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Transcriptional Enhancement of the Human Gene Encoding for a Melanoma-associated Antigen (ME491) in Association with Malignant Transformation

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A cloned DNA fragment (λR31) containing the human gene for melanoma-associated ME491 antigen was transfected into mouse fibroblast cell lines and the antigen expression was studied. Our preliminary observation of higher expression of the antigen in more malignant Ltk$^-$ cells and weaker expression in less malignant NIH3T3 cells tempted us to investigate the antigen expression in Harvey(H)-*ras*-transformed NIH3T3 cells. It was observed that malignant transformation of the λR31-transfected NIH3T3 cells by H-*ras* oncogene enhanced the antigen expression to some extent. Northern blot analysis suggested that the enhancement occurred at the transcriptional level. Nucleotide sequence analysis of the 5′-regulatory region of the ME491 antigen gene in λR31 identified a number of consensus sequence motifs for binding of transcription factors such as Sp1, AP-2 and polyomavirus enhancer binding proteins 2 and 3. A consensus sequence motif for binding of AP-1, known as a *ras*-responsive element, was not found in that region. The significance and possible involvement of the transcription factors in the enhancement of ME491 antigen expression are discussed.

Key words: Melanoma-associated antigen — Human gene — Gene regulation — Malignant transformation

Human melanoma has been an excellent model for studying multistep tumor progression[1-3] and several melanoma-associated antigens have been identified which demarcate each stage of the tumor progression.[4-6] ME491 antigen is among those antigens, and appears to represent early stages of melanoma progression.[7] ME491 antigen is not expressed in normal skin melanocytes. The antigen expression is weakly positive, if any, in benign nevus cells, and strongly positive in dysplastic nevus and radial growth phase of primary melanoma. The antigen expression, however, becomes weaker and even negative again as the melanocytic cells proceed to more malignant stages such as the vertical growth phase of primary melanoma and metastatic melanoma.[7] Mechanisms by which the antigen expression is regulated have yet to be elucidated. In order to examine this interesting problem, we have cloned human genomic DNA as well as complementary DNA (cDNA) encoding for ME491 antigen.[8] By transfecting one of the cloned DNA fragments (λR31) into mouse fibroblast cell lines, we have been studying regulatory mechanism(s) for the ME491 antigen expression. In the present study we show that the antigen expression mediated by λR31 is enhanced in association with malignant transformation of the cells.

MATERIALS AND METHODS

DNA Molecular cloning of λR31, a human genomic DNA fragment containing the ME491 antigen gene, was described previously.[8] Alignment of λR31 with a full-length ME491cDNA (pSe24-1) is shown in Fig. 1. pTK[9] and pSV2neo[10] were used as selection-marker genes for Ltk$^-$ and NIH3T3 cells, respectively. pT24 (activated Harvey(H)-*ras* oncogene)[11] was used to transform NIH3T3 cells to a fully malignant stage.[12-14]

Cell culture and DNA transfection Ltk$^-$ cells[15] and NIH3T3 cells[12-14] were cultured in Dulbecco's modified essential medium with 10% fetal calf serum. λR31 was transfected into the cells by the calcium phosphate co-precipitation method[15,16] with the selection-marker genes. Ltk$^+$ stable transfectants (Ltk/λR31) were selected by using HAT medium (hypoxanthine, 15 μg/ml; aminopterine, 1 μg/ml; and thymidine, 5 μg/ml).[8,16] NIH3T3 stable transfectants (3T3/λR31) were selected by using a neomycin derivative (G418, 400 μg/ml), and one of the clones (3T3/λR31-3) was further analyzed with respect to the effect of malignant transformation on ME491 antigen expression. The 3T3/λR31-3 cells were transformed to a fully malignant stage by H-*ras* oncogene[12-13] and selected by using a selection medium containing 5% new-born calf serum (3T3/λR31-3/H-*ras*).

Detection of ME491 antigen in the transfectants ME491 antigen expressed on the cell surface was detected by an immunological rosette formation assay as described pre-

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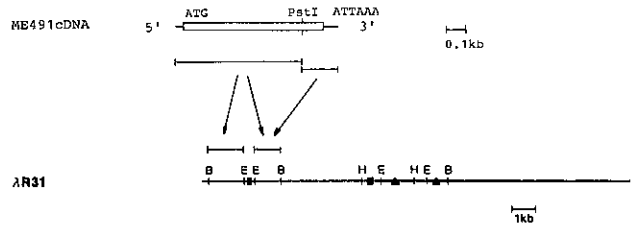


Fig. 1. Alignment between λR31 and ME491cDNA. A full-length ME491cDNA (pSe24-1) was fragmented into two portions by digestion with a restriction endonuclease *Pst*I. Each of the resulting fragments was labeled with ^{32}P and hybridized to λR31 digested with *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H). Arrows indicate positive hybridization. Closed boxes and triangles in λR31 represent human repetitive sequences of *Alu* family and of another family detected by probing with total human genomic DNA, respectively.

viously.[8, 16] In brief, cells in culture were incubated with phosphate-buffered saline (PBS) containing the monoclonal antibody against ME491 antigen (5 μg/ml)[7] for 1 h at room temperature. After washing three times with PBS, the cells were incubated with sheep red blood cells which had been coupled with goat anti-mouse IgG using chromium chloride as a coupling reagent.[17] After being washed with PBS again, ME491 antigen-positive cells were observed as those having sheep red blood cells on their surface.

ME491 antigen in the cytoplasm was detected by an indirect fluorescent antibody (FA) test after cells were dried and fixed with 95% ethanol at −20°C for 20 min. The fixed cells were incubated with the anti-ME491 monoclonal antibody (5 μg/ml) as a first antibody and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (1:50; Med. Biol. Lab. Co., Ltd., Nagoya) as a second antibody.

Northern blot analysis Cytoplasmic RNA was extracted according to a published procedure,[18] and applied to an oligo(dT) column to obtain poly(A)$^+$ RNA.[19] Five μg of poly(A)$^+$ RNA was denatured, fractionated by electrophoresis through 1.5% agarose/formaldehyde gel, and transferred to a nitrocellulose filter.[20] The filter was hybridized with ^{32}P-labeled ME491cDNA (pSe24-1) and ^{32}P-labeled rat α-tubulin cDNA[21] as described previously.[8, 20]

DNA sequence analysis λR31 was digested with appropriate restriction enzymes and desired fragments were subcloned into M13mp18 and M13mp19 vectors (Bethesda Research Lab., Inc., Gaithersburg, MD). Nucleotide sequences of the cloned DNA were determined by Sanger's dideoxynucleotide chain termination method.[22] Both strands were then sequenced.

RESULTS

Expression of ME491 antigen in λR31-transfected mouse fibroblast cell lines When λR31 was transfected into Ltk$^-$ and NIH3T3 cells, which otherwise were negative for ME491 antigen expression, higher expression of the antigen was observed in more malignant Ltk$^-$ cells than in less malignant NIH3T3 cells as determined by the immunological rosette formation assay (Table I) and an FA test (Figs. 2a and 2b). These observations led us to determine whether or not transformation of NIH3T3 cells to a fully malignant stage by H-*ras* oncogene might alter quantitatively the level of λR31-mediated ME491 antigen expression in those cells. Interestingly, ME491 antigen was enhanced to some extent when 3T3/λR31-3 was transformed by H-*ras* oncogene (3T3/λR31-3/H-*ras*) (Fig. 2b and 2d).

To determine whether or not the enhancement of ME491 antigen expression by H-*ras* oncogene occurred at the transcriptional level, Northern blot analysis was performed with poly(A)$^+$ RNA extracted from 3T3/λR31-3 and 3T3/λR31-3/H-*ras* as well as parental NIH3T3 cells and 3T3/H-*ras* as controls. As shown in Fig. 3, 3T3/λR31-3/H-*ras* contained a larger amount of ME491mRNA than did 3T3/λR31-3, suggesting that transcription of the ME491 antigen gene in λR31 was enhanced in association with H-*ras*-mediated malignant transformation.

Nucleotide sequence analysis of the 5′-flanking regulatory region of the ME491 antigen gene in λR31 Since λR31 could mediate ME491 antigen expression as described above, it was anticipated that λR31 contained at least some part, if not all, of the 5′-regulatory region essential for transcriptional initiation of the ME491 antigen gene. Alignment between λR31 and ME491cDNA (Fig. 1) suggested that the 5′-regulatory region of the ME491 antigen gene in λR31 was located very near the

Table I. Expression of ME491 Antigen Determined by Immunological Rosette Formation Assay

Cell	ME491 antigen
Ltk$^-$	−
Ltk/λR31	+
NIH3T3	−
3T3/λR31	−[b]
3T3/λR31-3[a]	−[b]
3T3/λR31-3/H-*ras*	+
3T3/H-*ras*	−

a) A clone derived from 3T3/λR31.
b) Too weak to be detected by this method.

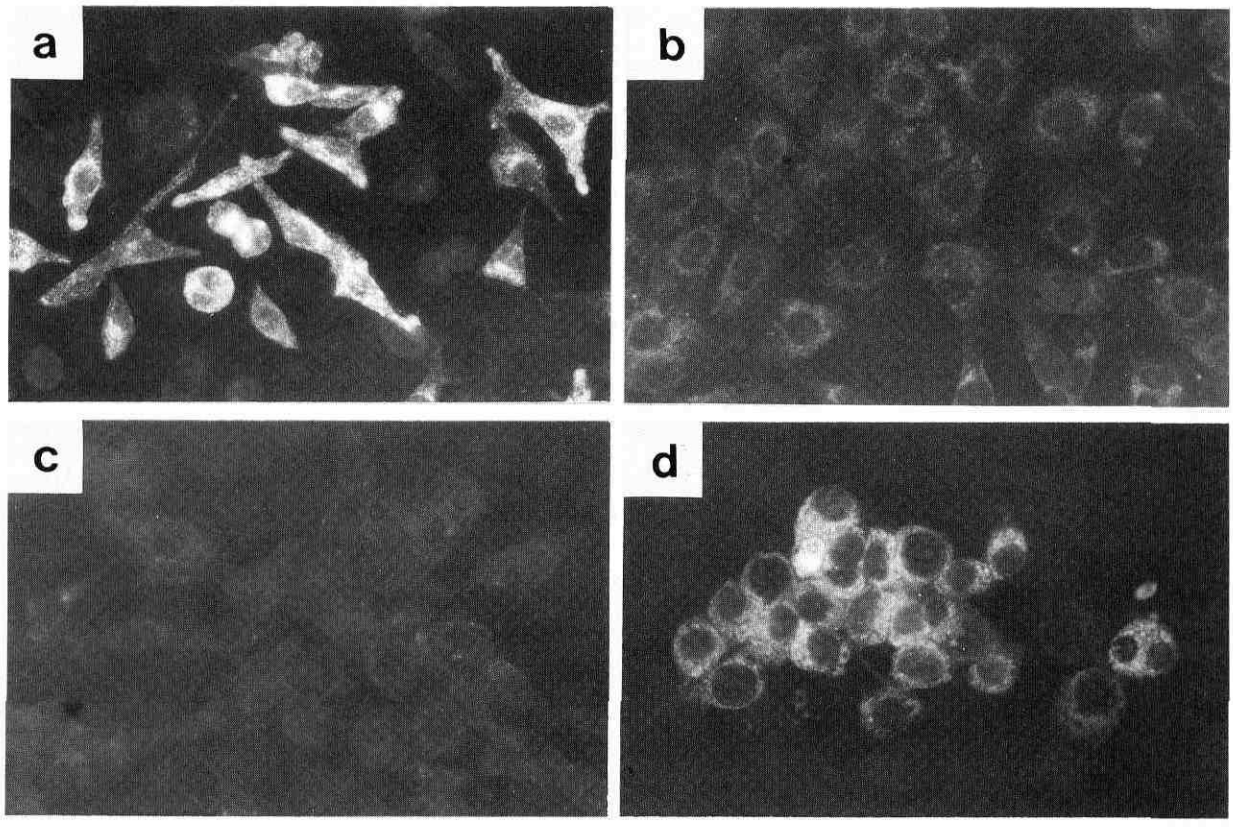
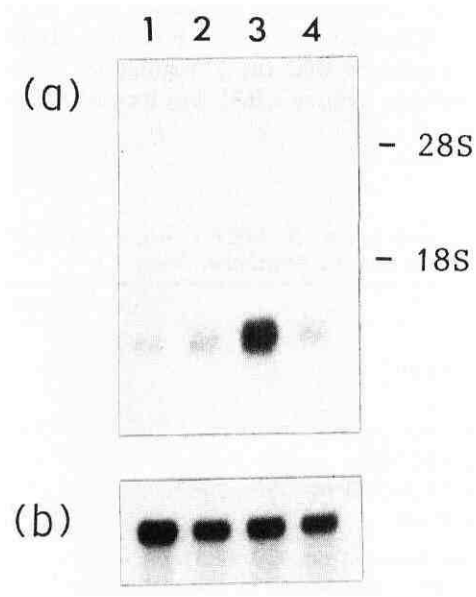


Fig. 2. Expression of ME491 antigen in λR31-transfectants determined by means of the FA test. (a) Ltk/λR31, (b) 3T3/λR31-3, (c) mock-transfected NIH3T3, and (d) 3T3/λR31-3/H-*ras*.



5′-end of the DNA fragment. Therefore, the nucleotide sequence of this portion beginning at the 5′-end of the cloned DNA was determined (Fig. 4). Comparison of the sequence with that of ME491cDNAs reported previously[8] and S1 nuclease mapping analysis (data not shown) identified the 5′-regulatory region of the ME491 antigen gene in λR31. It consisted of about 230 bp, and was very GC-rich. No TATA box was found in this region. Instead, there were many GC- and GC-like boxes. In this connection, a consensus sequence for binding of a transcription factor, Spl, $(^{GG}_{TA}GGCG^{GGGC}_{TAAT})$[23] was found along with the GC box (Fig. 4), which probably served

Fig. 3. Northern blot analysis of poly $(A)^+$ RNA obtained from λR31-transfectants and their parental cells. (lane 1) NIH3T3, (lane 2) 3T3/λR31-3, (lane 3) 3T3/λR31-3/H-*ras*, and (lane 4) 3T3/H-*ras*. (a) The nitrocellulose filter was hybridized with ^{32}P-labeled ME491cDNA (pSE24-1). (b) The same filter was hybridized with ^{32}P-labeled cDNA for rat α-tubulin[21] as a control.

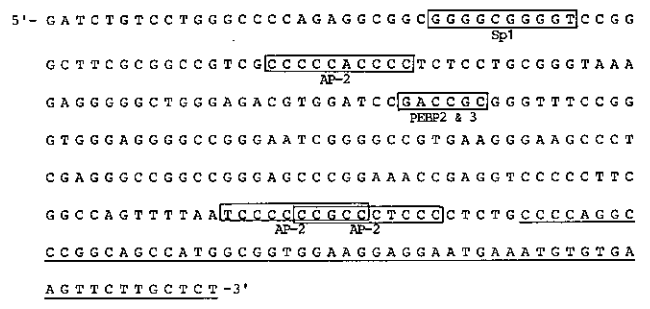


Fig. 4. Nucleotide sequence of the 5′-regulatory region of the ME491 antigen gene in λR31. Consensus sequences for transcription factors, Spl,[5] AP-2,[19] PEBP2 and 3[34] are boxed. The sequence identical with that of the ME491cDNA[17] is underlined.

as a signal for transcriptional initiation as in the case of H-*ras* protooncogene,[24] epidermal growth factor receptor gene[25] and so on.

A consensus sequence recognized by another transcription factor, AP-2, ($^{T}_{C}C^{C}_{G}CC^{A}_{C}N^{GCG}_{CGC}$)[26] was also found in this region (Fig. 4). In addition, there was a consensus sequence for polyomavirus enhancer binding proteins 2 and 3 (PEBP2 and 3) (GACCGC).[27,28] It appears interesting that PEBP3, previously referred to as D factor, has been reported to be present in NIH3T3 cells stably transformed by H-*ras* oncogene, but absent in parental NIH3T3 cells.[27]

A sequence motif for binding of AP-1 ($^{C}_{G}$TGACT$^{C}_{A}$A),[28-31] which is known as a *ras*-responsive element, was not found in this region.

DISCUSSION

In the present study we examined expression of human melanoma-associated ME491 antigen in cultured mouse fibroblast cells transfected with λR31, a cloned DNA fragment containing the ME491 antigen gene. An advantage of using mouse cells as the recipient was that antigen expression mediated by the transfected gene could be easily monitored because anti-ME491 monoclonal antibody reacted only with ME491 antigen of human origin, but not with the mouse counterpart (see "Results"). Therefore, it was possible, using this system, to examine gene expression mediated by different genomic clones containing possibly different 5′-regulatory regions of the gene. We used Ltk$^-$ and NIH3T3 cell lines as transfection recipients; the former is thought to be in a more malignant stage than the latter.[12-14] It was observed that ME491 antigen expression by λR31 was stronger in Ltk/λR31 cells than in 3T3/λR31 cells (Table I and Figs. 2a and 2b). We also observed that the antigen expression in 3T3/λR31-3 was enhanced in association with malignant transformation of the cells by H-*ras* oncogene (3T3/λR31-3/H-*ras*) (Table I and Figs. 2b and 2d), though to a lesser extent than Ltk/λR31. The H-*ras*-mediated enhancement was likely to occur at the transcriptional level as demonstrated by Northern blot analysis (Fig. 3). It has been reported that transcription of a number of cellular genes[31-34] as well as viral enhancers[28-30] was activated by *ras* oncogenes. In most cases, a sequence motif for binding of AP-1 has been considered to play an important role in the activation.[28,30,31] In order to see whether the AP-1 binding sequence motif is involved in the enhancement of ME491 antigen expression, the nucleotide sequence of the 5′-regulatory region of the ME491 antigen gene in λR31 was determined. In contrast to the cases described above, no AP-1 binding sequence was found in the region. This may suggest the possible existence of another *ras*-responsive sequence element. In this connection, a sequence motif (GACCGC) was found in the 5′-regulatory region of the ME491 antigen gene. The same sequence motif has been revealed to be a recognition site for polyomavirus enhancer binding proteins 2 and 3 (PEBP2 and 3).[27,28] Interestingly, PEBP3 was reported to be present in NIH3T3 cells stably transformed by H-*ras* oncogene, but not in the parental cells.[27]

In addition to the PEBP2- and PEBP3-binding sequence motif, two other possibly important sequence motifs were found in the 5′-regulatory region of the ME491 antigen gene (Fig. 4), one recognized by Spl ($^{GG}_{TA}$GGCG$^{GGGC}_{TAAT}$)[23] and the other recognized by AP-2($^{T}_{C}C^{C}_{G}CC^{A}_{C}N^{GCG}_{CGC}$).[26] While Spl is ubiquitous and is found in almost all types of cells, expression of AP-2 appears rather limited to certain cell types. The possibility remains, therefore, that the AP-2-binding sequence motif might be involved in the enhancement of the ME491 antigen expression.

We are currently analyzing enhancer and/or enhancer-like (positive regulatory) sequences in the 5′-regulatory region of the ME491 antigen gene by utilizing chloramphenicol acetyltransferase (CAT) assay. It was found by the CAT assay that the 5′-regulatory region contained a sequence motif exerting positive regulatory activity in H-*ras*-transformed NIH3T3 cells (to be published elsewhere). Further study along this line would help us understand the regulatory mechanism(s) for ME491 antigen expression.

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