the SV40 promoter was used (lane 3). Figure 7 also includes results of control assays in the absence (lane 1) and presence (lane 2) of the commercially purchased bacterial CAT enzyme. In contrast to FT02B cells, no SMP-2A promoter activity was detected in mouse fibroblast-derived L aprt- tk- cells (Figure 7, lane 10). Because the L cells transfected with pSV2CAT exhibited chloramphenicol acetyltransferase activity (Figure 7, lane 7), it is likely that some liver-specific factor is requied for expression of the SMP-2A gene and that this factor is absent or limiting in L cells. We conclude that the SMP-2A gene segment, which contains the start of transcription and the TATA-like element, as well as other promoter and regulatory sequences, functions as a promoter.

Discussion

The present study clearly demonstrates that the augmented expression of SMP-2 in aging male rats results primarily from the increase in the rate of transcription of the corresponding gene. Furthermore, SMP-2 is an androgen-repressible protein, distinct from the androgen-repressed protein of the prostate (Saltzman et al., 1987). The SMP-2 system thus provides a suitable model to study the molecular mechanisms of both hormonal repression and age-dependent modulation of specific gene activity. As a first step toward elucidation of the details of transcriptional control in SMP-2 expression, it is imperative to characterize the structure and promoter function of the 5′-flanking sequence of the SMP-2 gene.

The 2 clones for the SMP-2 gene, characterized in the present work, cover up to 2200 bases of the upstream flanking sequence, the first 2 exons, and a part of the second intron and represent homologous, yet separate, SMP-2 genes designated here as A and B. The existence of multiple genes encoding SMP-2 is also consistent with the complex signal pattern of the Southern blot of rat genomic DNA digested with any one

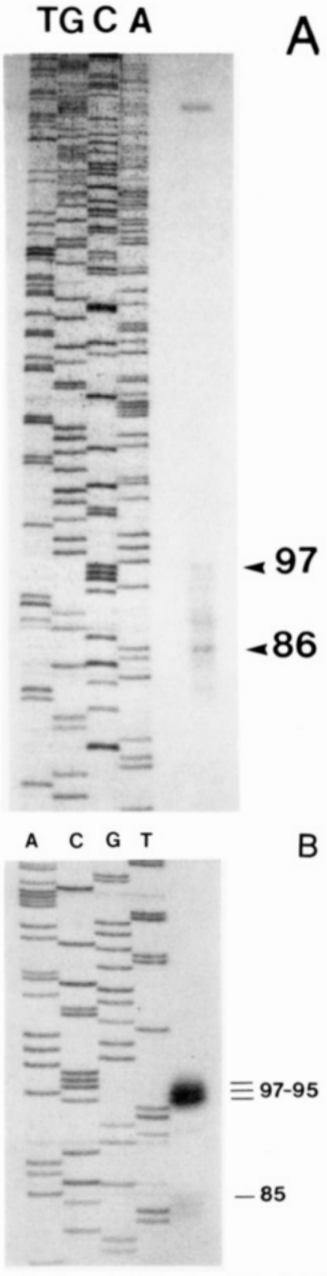


FIGURE 5: Determination of the transcription initiation site for the SMP-2 gene. (A) S1 nuclease analysis. The longest (97 bases) and the major (86 bases) protected bands are shown with arrows. The undigested probe is seen near the top of the gel. The lanes marked T, C, G, and A are a partial representation of a sequencing gel generated from a DNA fragment which is unrelated to the SMP-2 DNA sequence. The sequence ladder serves as the size marker for the protected DNA bands. (B) Primer extension analysis. The major bands are 97–95 bases in length. A minor band of 85 bases can also be seen. The sequence ladder on the left marked as A, C, G, and T serves as the size marker. The 30-nucleotide-long oligo-primer spans +68 to +97 bases of the SMP-2A gene.

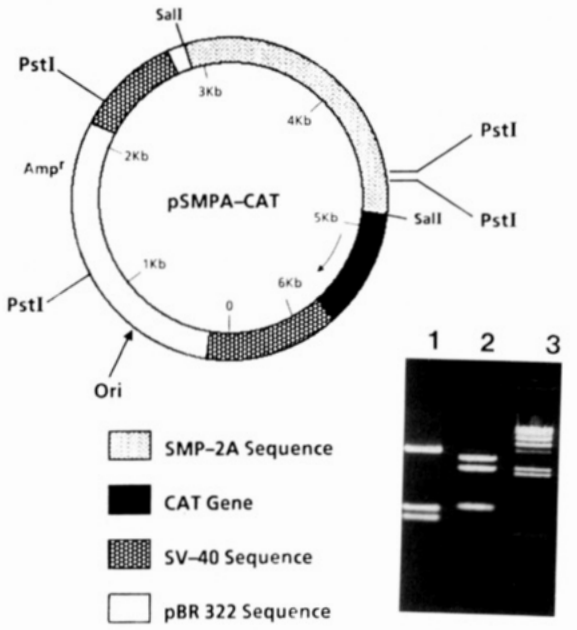


FIGURE 6: Restriction map of the construct pSMPA–CAT. The numbers indicate the distance in kilobases. The SMP-2A promoter with *Sal*I termini was fused to pSVOCAT-rev at the engineered *Sal*I site of the vector, as described under Materials and Methods. The inset represents the ethidium bromide stained gel of the *Pst*I-digested DNA fragments derived from pSMPA-CAT (lane 2) and pSMPA-rev-CAT in which the SMP-2A promoter is fused to the CAT gene in reverse orientation (lane 1). Lane 3 indicates molecular weight markers (λDNA-digested with *Hin*dIII).

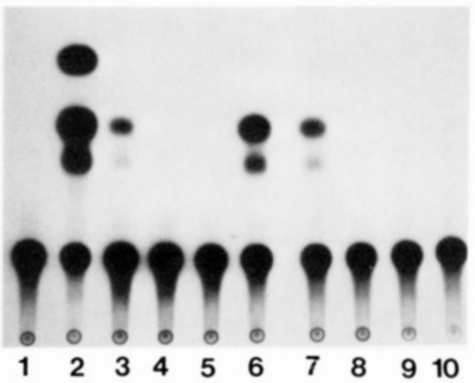


FIGURE 7: CAT enzymatic activities in the extracts of transfected cells. Lanes 3–6 show CAT activities of FT02B cells, whereas lanes 7–10 represent CAT activities of L aprt– tk– cells. Lanes are the following: 1, mock-transfected cell extract with no added enzyme; 2, extracts from mock-transfected cells plus bacterial CAT enzyme (Pharmacia, Piscataway, NJ); 3 and 7, extracts from pSV2CAT-transfected cells (positive control); 4 and 8, extracts from pSVO-CAT-rev-transfected cells; 5 and 9, extracts from pSMPA-rev-CAT-transfected cells; 6 and 10, pSMPA–CAT-transfected cells.

of several restriction endonucleases and hybridized with labeled SMP-2 cDNAs (B. Chatterjee, unpublished observation). The long stretch of alternate purine-pyrimidine bases within the second intron of SMP-2A (starting from +2554, there are 18 pairs of CA followed by 19 pairs of TA sequences) may signify the potential of this region to assume the Z-DNA configuration, capable of exerting a special regulatory influence on SMP-2 gene expression. Although many eukaryotic genes are known to contain purine-pyrimidine repeats, especially in the noncoding and/or intervening sequences of the genome, the precise regulatory role of such Z-DNA domains in gene expression has yet to be demonstrated.

Both SMP-2A and -B genes have the canonical "TATA" and "CAAT" upstream sequences within the usual distance (−20 to −30 bases for the TATA box and ca. −100 bases for the CAAT box) from the cap site assigned on the basis of S1 nuclease and primer extension analyses. Both of these genes contain the DNA sequences for several other putative cis-regulatory elements. Many liver-specific genes, such as *Xenopus* and mouse albumin, mouse α-fetoprotein, rat β-fibrinogen, and human α_1-antitrypsin, have been shown to contain (within some distance of 70 bases upstream of the TATA box) a consensus sequence, GNTNNTNNNNNC, which has been suggested to be a hepatocyte-specific cis-control element

(HP1 element). Gene transfer studies have shown that the HP1 element, in conjunction with the TATA box, constitutes the minimal promoter capable of supporting liver-specific expression of these genes (Ryffel et al., 1989). Both SMP-2A and B genes contain several HP1 elements (Table II). It should be noted that compared to other known liver-specific genes, the HP-1 consensus sequences in the SMP-2 gene are located much further upstream of the "TATA" box.

Germane to the observation of negative modulation of SMP-2 by androgens is the finding that several motifs for a half-palindrome of the androgen-response element (ARE) TGTTCT are present in both SMP-2A and SMP-2B genes. Most of the hormone-response elements are known to contain a perfect or near-perfect palindromic sequence. However, a half-palindrome estrogen-response element (ERE) within the ovalbumin gene promoter has been shown to confer estrogen responsiveness to this gene (Tora et al., 1988). Palindromic GRE and half-palindromic ERE sequences are also present in both of the SMP-2 genes. Other consensus sequences that are present within the SMP-2 genes include acute-phase response elements and an enhancer sequence described for another hepatic protein, antithrombin III. The presence of sequence motifs for acute-phase signals in the SMP-2 genes may have age-related regulatory implications. Since inflammations and other acute-phase triggers are expected to be on the rise during aging, reactivation of SMP-2 at old age may be preceded by DNA transcription factor interactions involving the acute-phase signal sequence.

The sequence motifs described above can only provide useful guides in the search for functional control elements in vivo in transcriptional modulation of the SMP-2 gene. In order to study the in vivo functionality, we have tested the transcriptional activity of one of the wild-type SMP-2 gene promoters (SMP-2A). Transfection of the hybrid CAT construct into rat hepatoma cells shows that this promoter segment is able to drive expression of the CAT gene. Promoter activity of this construct requires a liver-specific transcriptional factor(s), as transfection of this construct into mouse fibroblast cells (L aprt- tk-) failed to generate any CAT activity. The tissue-specific promoter functionality of the SMP-2A gene strongly suggests that this gene is transcribed in vivo.

In recent years, steroidal induction of gene activity has been extensively studied. However, repressive effects of steroid hormones have not received similar scrutiny. Only a few examples of glucocorticoid-mediated repression of transfected genes are currently available. These include glucocorticoid-mediated extinction of the genes for prolactin, proopiomelanocortin, and the α subunit of glycoprotein hormones (sakai et al., 1988; Akerblom et al., 1988; Drouin et al., 1987). Because of its high sensitivity, the androgenic repression of the SMP-2 gene offers a desirable model to study negative gene regulation by a steroid hormone. The promoter-CAT construct characterized in this study will greatly facilitate such an undertaking.

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