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CLONING AND STRUCTURAL CHARACTERIZATION OF A RABBIT GENOMIC DNA FOR α_1 ACID GLYCOPROTEIN[1]

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The gene for rabbit α_1 acid glycoprotein (AGP) has been isolated from a λ $EMBL_3$ genomic DNA library. Isolated clone contains a 12 Kbp fragment of rabbit genomic DNA. Restriction endonuclease mapping has localized the gene within a 4.2 Kbp fragment spanning two EcoRI sites. Southern blot analysis of the rabbit genomic DNA and its comparison with the cloned gene indicates that there is only one gene for AGP present per genome. DNA sequence analysis of the cloned gene indicates that the entire gene, TATA box to the polyadenylation signal, is located within the 4.2 Kbp region and contains six exons representing the full-length cDNA described earlier (1). The 5'-end of α_1-AGP gene sequences from rabbit, human, rat and mouse have been compared. Such analysis reveals two conserved regions located between -63 bp and -36 bp and -29 bp and -1 bp of putative transcription start site, which may play a role in transcriptional induction of this gene during acute response. In addition to this conserved domain, DNA sequence upstream of the major transcription start site contains a potential element for Sp1 binding and a 18 bp long palindrome sequence followed by a short repeating dinucleotide sequence, which may be important in the regulation of AGP gene induction.

Synthesis of α_1-acid glycoprotein in many mammalian hepatocytes is increased following the response to various stressful stimuli (2). Recent studies have indicated that nonspecific inflammatory stimulus by subcutaneous turpentine injection to rabbit elicit acute phase response resulting in a remarkable expression of AGP (1). Increased accumulation of AGP messenger RNA in rabbit liver cells suggests possible induction of transcriptional activity. Such an increase in AGP gene expression in rat, mouse and human hepatocyte cells by interleukin-1, tumor necrosis factor α, interleukin 6, leukemia inhibitory factor and glucocorticoids has been reported (3-6). Although the increased accumulation of AGP mRNA can be influenced by other factors such as transport of the mature mRNA out of the nucleus (7) and shortening of poly(A)-tail length (8), induction of transcriptional activity appears to be the major factor in AGP mRNA accumulation during acute inflammation. Growing evidence suggests that hepatocytes respond to acute phase signals produced by macrophages (9-13) and this results in the increased rate of transcription initiation of the acute

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phase genes. At least in part, the frequency of initiation is controlled by diffusible transacting transcription factors, binding to promoter and/or enhancer elements located upstream of transcriptional start sites (14-16). Coordinated action of multiple transcription factors are also evident in liver-specific expression of AGP. Existence of promoter-proximal and -distal glucocorticoid response element (GRE) in rat AGP gene and cooperativity between GRE receptor and other transcription factors (16, 17) which leads to the transcriptional activation of AGP suggests that a unique regulatory mechanism controls AGP gene expression. The exact mechanism of such synergistic interaction is not yet well understood. Nor do we know whether both distal and proximal GRE are simultaneously involved in the activation of AGP gene under acute condition. Over expression of AGP in rabbit liver suggests that a similar mechanism might be involved in the control of rabbit AGP gene expression.

To elucidate the molecular mechanism of induction of AGP gene in rabbit hepatocyte in acute response, we have cloned and characterized this DNA. In this paper, we describe unique structural features of AGP genomic DNA and discuss possible involvement of several structural elements in its expression.

MATERIALS AND METHODS

Isolation and characterization of a λ phage clone containing α_1-AGP gene

A rabbit liver λ $EMBL_3$ genomic library (18) was screened using random-prime labelled α_1-AGP cDNA (1) as probe. Among several positive clones one was further purified by low-density plating and DNA was isolated from the purified λ phage. The boundary of the AGP gene was determined by Southern hybridization and a restriction map was generated by single and double digestion with various restriction enzymes.

DNA sequence analysis

Various subclones were generated by ligating different fragments of AGP gene into the polylinker sites of pTZ19U plasmid (US Biochemical Corporation, Cleveland, Ohio). Double-stranded DNA suitable for sequencing was prepared following a method described earlier (19) and DNA sequences determined by dideoxynucleotide chain termination method (20) using sequenase sequencing kit (US Biochemical Corporation, Cleveland, Ohio). Sequences were determined from both sides using plasmid-specific primers as well as synthetic oligonucleotide primers from the known sequence of the genomic DNA.

Southern blot analysis of rabbit genomic DNA

AGP phage DNA (0.1 μg) and rabbit liver genomic DNA (15 μg) was digested with suitable restriction enzymes. Digested DNA was electrophoresed in a 0.75% agarose gel and then transferred onto a nitrocellulose membrane. The blot was hybridized in 5X SSC/50% formamide/2X Denhardt's solution/100 μg/ml denatured calf thymus DNA/0.1% SDS and radioactive AGP cDNA at 42°C for 15 h. Blots were washed with 0.2X SSC/0.1% SDS extensively and autoradiographed at -70°C with intensifier screens.

RESULTS AND DISCUSSION

Isolation and characterization of a recombinant λ phage containing the rabbit α_1-AGP gene

A λ $EMBL_3$ rabbit liver genomic library was screened with rabbit α_1-AGP cDNA as a probe. One positive clone designated as λg AGP5 was purified and isolated DNA was digested with restriction enzyme, SalI (unique restriction endonuclease cleavage site in λ$EMBL_3$) and products were analyzed on agarose gel. This indicated that the clone gene contains an insert of 12 Kbp size. Restriction mapping and Southern blot hybridization indicated that the α_1-AGP gene is located within two EcoRI fragments spanning a 4.2 Kbp region. A detail restriction map and organization of six exons and five introns of rabbit AGP gene is shown in Fig. 1. Comparison of this structural organization with that of rat (21) and human (22) reveals a very similar pattern indicating a structural conservation of AGP in human, rat and rabbit. For more detailed structural characterization complete nucleotide sequence of the 4.2 Kbp DNA was sought.

Gene copy number

To determine the total number of AGP gene, rabbit liver DNA was digested with various restriction enzymes, electrophoresed in a 0.75% agarose gel, transferred onto a nitrocellulose

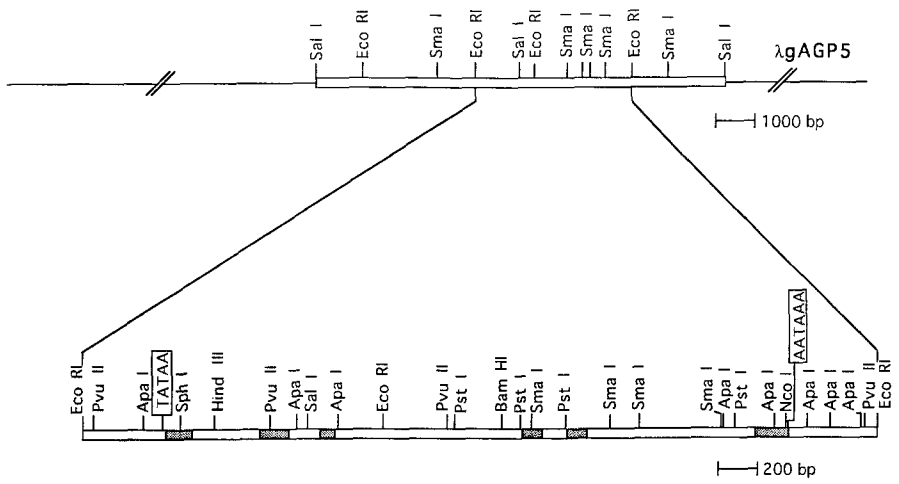

Figure 1. Restriction endonuclease digestion map of rabbit α_1-acid glycoprotein genomic clone. Rabbit liver genomic DNA fragment of 12-Kbp containing the AGP gene was cloned in λEMBL3 vector and selected by using rabbit liver cDNA as a probe. Location of the AGP gene into a 4231 bp fragment spanning two EcoRI sites was determined by Southern hybridization analysis. Detailed restriction map was generated using various restriction endonucleases. These restriction sites were used to generate subclones into plasmid vectors and used for DNA sequence determination. Relative position of six exons, shown by hatched boxes, were determined by DNA sequence analysis. Positions of TATA box and polyadenylation signal are shown. The direction of transcription is from left to right.

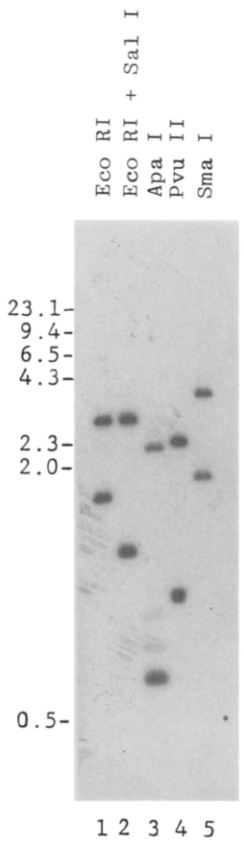

Figure 2. Southern blot analysis of rabbit liver genomic DNA. Genomic DNA (15 μg) was digested with various restriction endonucleases, electrophoresed on a 0.75% agarose gel, transferred on a nitrocellulose membrane and hybridized to a random-primed labelled AGP cDNA probe. Different RE used are indicated in each lane. Positions of molecular weight markers, obtained from HindIII digestion of λcI857 DNA, are shown. Digestion pattern of the genomic DNA seen here corresponds with the restriction map of the isolated clone λgAGP5. Short fragments of ApaI digestion, lane 3, ran off the gel.

membrane and hybridized to AGP cDNA probe (Fig. 2). Comparison of the size of resultant bands in Fig. 2 with the restriction map of isolated gene in λgAGP5 indicates that there is only one copy of AGP gene in rabbit genome. This data also indicates that no rearrangement has occurred during cloning and in bacteriophage propagation. Similar to our finding in rabbit, rat genome also contains a single AGP gene (21) while human and mouse genome has been found to contain multiple genes for AGP (22, 23).

In a separate experiment (data not shown) when genomic DNA was isolated from a different rabbit, we observed appearance of a 5.5 Kbp EcoRI band instead of 2.6 Kbp as seen in lane 1, Fig. 2. This indicates apparent loss of one EcoRI site as a result of intraspecies polymorphism at this locus. Such phenomenon is often observed in all eukaryotes.

DNA sequence analysis of AGP gene

To facilitate the sequencing, DNA was digested with suitable restriction enzymes and the fragments were subcloned in plasmid vector pTZ19U. Double-stranded DNA prepared from these subclones were sequenced from both sides using the plasmid-specific primers.

Results of DNA sequence of the 4.2 Kbp EcoRI-EcoRI fragment region of λgAGP5 is shown in Fig. 3. It also includes some DNA sequences further upstream of the EcoRI site. Comparison of the DNA sequence data with that of the cDNA which we have reported recently (1) shows that the cDNA sequence is confined to six exons and the nucleotide sequence of the exons are identical to that of the cDNA. Thus intraspecies polymorphism or replication error of lambda bacteriophage is absent at least in the exon region of the cloned AGP gene. Inspection of the sequences at splice junctions shows that they all contain the correct conserved sequence at the 5′ splice donor site which begins with the dinucleotide GT and the invariant AG dinucleotide at 3′ splice acceptor site (24). In addition, the donor and acceptor sites match quite well with the proposed consensus sequences of donor $^{GTATGT}_{G}$ and acceptor $(Py)_6$ NCAG (24). High conservation of sequences within the introns at the presumed splice junctions of AGP gene confirms that common mechanism of eukaryotic splicing of nuclear genes is involved in the maturation of AGP primary transcripts. Comparison of exons and introns of rabbit AGP gene with that of human (22) and rat (21) reveals a very similar pattern of arrangements.

In order to define the transcription start site we have previously performed a primer extension analysis (1). Comparison of the length of the primer-extended product with the genomic DNA sequence suggests that putative transcription start site, designated as +1 in Fig. 3, is 35 bases upstream of the ATG codon. This matches perfectly with the previously isolated cDNA for AGP (1). Also noteworthy is the presence of a potential TATA box located 24 bp upstream of the putative transcription start site. Very similar lengths of 5′ untranslated region and TATA box-transcription start site were also found in both rat and human AGP gene (21, 22). An additional TATA box is located further upstream at position -173 to -169 along with an apparent consensus sequence of a CAAT box at position -204 to -201. However, we do not have any indication regarding the possible use of this TATA box as the potential binding site for TFIID. Further work will elaborate the functional significance of this upstream TATA box. It is interesting to note, however, that similar structural features also exist in rat AGP gene (21). Sequence analysis has also revealed a characteristic GC box which is a potential binding site for transcription factor Sp1 (25). This element is located between nucleotides -84 and -67 and present as three tandem copies (Fig. 3). Unique structural feature also includes a large dyad symmetric sequence located between nucletotides -580 and -561 (underlined, Fig. 3) followed by a shortly repeated dinucleotide AG, -551 to -528 (broken underlined, Fig. 3), which resembles Z-DNA structure. It will be interesting to see whether these structural elements have any potential involvement in the regulatory expression of rabbit AGP gene.

-730 -666
CAGTACTGTTAGCCCCATTTTACAAGCTGAATTACTGGGGCTCTGAGAGTTATGGCACTTGTTCA
-601
AGGTCACCAGCTAGGAACTGGTAAAGCCAGGGAGTGCTGCAATCACCATCCGCTCCTGCTCTGAG
-536
GATTCTTCTGTCTCTTTCCTTCCCAAAAACTTTTTTGGGAGAGGCCAACAGACAGAGAGAGAGAG
-471
GGAGAGAGATACTAACAAAGTGATCTCCCATTGGCTGATTTGTTCCCTAAATGCCAGCAACAGGT
-406
AGGGCAGGGCTGGGCCAAAGCCAGGACCCTGGAATTCAATGAGGGTCTGCCATATGGGGGGGGGG
-341
GGGCAAGTACTACAGCTGCCATCACTGCTTCCAGGGTGCACCTGAGCAGGAAGCTGGCATCAGAA
-276
GCGGAGCTGGGACTCGAACTCGCACACTCCAGTATGGGATATGCACCTTCCAAGCGGTGTCCTAA
-211
TCACTGTGCCAAATCCCTGCCTCCCCACAAACAGATGGAGAGACAGGCTAGGCAGATCTGGCCCA
-146
GGACCTCAATCCACAGAGGCTCCGAGCTTGCCTGGAATATAAGGAGATGGGGAAATGGTCCTGTG
-81
TTGGGGGGTTGAGGGGAGAAACTCCGTGGATAGCATCACGGGTGGTCAGTGAGACCCCCTGGGGC
-16
GGGGGCGGGGGCGGCCCTCCCTGGGCCCCACCTGCCCTTCCCACAGCGCACTATAAAGGTGGCTG
+1 47
TGCCCTCCAGCCACC **AGCTCTGCCTGGCTCCAGCGCCTCTGTGTCTCAGC ATG GCC CTG CCC**
98
TGG GCC CTC GCC GTC CTG AGC CTC CTC CCT CTG CTG CAT GCC CAG GAC CCA
149
GCG TGT GCC AAC TTC TCG ACC AGC CCT ATC ACC AAT GCC ACC CTG GAC CAG
214
GTGAGTGTCGGGGCCAGGGTGGGCAGCCGCTGTCCCTCTCGCTGTGCTCAGCCCCTTCATCCTTG
279
CTCTGCCCCTCCCCTCCTGGTTCGCAGGATGAAATTCTGACAAGCTTGGGGAATCGGGGTGGCAC
344
TCCCCAGACTCCCTGCTGCACAGTCCCTCAACAAGGGACAATTCTGTGCAGGGCACGGAGAGGCC
409
ACTCGTGGGGGATGCCTGACCCCCGGCGTGAGCCCTGCCTGTCTGCTCCCCACCTGGGGCCTCCG
474
TTTCCCTGTTTGCAAATTGGATGGAAAGCTCTATCCAGATCTTGCGGCTGCCGACTGCCCCAGAC
532
TTAGCTCCGAGCCCCCGTCAGCGCCCCCTCTCCTCCCCAG **CTC TCC CAC AAG TGG TTT**
583
TTT ACC GCC TCG GCC TTC CGG AAC CCC AAG TAC AAG CAG CTG GTG CAG CAT
634
ACC CAG GCG GCC TTT TTC TAC TTC ACC GCC ATC AAA GAG GAG GAC ACC TTG
693
CTG CTC CGG GAG TAC ATA ACC AC GTGAGTCCCCACGCCAGCCCACCCAGGGCCCTGCTT
758
CCGAGCAAGTCCCTCCGTCCCCAGGGCCTTGGCCTTCCCAAGCGTGGAATGGGGAGGGTCGACTC
823
TAATCTCCACTCGTGCCTGACCGCTTTTCCTTGACCATCTTGCTGTTTGTCTCTCACCTCCCGTC
875
TGGCTGTAG **G AAC AAC ACG TGC TTC TAT AAC TCC AGC ATC GTG AGG GTC CAG**
932
AGA GAG AAT GGG ACC CTC TCC AAA CAC G GTGAGCCCTGGGCCCGAGGCAGGAGGCCT
997
CGACAAAGGCCCCGGAGGCTTCGGGCACGGAGAACATGGGACAGGCCGACTGTGATCACAGAGCA
1062
GCCAGCAGTTCTGACTTCTTACTCTGTGCCGGCTGCTGTTTGGAGGGCTTTGCATGAATGAACTC
1127
ACTTGATTCTCACAAACAATCAAGAGGACAAGGACAGAAACAGATCAGAGATGCAGTATCCTGCT
1192
CACACAGCTACGTCTTAGAACTAGAATTCAAACCCAAGGCTGCCTCCCCGTTTTCCTCTTCCTCT
1257
TCCTTAAACCACTGTTTAAACACTTTGGAAGCTATGGGTGGAGCCTATTTTTTAAAGGTGATTTT
1322
TTTTTAAAGATTTACTTATTTATTTGAAAGTCGTAGTTATACAGAGAGAGAAGGAGAGGCCAGAG
1387
AGAGAGGTCTTCCATCCGGCTGGTTCACTTCCCAATTGGCCGCAAATGATCAGAGCTGCGCTGAT

Figure 3. Nucleotide sequence of rabbit α_1-acid glycoprotein gene. DNA sequence of the AGP gene including six exonic regions, designated by bold letters, introns and the 5′ and 3′ flanking regions are shown. Transcription start site is indicated by the designation of +1. The putative TATA element and polyadenylation signal (AATAAA) are boxed. The underlined sequences in the 5′ flanking region upstream of the TATA element appears to contain unique structural features, which are discussed in Results and Discussion.

1452
CCGAAACCAGAAGCCAGGAGCTTCTTCCAGGTCTCCCACGTGGGTGCAGGGACCCAAGGGCTTGG
1517
GCCATCTTCTACTGCTTCCCCAGGCCATAGCAGAGAGCTGGATCAGAAGTGGAGCAGCTGGGACT
1582
CGAACCAGTATCTATATGGAATGGCGGCACTGCAGGCAGCAGACTTTACCCACTACGCCAAAGCG
1647
CTGGCCCTGGAGCCTATTTTTTTACACACCCACTACTCCCACAGAGACAGCACACACACACCCAT
1712
GGGGCTGACCAGACACCACTCAGCGGACCCCTGAGCCTCATCTCCCTCATCTGTGAAATGGGCAT
1777
GTAGGCCCTGACAGCCAGAGCCTATATTTATCACCAAGCGCTCAGTGCCAGCCTGGCACACAGCA
1842
GGGTTGGGCACGCCAGGACGGCTGGGATCCTTCCCTGGTGGTCCGTAGCCCTGGCACGCCAGGCC
1906
TCCTCCATTGAGATGGTGACCGTTGTGCCGTCTCCTCGTCTCACCGGAGCCTCTCTCCTGCAG **A**
1955
C GGC ATA CGA AAT AGC GTG GCC GAC CTG CTG CTC CTC AGG GAC CCC GGG
2006
AGC TTC CTC CTC GTC TTC TTC GCT GGG AAG GAG CAG GAC AAG GGA ATG TCC
2065
TTC TAC A GTAGGTGCCCCGGCAGCCACACCCCGCCCCCGGCCTGCCCTGACTCCACCCACC
2130
CTGGGGACTGGGCTCCCAGCCCCCTGGCCCTGCCTGGCCTCCCTCCTGGGCCTGCACACAGTTGC
2184
CTCGCTTCCCCTGCAG **CC GAC AAG CCC AAG GCC AGC CCG GAA CAA CTG GAA GAG**
2235
TTC TAC GAA GCC CTC ACG TGC CTG GGC ATG AAC AAG ACG GAA GTC GTC TAC
2295
ACT GAC TGG ACA AAG GTAAAGCCAGGGGTGGCCGTGCGGCGCAGGCAACCTCATAGGCCC
2360
AGAGAGGGAAAGTGGCTCAGCCCAGGCTGGGGAGAGAGGCAGGTGGCTTGCTTTGAAGGACCCAT
2425
CGCTGAGTCAGAGTCCCCGGGGCTGTGGGTCAGAGTCTGGCCGGGAGCAGTCCCTGTGAACTCGG
2490
GGTGCAGATGCCTAGGACCTGTCAGGCTTACAGGAAGGGAGCAGGCAGAACCAAGGGAGAGACAT
2555
GGCCCCACCCCACCCCCACAACTGTGTAACACCTGCATCGCGGAGAATCCAGGCTGCGGTTTTCC
2620
ATGTCACGCAAACTGCAGCAAGTCCCGGGTTTGTGAATCACCTGTGCACTGATCTCCTTACTACT
2685
TTTCTTTCTTTCACGCTGTGTTGAGTGAAAGGTTTTCCTTGGCATTGCCCTCGCCATCTTACCTG
2750
CAAATCCCCAGAGCCCATGAGCTGGTCACTGTTCCTCTGATGGATACACCTCGCTTTCATGACCA
2815
TTCAAACCCAGCGGTCCCCACCTGCTTGGCCTCTGCGTCCTCCCAGGACCACACCCCTTCCCACC
2880
TGGCTGCCATCCTAGAGCCCAGGGTTTAGGAGCCGCAGCCAACAGGGGTGGGTGCCGCTGGTGCA
2945
CAGCCTGCCTCCCCCACCAGCGGCAGAGACTGTCAGGAACCCAGCAGACACGCAGCGGGGGTTGG
3010
GGGAGGGGACGTGAAGGGATTGGGGTTGGGGAGTGAGCTGCCACACCCCAAGGGCTCAGGAAATG
3075
CCACTCGGTCCTTTGAGCCCCCGGGCCCCTATTTTAGGGGTGCCTAGACCAGGCTGCGGGAGGGA
3140
AATGACTTACTCGCCATGTTCCCTGCAGGGAGCGGCAGGGCTAGGGTTAGAACCCTGTCCCCTCA
3196
CTGCACTTGCTTGTCCCGTCTCCTAG **GAT CTG TGC GAG CCG CTG GAG AAG CAA CAC**
3250
GAG GAG GAG AGG AAG AAG GAA AAG GCA GAG TCA TAG GGCACAGCACCGGCTCCG
3315
GGACTCGGGGCCCACCCCCTGCACCTGCCTTTTTGTTTGTTTTGTAAATCTCTGTTCTTTCCCAT
3381
GGTTGCATCAATAAAACTGCTGGACCAGT CAGGTTGTCATTCATTCGTTCGTTCATTCATGGCGAA
3446
GCTGGTCTGGGGGGAGCCCCATCGGGTCCAGGATGAGCCCCGCCCCCTGGTTGGGAGGGCCCCTG
3511
CTGGAGTACCCAGCACATAGGCCAGGCTGGGTGCCAATCACCACTGTGCTGCGGGTGCCCCTGCA
3576
CCAGAAGCTGTGACTGGCAATCAGACATCGGGGAGGGGCCCGACAGCAGGCAGGAAGGCCTGGGA
3641
GGAGGCTGAGCCAGGCATCCTGGCTCGAGAGTTGCCCAGGCAAGGCGGGGTGATGCAGGGGACTT
3706
GGGGCCAAGGTCAGAGGGGCTGGGTCCCTCCATGCAAGTGTCCTCAGGGTGCCCACCTGAGACAC
3771
CTGGGAAATGATTTGGGGCCCTTGAGGTGCAGCTGAGGCTTGGCACACGCTGGTGGCACAGACGC
3792
TGGAGCCGGATGCTGGAATTC

Figure 3 - Continued

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                                REGION A
            -62                                    -36
Human       TTGCTGGGCT---CCAAG-TGACC-GCCCATA

            -63                                    -36
Rabbit      TCCCTGGGCC---CCACC-TGCCCTTCCCACA

            -64                                    -40
Rat1        CCCCTGG-CT---GCA-C--GCCCTTCCCACA

            -62                                    -34
Rat2        CCCCTGG-CG--ACGCCCATGCCCTTCCCACA

            -63                                    -33
Mouse       TCCCTGG-CTTCAGTCCCATGCCCTCCCCACA


                                REGION B
            -29                                    -1
Human       TATAAAGGTGACTGCACCCTGCAGCCACC

            -29                                    -1
Rabbit      TATAAAGGTGGCTGTGCCCTCCAGCCACC

            -33                                    -5
Rat1        TATAAAAGTCACTGCACTCTCCAGCCACC

            -27                                    +1
Rat2        TATAAAAGTCACTGCACTCTCCAGCCAC

            -26                                  +1
Mouse       CATAAATGTTGCTGCACCATCCAAACACC
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Figure 4. Comparison of DNA sequences at the 5′ flanking regions of AGP gene in different mammalian species. Region A represents DNA sequences upstream of the TATA box in each species. Region B represents DNA sequence of the TATA box and downstream region. Numbers indicate the position with respect to the transcription start site. The nucleotide sequences are taken from: human (22), rabbit (this paper), rat_1 (21), rat_2 (26), mouse (23).

Conserved sequences upstream and downstream of TATA box in AGP gene

To evaluate any possible regulatory role of DNA sequence element in and around the putative TATA box, we have analyzed this region by comparing the rabbit AGP sequence with that of human (22), rat_1 (21), rat_2 (26) and mouse (23). Comparative data in Fig. 4 reveals two regions of conserved sequences. Region A represents sequences upstream of TATA box while Region B contains that of downstream of TATA box. The extent of homology is quite striking. Recent studies on rat AGP gene have elucidated role of a DNA element located just upstream of this region as the site for glucocorticoid receptor and AGP/EBP, a C/EBP family member, binding region (14). Similar sequence element has also been found in mouse AGP gene (23). However, this motif is not present in the rabbit AGP gene, at least in this region of the genome. Sequence conservation of regions A and B (Fig. 4) may have some regulatory implication and probably warrants further attention.

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