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# GENOMIC STRUCTURE OF THE ME491/CD63 ANTIGEN GENE AND FUNCTIONAL ANALYSIS OF THE 5'-FLANKING REGULATORY SEQUENCES

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**SUMMARY:** Genomic structure of the ME491/CD63 antigen gene and promoter activity of the 5'-flanking regulatory sequences were studied. The antigen gene consists of eight exons that span 4 kilobase-pairs. Primer extension analysis with RNA from cultured human cells identified three major transcription initiation sites. The 5'-flanking region of exon 1 has features characteristic to promoters of many house-keeping genes and growth-regulating genes. The region is highly GC rich and contains potential binding sites for transcription factors such as Sp1 and ETF, but not a TATA box. The 5'-flanking sequence exerted strong promoter activity when linked to a reporter gene. Deletion mutant analysis of the 5'-flanking sequence has strongly suggested that a potential binding site for AP-1 plays an important role in positively regulating the gene expression. 

ME491 antigen was originally found as a stage-dependent melanoma-associated antigen, being most strongly expressed in early stages of melanoma progression such as dysplastic nevus and radial growth phase primary melanoma (1). cDNA clones encoding for the antigen were molecularly cloned and the sequence analysis revealed that the antigen might be classified in a novel group of proteins, having no significant homology with then reported proteins (2). Recently, cDNA clones encoding for CD63 and Pltgp40, both of which were independently identified as a surface marker of activated platelets (3, 4), were molecularly cloned and it has been proved that both are identical to ME491 antigen (5, 6). Although biological function of ME491/CD63 antigen is as yet unclarified, an attractive possibility has been raised that the antigen may exert a tumor-suppressing activity. The hypothesis is based on the following observations; (i) during the malignant progression of melanoma from radial growth phase to vertical growth and metastatic phases, intensity of the antigen expression was inversely correlated with increasingly expressed malignant phenotypes (1), and (ii) overexpression of the antigen suppressed malignant phenotypes of H-*ras* -transformed NIH3T3 cells (7). Data also have been accumulating that suggest the presence of a gene family on the basis of structural features. ME491/CD63 antigen shares significant sequence similarities with a tumor-associated

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Abbreviations: CAT, chloramphenicol acetyltransferase; PBL, peripheral blood leukocytes; TPA, 12-tetradecanoylphorbol-13-acetate.

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antigen CO-029 (8), a trematode parasite surface antigen Sm23 (9) and a target antigen of an antiproliferative antibody, TAPA-1 (10), all of which appear to have four transmembrane domains, leaving both amino and carboxy termini inside the cytoplasm.

In tissue samples, ME491/CD63 antigen expression is observed only in a limited number of cell types (1). On the contrary, the antigen is ubiquitously expressed in a wide variety of cells when cultured *in vitro*. However, it does not necessarily mean that the antigen expression simply correlates with cell growth, since actively growing malignant cells *in vivo* are often negative for the antigen expression. These observations suggest differential regulation of the antigen expression *in vivo* and *in vitro*. The regulatory mechanism(s) of this gene expression is an interesting subject to study. In our earlier study, we demonstrated that a cryptic promoter with enhancer and suppressor sequences was present in the first intron of the ME491/CD63 antigen gene and that the cryptic promoter was differentially activated or suppressed depending on the cell type (11, 12). In the present paper we describe whole structure of the antigen gene and functional features of the 5'-flanking authentic promoter and other regulatory sequences.

## MATERIALS AND METHODS

Molecular cloning of the ME491/CD63 antigen gene: A genomic library prepared from human peripheral blood leukocytes (PBL) DNA was a generous gift from Dr. Y. Tsujimoto, Biomedical Research Center, Osaka University. The library was screened with [$^{32}$P]-labeled ME491/CD63 antigen cDNA (pSe24-1) in a stringent condition (2, 11).
DNA sequencing analysis: Restriction enzyme-digested genomic fragments were subcloned into M13mp18 or M13mp19, and sequenced by the dideoxy chain termination method (13) using Sequenase version 2.0 DNA sequencing kit (United States Biochem. Corp.).
Primer extension analysis: Total cellular RNA was extracted from human cell lines WM1158 (melanoma), HeLa (cervical carcinoma) and FL (amniotic membrane). Synthetic oligonucleotide designated E2N (5'-CATTCCTCCTTCCACCGCCAT-3') was used as a primer. E2N is complementary to a portion of ME491/CD63 antigen mRNA that corresponds to the extreme amino-terminus of the antigen. Detailed procedures for primer extension analysis were described previously (12).
Determination of promoter activity by chloramphenicol acetyltransferase (CAT) assay: DNA fragments containing various lengths of the 5'-upstream portion of the ME491/CD63 antigen gene were blunt-ended and subcloned into the promoter-less CAT plasmid pSV00CAT (14) in the proper orientation. Two μg of each promoter-CAT construct was transfected into WM1158 human melanoma cells or H-*ras*-transformed NIH3T3 (3T3-Hras) cells by DEAE dextran method (12, 15). pSV2CAT was used as a positive control (16). After cultured for 48-60 h, cell extracts were obtained and CAT assays were performed as described previously (12, 16).

## RESULTS AND DISCUSSION

### Genomic structure and nucleotide sequence of the ME491/CD63 antigen gene.

Two overlapping clones, designated λ1 and λ2, were isolated from a human genomic library by probing with [$^{32}$P]-labeled pSe24-1. To analyze the structure of the ME491/CD63 antigen gene, 3.2-kilobase (kb) *Bam*HI fragment that hybridized to pSe24-1 and the 5'-flanking 1.2-kb *Bam*HI fragment were subcloned from λ2, and nucleotide sequence was determined (EMBL accession no. X62654). A computer comparison with the antigen cDNA sequence (2) allowed the identification of a total of eight exons that span 4 kb (Fig. 1). Intron-exon boundary sequences mostly agreed with consensus sequences of both 5' and 3' splice junctions (17-19) (Table 1). The branchpoint consensus sequence (17, 20) were also found between 23-bp and 36-bp upstream of the splice junction (data not shown).

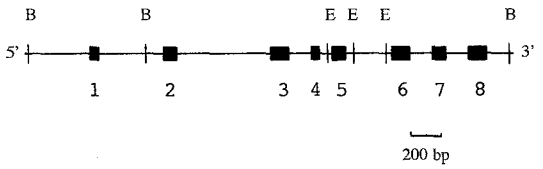


**Fig. 1.** Schematic representation of exon-intron structure of the ME491/CD63 antigen gene. Solid boxes represent exons. Each numeral denotes the exon number. B, *Bam*HI; E, *Eco*RI.

Exon 1 encodes exclusively for the 5'-untranslated sequence of the antigen mRNA. The translation initiation and termination codons are located in exons 2 and 8, respectively. The entire translated sequence encoded by the above exons is identical to that of the antigen cDNA obtained from the human melanoma cell line WM1158 (2), megakaryoblast cell line MEG-01 (5) and monocytic cell line U-937 (6). The results indicate that all of the above cell lines express non-mutated prototype ME491/CD63 antigen.

**Identification of the 5'-end of the ME491/CD63 antigen mRNA.**

To identify a transcription initiation site(s), primer extension analysis was performed using the primer E2N that anneals to a portion corresponding to the amino terminus of the antigen. With RNA samples obtained from human cell lines FL, HeLa and WM1158, major extended products of 90-, 111- and 152-bases were observed (Fig. 2). Based on the result together with the sequence data, three major transcription initiation sites were determined (see Fig. 3).

**Table 1.** Intron-exon boundary of the ME491/CD63 antigen gene[a]

| Exon (nt. no.[b]) | Nucleotide sequence |
|---|---|
| 1 (1-58) | TAGAGAGCCCCGGAGCCGCGGCGGGAGA.....GAAGCCCAG gtgagg |
| 2 (659-735) | cctctgccccag GCCCGGCAGCCATGGCG...GCCTTTTGC gtgagt |
| | M A A F C |
| 3 (1671-1859) | attttctcttag GCCTGTGCA.....ATGATCACG gtgcgt |
| | A C A M I T |
| 4 (2044-2118) | ttgctcctgcag TTTGCCATC.....AGAGATAAG gtaagc |
| | F A I R D K |
| 5 (2213-2308) | ccgtactctcag GTGATGTCA.....CAGGCAGAT gtgagt |
| | V M S Q A D |
| 6 (2746-2886) | tccctcccccag TTTAAGTGC.....CATAAGGAG gtaggg |
| | F K C H K E |
| 7 (3111-3194) | tgccaccttcag GGCTGTGTG.....TTTGTCGAG gtaaga |
| | G C V F V E |
| 8 (3381-3533) | cgggccttctag GTTTTGGGA.....GTGATGTAG..ATTAAA.. |
| | V L G V M End |

[a] Nucleotide sequences at the 5'- and 3'-boundaries of the eight exons are listed. Introns are in lowercase letters and exons in capital letters. Deduced amino acid sequences are written below each codon. The translation initiation and termination codons are underlined. A polyadenylation signal (ATTAAA) is located near the 3'-end of exon 8.

[b] Nucleotide residue +1 denotes the most downstream one of the major transcription initiation sites determined by primer extension analysis.

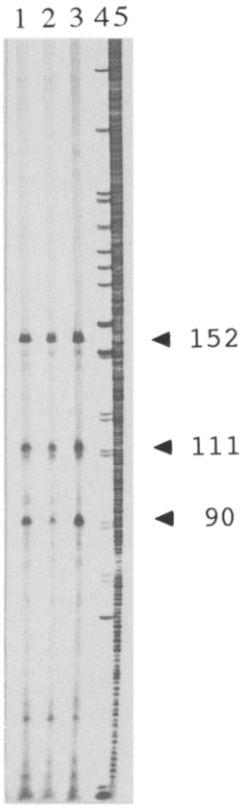


**Fig. 2.** Determination of the 5'-end of ME491/CD63 antigen mRNA by primer extension analysis. A representative result using a primer (E2N) is shown. Lane 1, FL cell RNA; lane 2, HeLa cell RNA; lane 3, WM1158 cell RNA; lane 4, size marker (pBR322 DNA digested with *Msp*I); lane 5, one-base ladder. Major extended products are shown by arrowheads.

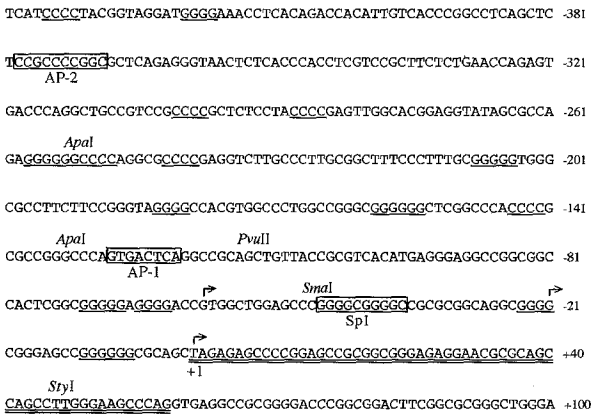


**Fig. 3.** Nucleotide sequence of the 5'-flanking region of the ME491/CD63 antigen gene. Major transcription initiation sites determined by primer extension analysis are depicted by arrows and the most downstream one is numbered as +1. The sequence of exon 1 is double-underlined. Potential binding sites for transcription factors Sp1, AP-1 and AP-2 are boxed, and those for ETF are underlined. Restriction sites for *Apa*I, *Pvu*II, *Sma*I and *Sty*I are shown.

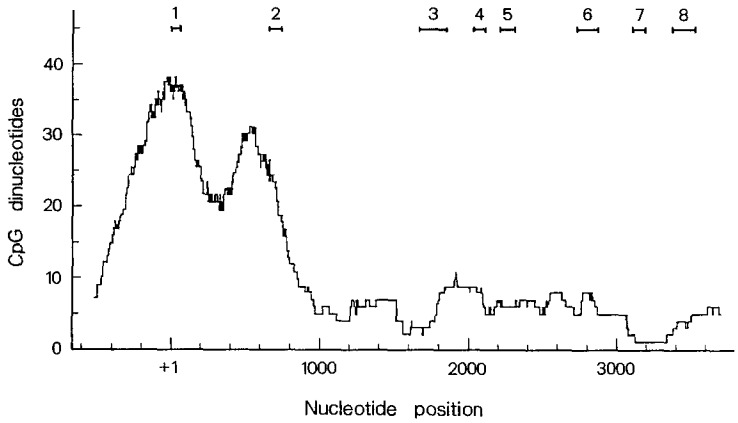


**Fig. 4.** Distribution of CpG dinucleotides along the ME491/CD63 antigen gene. The frequency of the dinucleotide CpG in a window of 300 bp is plotted along the gene sequence, where nucleotide residue +1 denotes the most downstream one of the major transcription initiation sites determined by primer extension analysis. Relative positions of eight exons are shown at the top.

**Promoter of the ME491/CD63 antigen gene.**

As shown in Fig. 3, nucleotide sequence of the 5'-flanking region of exon 1 exhibits interesting features characteristic to promoters of house-keeping genes and growth-regulating genes (21-26). While not having a "TATA" or "CAAT" consensus sequences, the promoter of the ME491/CD63 antigen gene is highly GC rich (82% between nucleotides -200 and -1), and contains potential binding sites for transcription factors such as Sp1 and ETF (27-29). The sequence motif from nucleotide -129 to -122 is a potential binding site for AP-1, which has been known as the *ras*- and phorbol ester-responsive enhancer element (30-32). The antigen gene promoter is also particularly rich in the dinucleotide sequence CpG, forming a "CpG island." (Fig. 4). "CpG islands" have been found at transcription initiation sites of many house-keeping genes (21, 22). There is an additional "CpG island" in the first intron of the antigen gene, though somewhat smaller than the other. We have reported the presence of a cryptic promoter in the first intron that appears to be differentially activated or suppressed depending on the cell type (12). The localization of the cryptic promoter exactly matches that of the second "CpG island." Taken together, the results may suggest a possibility that the cryptic promoter is involved in the regulation of the gene expression, either cooperating or competing with the authentic promoter.

Promoter activity of the 5'-flanking sequence was tested by CAT assay. A 704-base-pair (bp) *Bam*HI-*Sty*I fragment (nucleotide residues from -656 to +48) and its 5'-deletion fragments, 183-bp *Apa*I-*Sty*I (-135 to +48), 161-bp *Pvu*II-*Sty*I (-113 to +48) and 94-bp *Sma*I-*Sty*I (-46 to +48) fragments, were introduced into pSV00CAT in the proper orientation relative to the CAT gene (704BStyCAT, 183ApCAT, 161PvCAT and 94SmCAT, respectively) (see Fig. 5a), and transfected into 3T3-Hras and WM1158 cells. The 183-bp *Apa*I-*Sty*I fragment exerted strong promoter activity in both cells tested, the activity being practically the same as that of the 704-bp *Bam*HI-*Sty*I fragment (Fig. 5b). On the other hand, promoter activity of the 161-bp *Pvu*II-*Sty*I fragment was much weaker than that of the 183-bp *Apa*I-*Sty*I fragment. These results strongly suggest that the potential AP-1 binding sequence in the 183-bp *Apa*I-*Sty*I

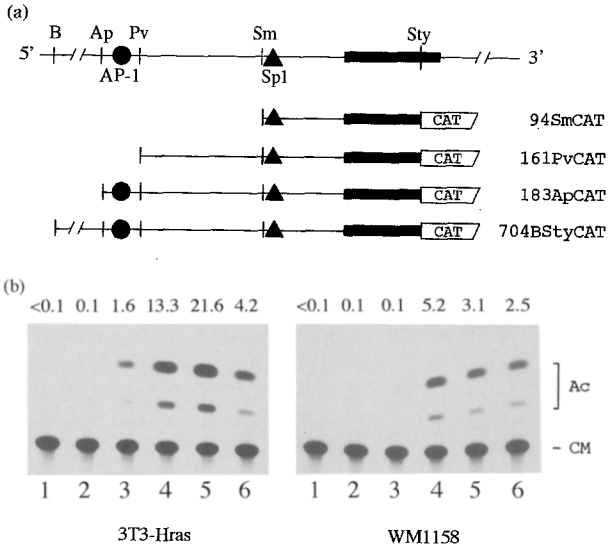


**Fig. 5.** Functional analysis of the promoter of the ME491/CD63 antigen gene by CAT assay. (a) Schematic representation of the 5'-flanking fragments and CAT constructs for promoter analysis. A solid box represents exon 1. Potential binding sites for transcription factors AP-1 and Sp1 are depicted by a solid circle and triangle, respectively. B, *Bam*HI; Ap, *Apa*I; Pv, *Pvu*II; Sm, *Sma*I; Sty, *Sty*I. (b) A representative result of CAT assay using 3T3-Hras and WM1158 cells. Lane 1, pSV00CAT; lane 2, 94SmCAT; lane 3, 161PvCAT; lane 4, 183ApCAT; lane 5, 704BStyCAT; lane 6, pSV2CAT. Acetylated (Ac) and non-acetylated forms of chloramphenicol (CM) are denoted. Acetylation ratios (%) are shown at the top of each lane.

fragment plays an important role in positively regulating the gene expression, and also agrees with our unpublished observation that HL 60 promyelocytic leukemia cells showed enhanced expression of ME491/CD63 antigen after treated with 12-tetradecanoylphorbol 13-acetate (TPA), since the AP-1 binding sequence is known as a TPA-responsive enhancer (30, 31).

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