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Activation and Suppression of a Cryptic Promoter in the Intron of the Human Melanoma-associated ME491 Antigen Gene

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A deletion mutant of the human melanoma-associated ME491 antigen gene starting at the first intron (λR31) differentially mediates the antigen expression depending on the cell type. Cryptic promoter activity residing in a 270-base-pair (bp) fragment of the first intron was examined by primer extension analysis and recombinant chloramphenicol acetyltransferase (CAT) assay. The cryptic promoter, further localized within a 153-bp fragment (fr153BN), exerted its effect in Ltk⁻ and H-*ras*-transformed NIH3T3 (3T3-Hras) but not in parental NIH3T3 cells. The results suggested that the cryptic promoter was associated with a novel *ras*-responsive positive regulatory element, since fr153BN did not contain an AP-1-binding sequence motif, known as the *ras*-responsive enhancer element. The cryptic promoter activity of fr153BN was suppressed by an upstream 121-bp fragment (fr121SB) which contained a consensus sequence motif for binding of a repressor protein, GC factor, and regions showing sequence similarity with putative *cis*-acting repressor elements found in the vimentin gene. The degree of the suppression was greater in 3T3-Hras than in Ltk⁻ cells. These positive and negative regulatory elements may be differentially involved in the regulation of ME491 antigen expression depending on the cell type.

Key words: Cryptic promoter — Positive regulatory element — Negative regulatory element — Intron-mediated transcription initiation — Melanoma-associated antigen

ME491 antigen is a highly glycosylated membrane protein with four putative transmembrane domains.[1] The antigen is well conserved among a wide range of species,[1] and appears to belong to a family of cell surface antigens that includes the human tumor-associated antigen CO-029[2] and Sm23 antigen of the human trematode parasite *Schistosoma mansoni.*[3] Atkinson *et al.*[4,5] reported that ME491 antigen expression was associated with early stages of melanoma progression. While not expressed in normal skin melanocytes, the antigen was strongly expressed in dysplastic nevi and radial growth phase primary melanoma. Interestingly, the antigen expression then declined significantly, or sometimes even disappeared, as melanoma cells progressed to more malignant stages such as vertical growth phase primary melanoma and metastatic melanoma.[5] Besides the tumor cells of melanocytic lineage, some adenocarcinomas showed strong expression of this antigen.[5] As for normal cells, tissue macrophages were consistently strongly positive.[5] Regulation of this gene expression is an interesting subject, and its elucidation may give us a clue to understand a common event(s) occurring in those cells.

The mouse counterpart for the ME491 antigen gene is transcribed in mouse cell lines such as Ltk⁻ and H-*ras*-transformed NIH3T3 cells as detected by Northern blot analysis using the cloned human gene as a probe.[1,6] The results suggest that regulatory mechanisms for the mouse gene expression are functioning in those cells. Furthermore, the mouse gene product shares high sequence similarity with human ME491 antigen (79% homology in amino acid sequence), implying a high degree of gene conservation as well. Therefore, it seems likely that the mouse cell system can be utilized for the study of ME491 antigen gene expression. We previously reported that a human genomic DNA clone containing a deletion mutant of the ME491 antigen gene which lacked its authentic promoter and the first exon (λR31) mediated a considerable degree of ME491 antigen expression when transfected into Ltk⁻ cells (Ltk/λR31), while the same DNA hardly mediated the antigen expression when transfected into NIH3T3 cells (3T3/λR31-3).[1,6] We also observed that the otherwise bare expression of the antigen in 3T3/λR31-3 was enhanced when the cells were transformed by H-*ras* oncogene (3T3/λR31-3/Hras).[6] These observations suggest the possible presence of a cryptic promoter(s) in the first intron and its differential activation depending on the cell type. The idea of the cryptic promoter may also apply to our unpublished observation that there is a cluster of CpG dinucleotides called the 'CpG island' in the first intron, as well as at the authentic promoter region, of the ME491 antigen gene; 'CpG islands' are usually found at the transcription initiation sites of many house-keeping genes.[7,8] In the present study we provide evidence that ME491 antigen mRNA transcription initiates at different sites within the first intron in Ltk/λR31 and 3T3/λR31-3/Hras. We then demonstrate that the first intron contains a cryptic promoter apparatus with a *ras*-responsive positive regulatory

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element, and that the cryptic promoter is suppressed by an upstream fragment containing a consensus sequence for binding of certain repressor proteins such as GC factor (GCF).[9]

MATERIALS AND METHODS

Primer extension analysis Total cellular RNA was extracted from Ltk/λR31 and 3T3/λR31-3/Hras as described previously.[6] A synthetic 21 mer (5′-CATTCCTCCTTCCACCGCCAT-3′) complementary to ME491 antigen mRNA was labeled with $[\gamma\text{-}^{32}P]$ATP and T4 polynucleotide kinase. The labeled oligonucleotide was precipitated with 50 μg of total RNA and then dissolved in 20 μl of hybridization buffer containing 50% formamide/400 m*M* NaCl/1 m*M* EDTA/40 m*M* PIPES (pH 6.4). Samples were heated at 80°C for 15 min and allowed to hybridize at 30°C overnight. Following hybridization, the samples were ethanol-precipitated and dissolved in 30 μl of reverse transcriptase buffer containing 1 m*M* dNTP's/100 m*M* KCl/10 m*M* $MgCl_2$/20 m*M* 2-mercaptoethanol/50 m*M* Tris-HCl (pH 8.0). To each sample, 50 U of reverse transcriptase was added, and the mixture was incubated at 40°C for 30 min. The reaction products were ethanol-precipitated, subjected to electrophoresis through a 5% polyacrylamide/8 *M* urea gel, and visualized by autoradiography.

Recombinant chloramphenicol acetyltransferase (CAT) gene constructs and CAT assay λR31 contained a deletion mutant of the ME491 antigen gene starting at the first intron. DNA fragments corresponding to the intron were introduced into pSV00CAT[10] to determine their promoter activities (see Fig. 2). pSV2CAT[11] was used as a positive control. Five μg of each CAT construct was transfected into Ltk$^-$ cells by the calcium phosphate co-precipitaion method,[12, 13] and into H-*ras*-transformed NIH3T3 (3T3-Hras) and the parental NIH3T3 cells by the DEAE dextran method.[14] Transfection efficiency was monitored by co-transfecting the cells with each CAT construct and 2 μg of pSV2ME491, an expression plasmid for ME491 antigen under the control of SV40 early promoter/enhancer. ME491 antigen-positive cells, which were detected by an indirect fluorescent antibody method using monoclonal antibody against the antigen, usually amounted to 1–5% of total cells and the values did not vary significantly with different CAT constructs in each experiment (data not shown).

After culture for 48–60 h, the cells were harvested, disrupted by a sonicator (160 W, 2 min; UCD-200T, Cosmo-Bio Co., Ltd., Tokyo) in 250 m*M* Tris-HCl (pH 7.8) and centrifuged at 10,000 rpm for 5 min to obtain cell extracts. Protein concentration of the cell extracts was adjusted so that every sample had the same concentration in each experiment, and each sample was assayed for CAT activity as described previously[11] with minor modifications.

RESULTS

Intron-mediated transcription initiation of the ME491 antigen gene in Ltk$^-$ and 3T3-Hras cells transfected with λR31 λR31 mediated ME491 antigen expression in Ltk/λR31 and 3T3/λR31-3/Hras cells as reported previously.[6] To exclude the possibility that the antigen mRNA was transcribed from EMBL3 sequence flanking the deleted ME491 antigen gene, λR31 was treated with *Sal*I, which separated the human sequence from long and short arms of EMBL3 vector, leaving the human sequence intact. When transfected into Ltk$^-$ and 3T3-Hras cells, *Sal*I- treated λR31 mediated ME491 antigen expression to the same degree or more than that mediated by untreated λR31 (data not shown). The results suggested that the antigen mRNA was transcribed from a cryptic promoter(s) present in the first intron, but not from the EMBL3 sequence.

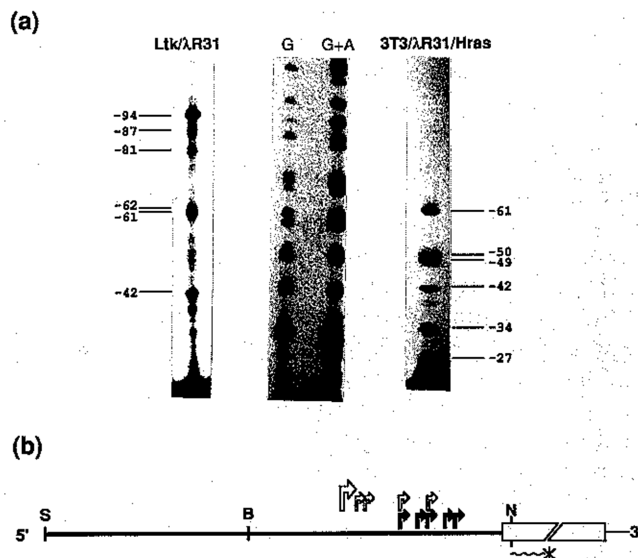

Fig. 1. (a) Primer extension analysis of ME491 antigen mRNA transcribed from a deletion mutant of the antigen gene in λR31. Maxam-Gilbert sequence reactions (G, G plus A) with fr270SN were run for accurate sizing. Numbers beside the extended products represent the distance from the adenine nucleotide of the translation initiation ATG codon.[1] (b) Schematic representation of the transcription initiation sites. A large arrow and small arrows in the upper column indicate the major and minor initiation sites in Ltk/λR31 cells, respectively, and thin arrows in the lower column indicate the initiation sites in 3T3/λR31-3/Hras cells. A solid line and an open column represent the first intron and the second exon, respectively. A wavy line with an asterisk beneath the exon represents a synthetic 21 mer used in primer extension analysis. B, *Bam*HI; N, *Nco*I; S, *Sal*I.

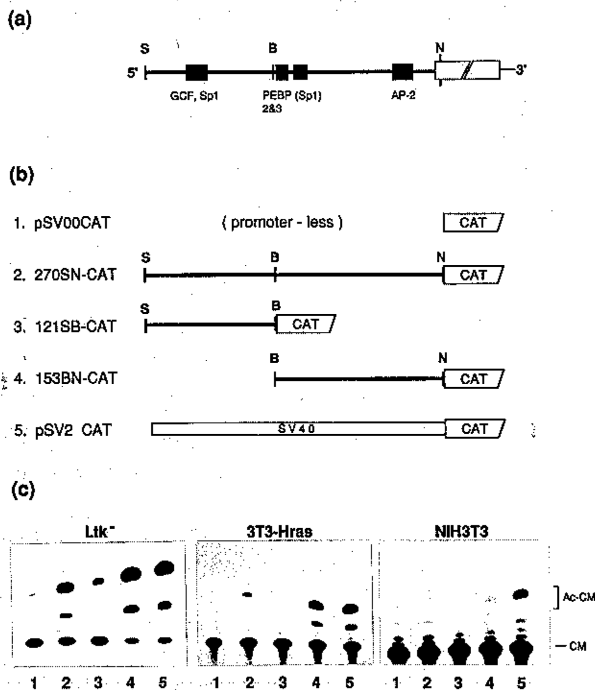

Fig. 2. (a) Localization of transcription factor-binding motifs in fr270SN. Closed boxes represent sequences that match consensus motifs for binding of transcription factors. Spl in parenthesis indicates a sequence motif which fits a consensus sequence reported by Ishii *et al.*[15] but not by other investigators.[16] An open box represents a portion of the second exon. B, *Bam*HI; N, *Nco*I; S, *Sal*I. (b) Schematic representation of CAT constructs. fr270SN, fr121SB and fr153BN were introduced into pSV00CAT in the proper orientation relative to the CAT gene to generate 270SN-CAT, 121SB-CAT and 153BN-CAT, respectively. (c) Representative results of CAT assay with Ltk$^-$, 3T3-Hras and NIH3T3 cells. Lane 1, pSV00CAT; lane 2, 270SN-CAT; lane 3, 121SB-CAT; lane 4, 153BN-CAT; lane 5, pSV2CAT. Spots corresponding to acetylated form (Ac-CM) and nonacetylated form (CM) of chloramphenicol are shown.

Primer extension analysis of mRNA from Ltk/λR31 cells identified a major transcription initiation site 94-bp upstream of the translation initiation ATG codon and a number of minor mRNA start sites downstream of the major start site (Fig. 1). On the other hand, equally utilized transcription initiation sites were identified in 3T3/λR31-3/Hras cells between 27 bp and 61 bp upstream of the ATG codon, only some of which were shared with Ltk/λR31 (Fig. 1). It thus appeared that the intron-mediated transcription started at different positions depending on the cell type, suggesting differential involvement of transcription factors among different cells.

Differential activation and suppression of a cryptic promoter present in the intron We then analyzed functional aspects of the cryptic promoter(s) in the intron by CAT assay. A 270-bp *Sal*I-*Nco*I fragment (fr270SN) spanning from the extreme 5′-end of the first intron in λR31 to the 5′-terminal portion of the second exon was first obtained. We previously reported that the intron contained consensus sequences for binding of various transcription factors such as Sp1,[15,16] AP-2[17-19] and polyomavirus enhancer binding proteins (PEBP) 2 and 3.[20,21] It was also revealed that fr270SN contained a sequence motif (CGGCGGGGCG) which fitted a consensus sequence for binding of a recently identified negative regulatory protein, GCF.[9] Localizations of those transcription factor-binding motifs are shown in Fig. 2a. fr270SN was further divided into two fragments (fr121SB and fr153BN) by *Bam*HI digestion. The fragments were made blunt-ended and introduced into pSV00CAT in the proper orientation to determine their promoter activities (270SN-CAT, 121SB-CAT and 153BN-CAT) (Fig. 2b). In Ltk$^-$ cells, fr270SN exerted a considerable degree of promoter activity (Fig. 2c). Interestingly, promoter activity of fr153BN, which corresponded to the 3′-half of fr270SN, was stronger than that of fr270SN itself, suggesting that a repressor sequence was present in the 5′-half of fr270SN (fr121SB) and was functioning in Ltk$^-$ cells. In 3T3-Hras cells, fr270SN exerted distinct but weaker promoter activity than in Ltk$^-$ cells. Again, fr153BN exerted stronger activity than fr270SN in this cell type. Promoter activity was hardly detected in NIH3T3 cells either with fr270SN or fr153BN. Thus, the promoter activity of fr270SN determined by CAT assay was considerably strong in Ltk$^-$, weak but distinct in 3T3-Hras and negligible in NIH3T3 cells.

Table I. Suppression of Cryptic Promoter Activity of fr153BN by an Upstream Repressor Sequence(s) Present in the 5′-Half of fr270SN

CAT construct	Relative CAT activity[a]	
	Ltk$^-$	3T3-Hras
270SN-CAT	0.27±0.16	0.09±0.05
121SB-CAT	0.06±0.02	0.01±0.01
153BN-CAT	1.00	1.00

a) CAT activities obtained with pSV00CAT were subtracted from those obtained with the test CAT constructs and the value with 153BN-CAT was set as 1. Mean values obtained from three separate experiments are shown.

To verify that fr153BN exerted stronger promoter activity than fr270SN, we repeated the experiments three times with Ltk$^-$ and 3T3-Hras cells using different batches of CAT constructs. Table I shows mean values of relative CAT activities induced by the test constructs. In both cells tested, promoter activity of fr153BN was stronger than that of fr270SN. The difference in the promoter activity between the two fragments was greater

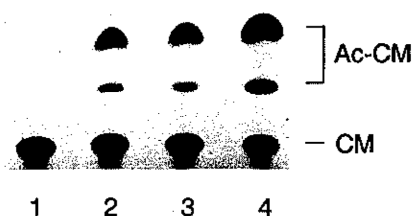

Fig. 3. Bidirectional expression of cryptic promoter activity residing in fr270SN. Promoter activity was assayed with Ltk⁻ cells. Lane 1, pSV00CAT; lane 2, 270SN-CAT, lane 3, 270SN-R-CAT; lane 4, pSV2CAT. Spots corresponding to acetylated form (Ac-CM) and nonacetylated form (CM) of chloramphenicol are shown.

in 3T3-Hras than in Ltk⁻ cells. These results suggested that the cryptic promoter in fr153BN was suppressed by an upstream repressor sequence(s) present in the 5′-half of fr270SN (fr121SB), and that the repressor exerted its activity more strongly in 3T3-Hras than in Ltk⁻ cells.

When fr270SN was introduced into pSV00CAT in the reverse orientation relative to the CAT gene (270SN-R-CAT), practically the same degree of promoter activity was observed compared with 270SN-CAT in which fr270SN was placed in the proper orientaion (Fig. 3). The results indicated a bidirectional character of the cryptic promoter.

DISCUSSION

λR31 contains a deletion mutant of the human melanoma-associated ME491 antigen gene that lacks its authentic promoter and the first exon, and yet mediates the antigen expression when transfected into Ltk⁻ and 3T3-Hras cells.[1, 6] The present study revealed that the antigen mRNA transcription started at different sites within the first intron in two types of stable transfectants, Ltk/λR31 and 3T3/λR31-3/Hras (Fig. 1). The results indicated that the intron contained a cryptic promoter apparatus which differentially regulated transcription initiation depending on the cell type. We then analyzed by CAT assay functional aspects of the cryptic promoter and related regulatory sequences present in the first intron of the deleted gene. It was revealed that fr270SN, which contained the entire sequence of the intron and a small portion of the second exon, exerted promoter activity in Ltk⁻ and 3T3-Hras cells, more prominently in the former than in the latter (Fig. 2). Promoter activity of fr270SN was hardly detected in NIH3T3 cells. These results were in good accordance with the previous observation that λR31 mediated considerable degrees of ME491 antigen expression in Ltk/λR31 and 3T3/λR31-3/Hras, but very little in 3T3/λR31-3 cells.[6] The differential expression of the cryptic promoter activity residing in fr270SN might be due to differential expression of various transcription factors that bound to the regulatory sequences.

The cryptic promoter appeared to have an ability to act bidirectionally, since fr270SN exerted promoter activity irrespective of its orientation relative to the CAT gene (Fig. 3). Bidirectional transcription has been reported in a number of genes, including the c-H-*ras* gene,[22] the SV40 early and late promoters,[23] the dihydrofolate reductase gene[24, 25] and human mitochondrial promoters,[26] all of which are associated with GC-rich sequences. Although the significance of such bidirectional promoters is not yet clear, the possibility should be considered that the cryptic promoter in the intron could generate anti-sense RNA that interferes with normal mRNA under certain conditions.

The cryptic promoter apparatus was further localized in fr153BN, which corresponded to the 3′-half of fr270SN, and was found to be active in Ltk⁻ and 3T3-Hras but not in NIH3T3 cells (Fig. 2). Comparison between 3T3-Hras and NIH3T3 cells in particular suggested the involvement of a *ras*-responsive regulatory element in fr153BN (see lanes 4 of both cells in Fig. 2c). In fr153BN, possibly important sequence motifs were found that matched the consensus sequences for binding of Spl, AP-2 and PEBP2 and 3 (Fig. 2a), but not the *ras*-responsive AP-1-binding motif.[27-29] Among the above sequence motifs, the Spl-binding sequence, which is located just upstream of the most 5′ transcription initiation site in Ltk/λR31 (Fig. 2a), seems to be the most likely candidate for the cryptic promoter. In addition, a *ras*-responsive positive regulatory sequence(s) in fr153BN should be functioning in 3T3-Hras cells, since the Spl-binding motif itself is not *ras*-responsive.

Interestingly, promoter activity of fr153BN was stronger than that of fr270SN and the difference between them was greater in 3T3-Hras than in Ltk⁻ cells (Table I). These results suggest that a repressor sequence is present in fr121SB, the 5′-half of fr270SN, and suppresses the cryptic promoter activity of fr153BN to various extents depending on the cell type. In this connection, we observed in the 3T3-Hras cell system that the authentic promoter of the ME491 antigen gene, which contained Spl binding motifs but not a TATA box, was suppressed by the presence of an intron region containing fr121SB (to be published elsewhere). fr121SB contains a consensus sequence motif for binding of a repressor protein GCF.[9] GCF has been reported to suppress transcription from the human epidermal growth factor receptor (EGFR) promoter,[9] which lacks a TATA box but contains GC-rich sequence motifs to interact with Spl and EGFR-specific transcription factor (ETF).[15, 30, 31] fr121SB also contains regions that show sequence similar-

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                                              (C)    (C)(C)(C)
Consensus GCF-binding sequence             N N G C G G G G C N
                                           | | | | | | | | | |
fr121SB (-233 to -218)            C A G A G G C G G C G G G G C G
                                  | | |     | | |     |   |
Chicken vimentin (-607 to -595)   C A G G A G C G C T G T G


fr121SB (-190 to -177)            C A C C C C T C T C C T G C
                                    |     | |   | | | | | | |
Chicken vimentin (-581 to -568)   G A T G C C C C T C C T G C
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Fig. 4. Sequence similarity of fr121SB in the ME491 antigen gene with GCF-binding sequence and *cis*-acting repressor elements in the chicken vimentin gene. Sequence similarity is indicated by dashed lines. Nucleotide positions of fr121SB and the vimentin gene repressor elements are indicated relative to the translation initiation site[1, 6] and the transcription initiation site,[32, 33] respectively. N in the GCF-binding sequence[9] indicates any nucleotide.

ity with two putative *cis*-acting repressor elements found in the 5′-flanking region of the chicken vimentin gene[32, 33] (Fig. 4). GCF and/or other repressor-binding proteins differentially suppress both cryptic and authentic promoters of the ME491 antigen gene and thereby regulate the gene expression depending on the cell type.

The question should be addressed as to whether or not the intron-mediated transcription initiation actually occurs in a situation(s) other than in transfection experiments. Primer extension analysis of mRNA from ME491 antigen-positive human cell lines identified a number of minor transcription initiation sites, some of which might correspond to the intron-mediated initiation (data not shown). However, we could not exclude the possibility that all of those minor initiation sites were located in upstream exons, including as yet unidentified ones. Meanwhile, we have noticed that there is a cluster of CpG dinucleotides, so-called 'CpG island,' around the cryptic promoter region in the first intron (to be published elsewhere). 'CpG islands' are usually found at the transcription initiation sites.[7, 8] It is therefore possible that intron-mediated transcription initiation occurs under some circumstances, i.e., in tumor cells, including particular stages of melanoma in which normal gene expression is deregulated. Further study is needed on this point.

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Note added in proof: After submission of the manuscript, we have learned that a platelet activation antigen CD63/Pltgp40 was molecularly cloned and found to be identical to ME491 antigen.[1,2]

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