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Murine DNA Polymerase β Gene: Mapping of Transcription Initiation Sites and the Nucleotide Sequence of the Putative Promoter Region

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The nucleotide sequence of the region (total, 2,512 base pairs [bp]) from intron 2 to the 5′-flanking region was determined for the mouse DNA polymerase β genomic clone, and the 300-bp region from intron 1 to the 5′-flanking region was also sequenced for the rat clone. At 51 bp upstream from the ATG codon which was previously suggested to be the translation initiation codon for the rat cDNA sequence, we found another ATG in the same reading frame in both mouse and rat genes. Three major transcription initiation sites (cap sites) each for rat and mouse DNA polymerase β mRNAs were localized precisely by primer extension analysis at 51, 41, and 0 bp upstream from the first ATG codon, suggesting that this codon is used for translation initiation. The 400-bp region around exon 1 was extremely G+C rich (about 70%). Although neither a TATA box nor a CAAT box was found within the 500-bp region upstream of the 5′-most cap site, triple repeats of 5′-CCGCCC were found within the 100-bp region flanking the cap site.

DNA polymerase β purified from various animal cells consists of a single polypeptide of about 40,000 daltons (32, 33, 39), and its structure is highly conserved through the course of evolution (4, 33, 34), suggesting some vital function in the maintenance of living cells. The reaction properties of DNA polymerase β are well suited for an efficient gap-filling reaction (17, 37), and repair synthesis of damaged DNA has been considered to be at least one of the roles of this enzyme. DNA polymerase β is expressed in most growing and resting cells throughout various developmental stages (10, 16, 19), and its level does not fluctuate very much during the cell cycle (5, 31). These properties are in sharp contrast to those of some other housekeeping enzymes such as DNA polymerase α (5, 10, 16, 24, 31) or dihydrofolate reductase (DHFR) (8) whose levels vary with different physiological conditions and cell cycle stages. The following two questions remain to be resolved. (i) What cellular factor(s) regulates the expression of the DNA polymerase β gene? (ii) What structural features of the transcription regulatory region of the gene contribute to this highly constitutive expression of DNA polymerase β? We have isolated a cDNA clone for rat DNA polymerase β (41) and used it to assign the human DNA polymerase β gene to chromosome 8 (18).

In this study, we isolated rat and mouse genomic clones containing the DNA polymerase β gene sequence and focused our attention on the 5′ upstream region of the gene which is supposed to contain the promoter. Mapping of transcription initiation sites defined the putative transcription regulatory region, and nucleotide sequence analysis revealed structural features of this region of the DNA polymerase β gene.

DNAs extracted from mouse and rat cells were analyzed by Southern hybridization with nick-translated pUC9-10F, which is specific for the 5′ side of DNA polymerase β, pUC19-10SL, which is specific for the 3′ side of the cDNA, or a mixture of both probes (Fig. 1). A 7.6-kilobase (kb) band of rat DNA was detected with the 5′-side-specific probe (Fig. 1B, lane 2), while 6.7- and 5.1-kb bands were detected with the 3′-side-specific probe (Fig. 1B, lane 4). With mouse DNA, 7.1- and 5.9-kb bands were detected with the 5′-side-specific probe (Fig. 1B, lane 1), while doublet bands of 3.0 kb were detected with the 3′-side probe (Fig. 1B, lane 3). Previously, we observed that a single *Bam*HI fragment hybridized with a pUC9-10F probe in both mouse and rat DNA, suggesting the occurrence of a single copy of the DNA polymerase β gene per genome (18). Thus, both mouse and rat DNA polymerase β genes seem to distribute through about 19 kb of each genomic DNA. From the gel region containing DNA fragments that gave positive signals in the Southern analysis (30) (Fig. 1B, lanes 1 and 2), DNA was electrophoretically eluted and used for the construction of a genomic library in λgtWES · λB vector (gift from Y. Nishida, Aichi Cancer Center Research Institute) as described previously (15). Positive plaques were selected by plaque hybridization (2) with nick-translated (26) pUC9-10F DNA as a probe. The positive clone containing the 7.1-kb mouse DNA was designated λMGβ01, and that containing the 5.9-kb DNA was designated as λMGβ02. The positive clone containing the 7.6-kb rat DNA was designated λRGβ01.

To determine the nucleotide sequence of the mouse genomic clone, we constructed a series of unidirectional deletion derivatives proceeding from the *Hin*dIII site toward the *Pst*I site as described previously (12) (Fig. 1C, arrows), and the nucleotide sequence of the individual clone was determined by the dideoxy sequencing method (28, 36). The total region of 2,512 base pairs (bp) including a 642-bp region upstream from the 5′-most cap site, exon 1 (111 bp), intron 1 (194 bp), exon 2 (58 bp), and a part of intron 2 (1,506 bp) was sequenced (Fig. 2A). For simplicity in describing the sequences, we used a numbering scheme in which +1 denotes

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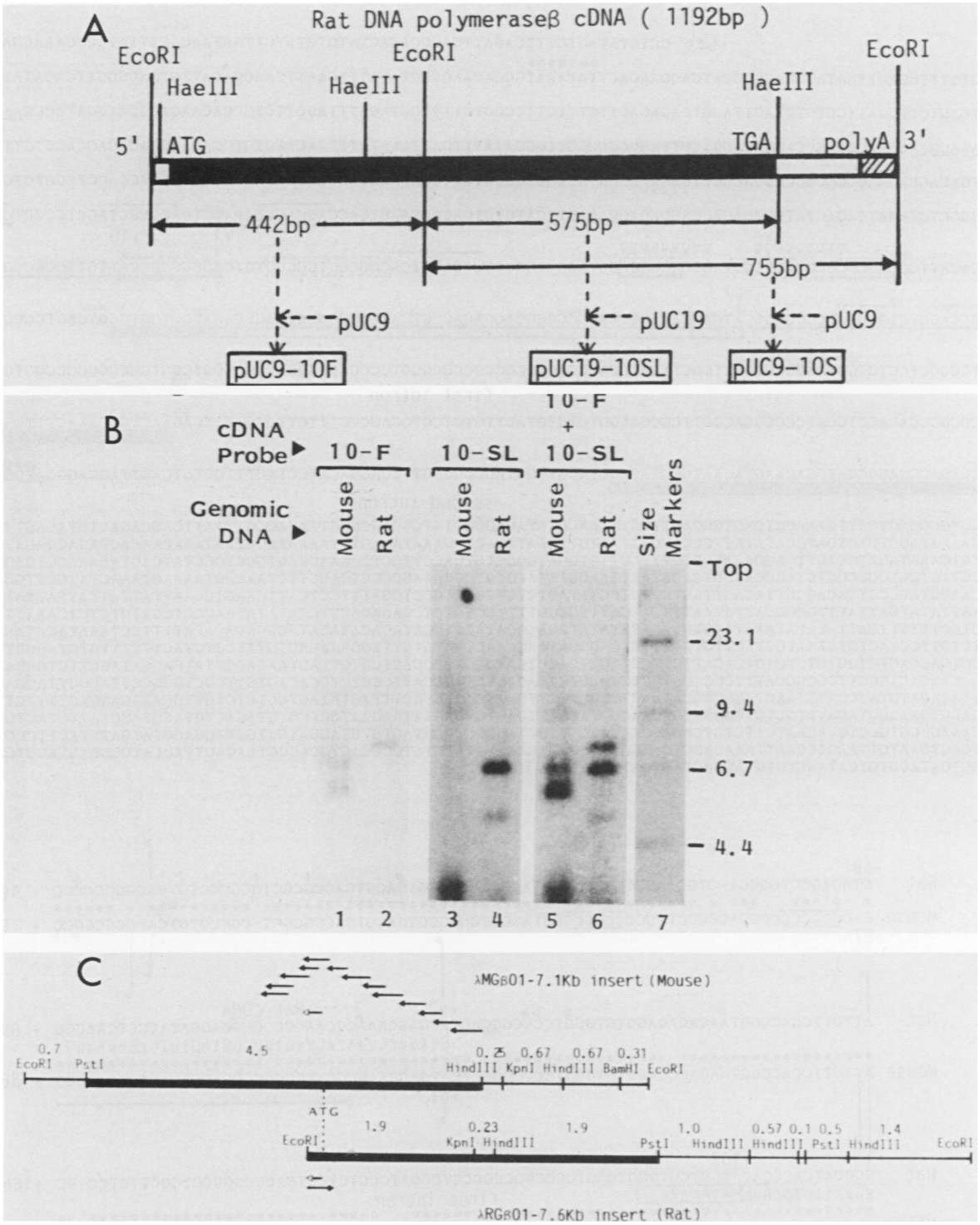

FIG. 1. Southern blot hybridization analysis of DNAs from mouse and rat. (A) Structure of rat DNA polymerase β cDNA and subcloning of 5′-side and 3′-side fragments into pUC9 or pUC19 vector. 5′-side 442-bp and 3′-side 755-bp fragments were subcloned into pUC9 to construct the plasmids pUC9-10F and pUC9-10S, respectively. In addition, the 3′-side 575-bp fragment was cloned to construct pUC19-10SL. pUC9-10F and pUC19-10SL were used as probes in the Southern hybridization. (B) Autoradiogram showing results of Southern blot hybridization analysis. Genomic DNAs from mouse spleen (lanes 1, 3, and 5) and rat spleen (lanes 2, 4, and 6) were digested with *Eco*RI, fractionated by 0.6% agarose gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized with ^{32}P-labeled pUC9-10F (lanes 1 and 2), pUC19-10SL (lanes 3 and 4), or mixture of the two probes (lanes 5 and 6). The size markers (in kilobases) were *Hin*dIII-digested and 3′-end-labeled λ DNA. Exposures were 16 h for lanes 1 and 2 and 36 h for lanes 3 to 7. (C) Restriction maps and sequencing strategy for genomic clones containing the 5′ region of mouse and rat DNA polymerase β genes. Restriction mapping was performed by digesting subclones pUC19-MGβ01 and pUC19-RGβ01 with restriction endonucleases and submitting the fragments to gel electrophoresis. Nucleotide sequences were determined by the Sanger method (31, 40) with sequential deletion derivatives of pUC19-MGβ01A containing the *Pst*I-*Hin*dIII 4.5-kb fragment (thick bar) for the mouse gene with reverse 17mer universal primer (◀—) or synthetic 15mer primer I (◁—). pUC19-RGβ01A containing the *Eco*RI-*Pst*I 4.03-kb fragment (thick bar) for the rat gene was also sequenced with normal 17mer universal primer (—▶) or synthetic 15mer primer I (◁—).

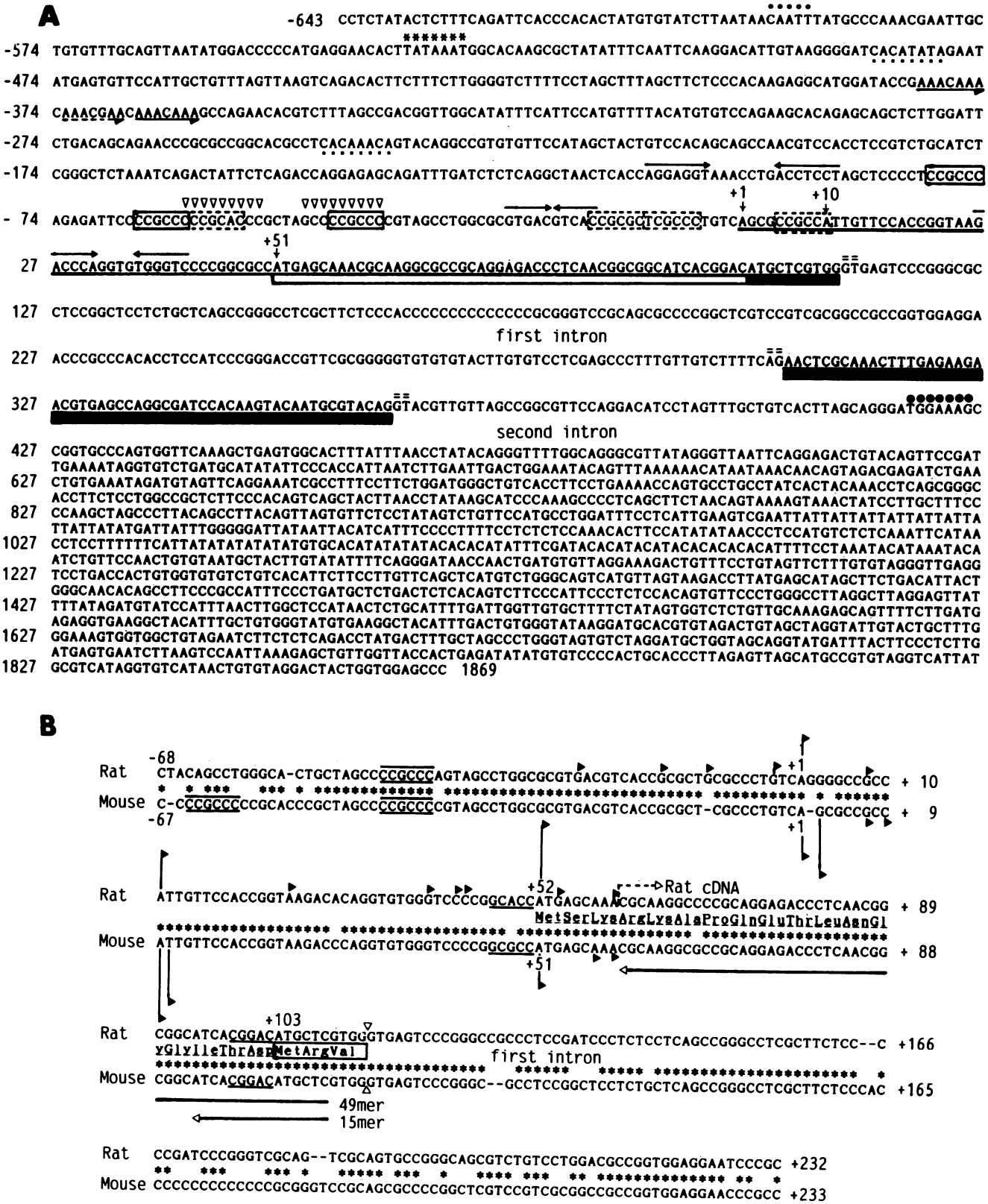

FIG. 2. Nucleotide sequence of the 5′ upstream region, exon 1, intron 1, exon 2, and a part of intron 2 of the mouse DNA polymerase β gene and transcription initiation sites for rat and mouse DNA polymerase β mRNAs. (A) Nucleotide sequence. Nucleotide +1 denotes the 5′-most proximal major cap site, and residues preceding it are indicated by negative numbers. Three major cap sites determined by primer extension analysis (see Fig. 3) are indicated by numbered vertical arrows. The untranslated region of the mRNA is indicated by a solid line below the nucleotides. The protein-coding region starting with the first ATG codon is indicated by an open bar, and that starting with the

the position of the 5'-most cap site. The rat clone contained only 68 bp of upstream sequence from the 5'-most cap site. The nucleotide sequence of this region along with that of exon 1 (112 bp) and a part of intron 1 (120 bp) was determined (Fig. 2B). Nucleotide sequences of this region were highly conserved between the rat and the mouse; exon 1 and the 50-bp upstream region were almost identical (Fig. 2B).

Sequences around the intron-exon boundaries are consistent with the splicing signals reported (3). From the sequence analysis of the rat DNA polymerase β cDNA (41), the ATG at position +103 was suggested to be the translation initiation codon for the DNA polymerase β polypeptide (41). In rat genomic DNA, we found another ATG in the same reading frame at position +52 which was not containd in the rat cDNA (Fig. 2B). Similarly, two ATGs were found at the corresponding positions of the mouse genome (Fig. 2B).

Up to position −535 (Fig. 2A), there is no TATA box, which is typically located between −20 and −30 and has the consensus sequence $TATA^{A}_{T}A^{A}_{T}$ (3). Similarly, up to −597, there is no canonical CAAT box, which is usually found at −70 to −80 (3). Instead, a specific 6-bp sequence, 5'-CCGCCC, is repeated three times at positions −44, −65, and −80 (Fig. 2A). Five other similar sequences which match five of six nucleotide residues of 5'-CCGCCC are distributed between −260 and +15. Triple tandem repeats of 5'-AAACAAA, although one of them is imperfect, start at −381, and three inverted repeats at −110, −25, and +26 were also found. It is interesting that there is a sequence, 5'-TGGAAAG, in intron 2 which completely matches the enhancer core sequence of simian virus 40 (SV40) (14, 38).

To identify the 5' termini of DNA polymerase β mRNA, we prepared α-^{32}P-labeled 49mer primer (Fig. 2B) as follows. A synthetic 55mer (5'-CGCAAGGCCCCGCAGGCGAC CTCAACGGCGGCATCACGGACATGCTCGTGGAA-3') was hybridized with the synthetic 15mer primer I (5'-GAGCATGTCCGTGAT-3') which is complementary to the underlined region of the 55mer, and the primer was extended with Klenow fragment in the presence of [α-^{32}P]dCTP to synthesize a 49mer. The 49mer was isolated and hybridized with either rat or mouse poly(A)$^+$ RNA. The 49mer primer was extended with avian myeloblastosis virus reverse transcriptase, and the products were size analyzed by gel electrophoresis under denaturing conditions followed by autoradiography (27). ^{32}P-labeled DNA fragments produced in the dideoxy sequencing reaction with the template DNA which contained the same sequence as the mRNA and the 15mer primer I were run in parallel, allowing precise mapping of the cap sites.

Three major bands were detected with rat mRNA at apparent nucleotide locations of −1, +10, and +51 (Fig. 3B). However, the mobility of the primer extension products was slower by one nucleotide equivalent than the correct position. In fact, unextended 49mer (Fig. 2B), which was expected to migrate at position +61, actually migrated at position +60 (Fig. 3D). This one-nucleotide difference might have resulted from either the high concentration of nucleic acids in the primer extension reaction or the 3'-dideoxy terminal structure of the DNA products in the sequencing reaction. Thus, by adding one nucleotide to the apparent positions on the gel for correction, the three major cap sites of rat mRNA were mapped at the positions +1, +11, and +52 (Fig. 2B). All these start sites resemble a consensus start site sequence, 5'-PyAPyPyPyPyPy, in which the 5' end of the mRNA corresponds to A (3). Several minor start sites including four sites further upstream than +1 were also detected (Fig. 3B). The 5' termini of mouse DNA polymerase β mRNAs were mapped at the almost identical positions (+1, +10, and 51) as in rat mRNA (Fig. 3A), although two additional strong sites at +2 and +11 were present. These two sites are, however, not consistent with the consensus start site sequence (Fig. 2B), and therefore, they could be due to some degradation of mRNA during isolation process.

We mapped three major transcription initiation sites at nucleotide positions +1, +11, and +52 for DNA polymerase β mRNA on the mouse and rat genes. There are two ATG codons in the same reading frame at +52 and +113 (Fig. 4). Two frames started with these different ATG codons are able to code for proteins of 38,221 and 36,375 daltons, respectively. The former has 17 more amino acid residues than the latter. For the following reasons, we suggest that the first ATG is the true initiation codon. (i) Four of five nucleotide residues preceding the first ATG and three of five nucleotide residues preceding the second ATG match the consensus initiation sequence 5'-$CC^{A}_{G}CC$ for translation (13), suggesting that the first ATG codon is slightly preferred for translation initiation. (ii) The length of the 5' untranslated region of eucaryotic mRNA is usually in the range of 20 to 80 nucleotides (13), and in most cases translation starts at the 5'-most proximal AUG triplet (13). (iii) The size of protein produced in *Escherichia coli* carrying a recombinant plasmid in which 1,005 bp of coding sequence starting with the first ATG were placed under the control of *E. coli lac* operon regulatory sequence was indistinguishable from that of the purified rat DNA polymerase β protein (unpublished data).

Nucleotide sequence analysis of the region upstream from the transcription initiation site of the DNA polymerase β gene revealed some characteristic features of mammalian

second ATG is shown by a solid bar. The coding region of the latter is common with that of the former. Consensus sequences at exon-intron boundaries are indicated by = above nucleotides. The repeated sequence 5'-CCGCCC is enclosed with a solid box, and the repeated sequences whose five of six residues match 5'-CCGCCC are enclosed with dashed boxes. The conventional TATA- and CAAT-like sequences are indicated by asterisks and open circles, respectively. Three inverted repeats are shown by pairs of arrows (head to head) above the nucleotide sequences. Triple direct repeats are shown by arrows below the nucleotide sequences (head and tail). The repeated sequences whose seven of eight residues match the repeated sequence CACAAAATA of the DHFR gene promoter region are indicated by closed square dots below the nucleotide sequences. The sequences whose seven of nine residues match the sequence 5'-GCCACACCC (CACCC-box) of the β-globin gene promoter region are shown by open triangles above the nucleotide sequences. (B) Transcription initiation sites. Nucleotide sequences of both rat and mouse DNA regions containing the 5' upstream region, exon 1, and a part of intron 1 are shown. Matched nucleotides are marked by asterisks. Vertical lines with arrowheads and arrowheads alone indicate the locations of major and minor transcription initiations, respectively. The length of lines shows the relative abundance of the initiations. The 5'-most proximal major sites were numbered as +1. The protein-coding region starting with the first ATG is underlined with a dashed line, and that starting with the second ATG is enclosed with a box. The five nucleotides immediately preceding the first and second ATGs are underlined to compare with translation initiation consensus sequence 5'-$CC^{A}_{G}CC$. The hexanucleotide repeats, 5'-CCGCCC, are shown between two lines above and below the nucleotide sequences. Sequences corresponding to the synthetic 15mer primer I used for both sequence analysis and the synthesis of the 49mer primer and the 49mer primer used for primer extension analysis are shown by arrows with open heads.

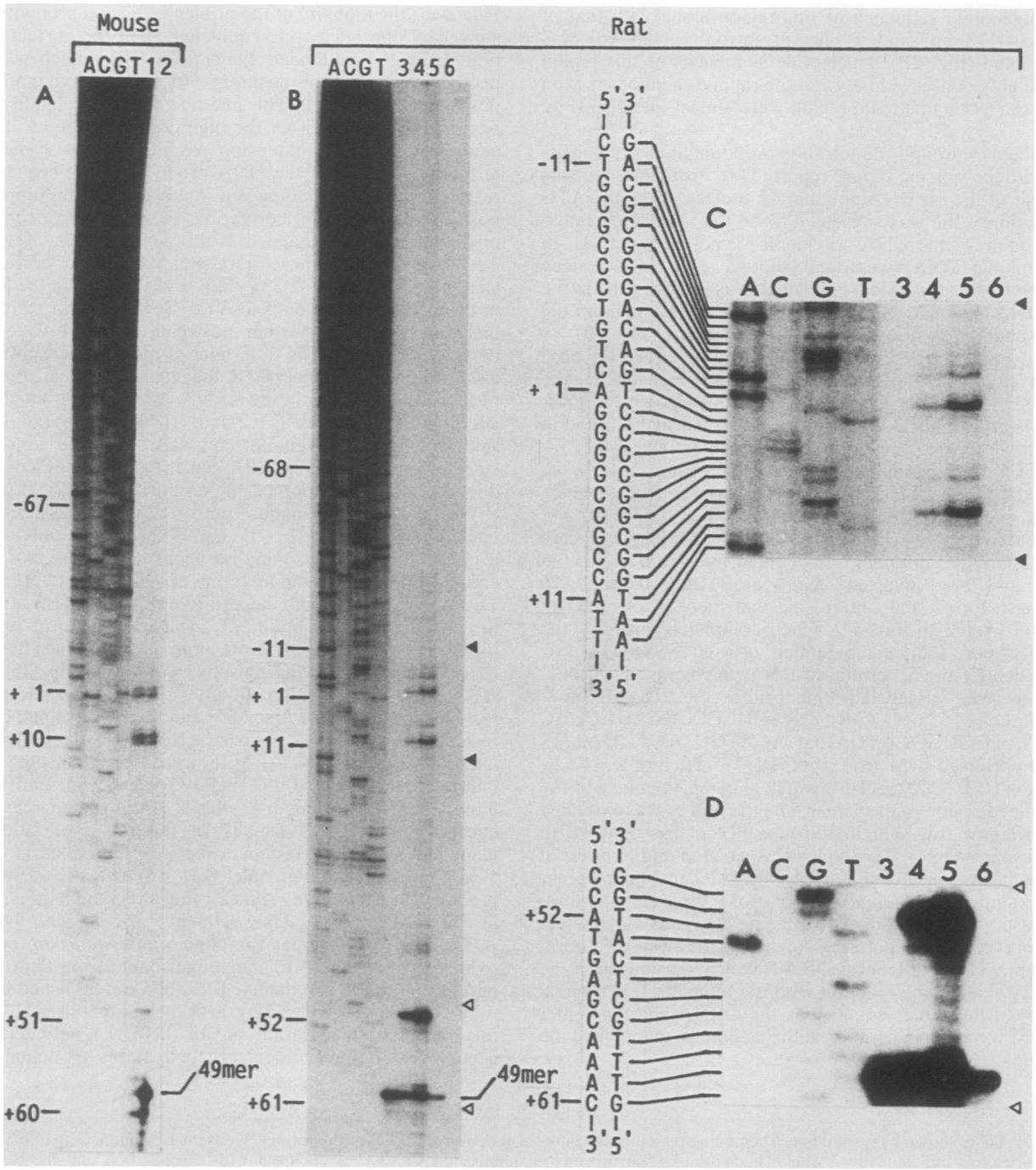

FIG. 3. Mapping of transcription initiation sites by primer extension analysis. The ^{32}P-labeled 49mer primer complementary to the coding sequence of the region for the 5′ part of DNA polymerase β mRNA (Fig. 2B) was hybridized with either mouse (lanes 1 and 2) or rat (lanes 3 and 5) poly(A)$^+$ RNA and extended by avian myeloblastosis virus reverse transcriptase for 1 h (lanes 1 and 4) or 2 h (lanes 2, 3, 5, and 6). The reaction mixture for lane 3 did not contain reverse transcriptase, and the reaction mixture for lane 6 did not contain poly(A)$^+$ RNA. To align the extended products with the genomic DNA sequence, we performed a parallel dideoxy sequencing reaction using the synthetic 15mer primer I whose sequence is identical to the 5′ portion of the 49mer primer in combination with the pUC19-MGβ01A DNA for the mouse (mouse lanes A to T) or the pUC19-RGβ01A DNA for the rat (rat lanes A to T) as the template. The products of the sequencing reaction and the primer extension reaction were analyzed by electrophoresis in the same gel under denaturing conditions followed by autoradiography. The autoradiograms were exposed for 2 weeks for panel A, for 1 week for panels B and C without an intensifying screen, and for 1 week for panel D with the screen. The numbers on the left side of the gel indicate the nucleotide positions from the 5′-most proximal major transcription initiation site which was defined as +1. The position of the 49mer primer is also indicated.

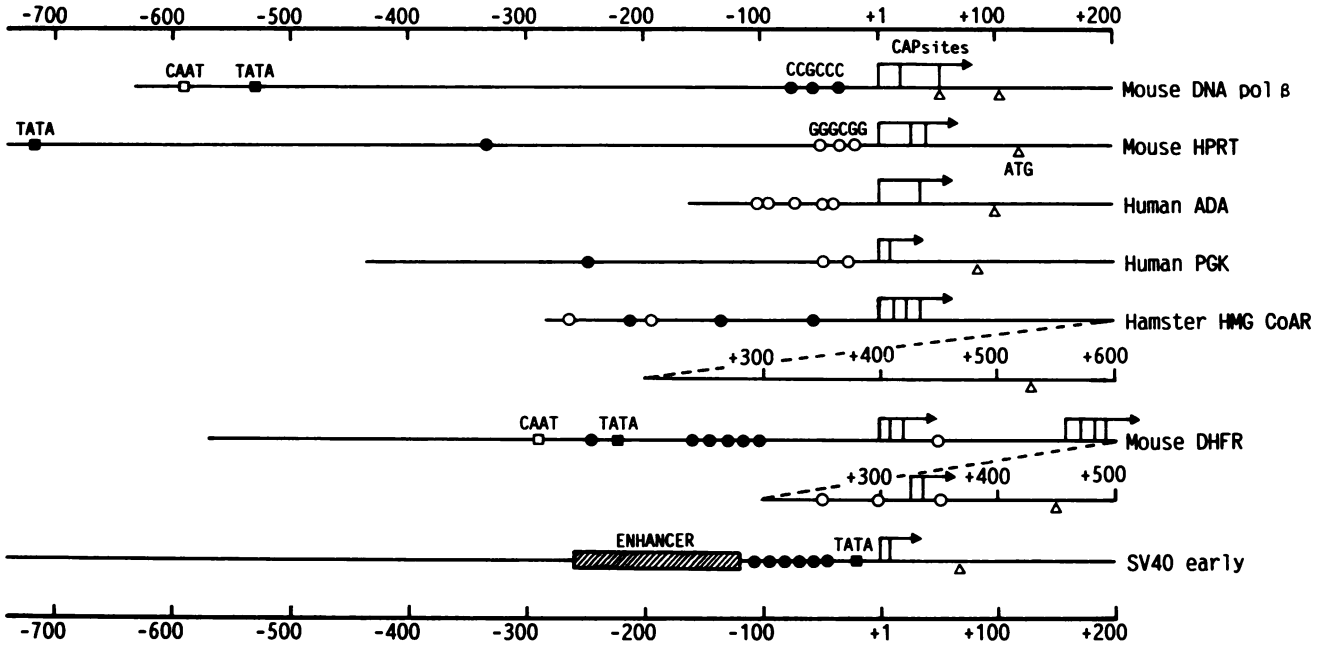

FIG. 4. Diagrammatic view of the 5′ upstream regions of several mammalian housekeeping genes and the SV40 early gene. The numbering of the nucleotides is relative to the 5′-most proximal transcription initiation site. Major sites for transcription initiation are indicated by vertical lines in combination with arrows. The positions of translation initiation codon ATG are shown by open triangles. The hexanucleotide motif 5′-CCGCCC and its reverse sequence 5′-GGGCGG are indicated by closed circles and open circles, respectively. The CAAT and TATA boxes are shown by open and closed squares, respectively. The region of the SV40 enhancer sequence is also indicated. The abbreviations used for the enzymes are: DNA pol β, DNA polymerase β; HPRT, hypoxanthine phosphoribosyltransferase; ADA, adenosine deaminase; PGK, 3-phosphoglycerate kinase; HMG CoA R, 3-hydroxy-3-methylglutaryl coenzyme A reductase.

housekeeping genes, which can be summarized as follows. (i) Conventional transcription regulatory sequences such as TATA and CAAT boxes are absent in the appropriate upstream region from the cap sites (20, 21, 25, 29, 35), but both TATA and CAAT boxes are located in a region more than 500 bp upstream from the 5′-most cap site. With the mouse DHFR gene, both the CAAT-TATA region and the downstream region containing 48-bp repeats function as independent promoter elements (20). The existence of a far upstream TATA box is also reported in the mouse hypoxanthine phosphoribosyltransferase gene (21). (ii) An extremely G+C-rich sequence exists in the 5′-flanking region. The 5′-flanking regions of known housekeeping genes are highly G+C rich, ranging from 65 to 85% (21, 22, 25, 26, 29). Notably, with the DNA polymerase β gene, not only the 5′-flanking region but also exon 1 and even intron 1 are G+C rich (about 70% overall). (iii) GC hexanucleotide motifs are found. The hexanucleotide sequence 5′-CCGCCC repeats six times in the SV40 early gene promoter region (9) and has been proved to be essential for its transcription (1, 11). The same sequence or the sequence in the opposite orientation (5′-GGGCGG) has been found in the upstream region of various housekeeping genes (20, 21, 25, 29, 35). These motifs are clustered in the regions within 300 bp upstream from the 5′-most transcription initiation sites (Fig. 4), although the location and number of these motifs are not completely identical among different genes. HeLa cell transcription factor Sp1 that stimulates the in vitro transcription of the SV40 early promoter binds to a specific SV40 early gene promoter region containing the 5′-CCGCCC sequence (6). This factor, in fact, interacts with cellular sequences containing 5′-CCGCCC in the promoter region of cloned monkey genomic DNA and the mouse DHFR gene as well as several viral promoters other than those of SV40 (7). Thus, the widespread existence of the hexanucleotide motifs suggests that Sp1 provides a basal transcription level that could be modulated by other positive or negative regulatory factors.

There are two sequences starting at −486 and −255 (Fig. 2A) where seven of eight residues match the sequence 5′-CACAAATA which is present in 48-bp triple repeats of the mouse DHFR gene promoter element (20). Other regions starting at −60 and −47 (Fig. 2A) share seven of nine residues with the 5′-GCCACACCC sequence which functions as a promoter element for mammalian β-globin genes (23). Although they are located far upstream, the CAAT and TATA boxes of the DNA polymerase β gene could be functional under some special physiological conditions just as for the DHFR gene (20). Furthermore, we found a sequence, 5′-TGGAAAG, in intron 2 which perfectly matches the SV40 enhancer core sequence (38). Thus, the transcription regulatory region of DNA polymerase β appears to be a combination of various types of transcription regulatory elements. Expression of a number of housekeeping genes is cell cycle regulated, usually high in the G1 to early S phase (5, 20). However, the expression of DNA polymerase β appears almost constant throughout the cell cycle and various developmental stages (5, 16, 19, 31). Coordinate functioning of a set of various regulatory elements might be responsible for this type of very constitutive expression of genes such as DNA polymerase β.

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