# Clonal Diversity of the Expression of MHC Class I Antigens and Endogenous Retroviral Antigens on the Oncogene-Induced Cell Transformation of BALB3T3 Cells-Different Regulatory Mechanisms for the Expression of Antigens

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## ABSTRACT

The effect of the activated oncogene transfection on the expression of MHC class I H-2K$^d$, D$^d$ and L$^d$ and endogenous retroviral antigens was analyzed on 21 clones independently derived from the same parental BALB3T3. These clones were obtained by transfecting BALB3T3 with EJ-ras, PyMT, c-myc, and v-src oncogenes. The expression of MHC class I H-2L$^d$ and H-2K$^d$ antigens was reduced in clones with high anchorage independent growth potential. However, clones with low or no anchorage independent growth potential displayed almost the same degree of expression as parental BALB3T3. The H-2D$^d$ antigen was for the most part conserved. The reduced expression of H-2K$^d$ antigen was completely restored by IFN treatment, whereas that of H-2L$^d$ antigen was only partially restored. Meanwhile, MuLV and MMTV antigens were expressed on parental BALB3T3. These antigens was down-modulated in all of the EJ-ras and in one of the v-src-transfected clones. However, all the PyMT-transfected clones expressed the antigens as much as parental BALB3T3 even in the clones with high anchorage independent growth potential. These data suggest that the MHC class I antigen expression is modulated with the transforming process of cells, whereas the endogenous retroviral antigen expression might be dependent upon the transfected oncogenes rather than the transforming phenotype of the cells.

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[2] Abbreviations used are: CTL, cytotoxic T lymphocytes; FACS, fluorescein activated cell sorter; FITC, fluorescein isothiocyanate; IFN, interferon; Ig, immunoglobulins; mAb, monoclonal antibody; MHC, major histocompatibility complex; MMTV, murine mammary tumor virus; MuLV, murine leukemia virus; PBS, phosphate-buffered saline; PyMT, polyoma middle T.

**Key word:** Class I antigen, Retroviral antigen, Transformation

## INTRODUCTION

The expression of mouse MHC[2] class I genes and their products reveals complex pattern of regulations(1). Although MHC class I antigens are expressed on almost all mouse cells, it has been demonstrated that certain spontaneous tumors express only a reduced level of MHC class I antigen(2, 3). Since CTLs can recognize the foreign antigens in the context of self MHC class I molecules for the target cell lysis, the loss of expression of the MHC class I antigens may cause an escape from the immunesurveillance by host CTLs(4, 5). In contrast, some studies have shown that an enhanced expression of MHC class I antigens could follow a virus-induced transformation such as SV 40(6)or polyoma virus(7), or K-ras-induced transformation(8). Thus the relationship between the cell transformation and the modulation of expression of MHC class I antigens seems to be complicated. It appears that these discrepancies could result from a comparison of cells with different cell origins. This drawback should be eliminated by using the same parental cells and their transformed clones.

There have also been debates as to the relationship between cell surface expression of endogenous retroviral gene products and the cell transformation. The cell transformation is associated with the activation of several genes, and one of these might be the activation of endogenous provirus. Alterations of these cell surface antigens after cell transformation must be involved in some definite mechanisms that regulate the transformation. In order to clarify these mechanisms, it is important to compare cell surface antigens expressed on transformed cells with those expressed on non-transformed cells using the exactly same parental cell line(9, 10).

In this paper, we used BALB3T3 clone # 5 as a recipient line of cells for the activated oncogene transfection. This clone shows the strict phenotype for non-transformed cells such as contact inhibition, anchorage dependency and serum dependency. We demonstrated the clonal diversity of the expression of MHC calss I antigens and retroviral antigens on oncogene-induced BALB3T3 transformed clones (transformants) as well as non-transformed clones (transfectants). The data suggest the presence of different modulatory mechanisms for the expression of these antigens.

## MATERIALS AND METHODS

*Cells*

At first, we obtained BALB3T3 clone # 5 by single cell cloning of BALB3T3

(10). This clone displayed strict phenotypes for the non-transformed cells such as anchorage dependency, contact inhibition, and high serum dependency. The cells were grown in Eagle's modified culture medium supplemented with 5% fetal calf serum and 292 $\mu g/ml$ of L-glutamine. This clone was employed as a parental BALB3T3 recipient line for the oncogene transfection to obtain transformants and transfectants.

*Oncogenes*

The oncogenes used in this study were previously mentioned in our papers(9, 10, 11). Briefly, we used 1) 6.6 kb EJ-ras that was separated by Bam HI cleavage of the genomic activated -H-ras, 2) recombinat mouse c-myc cloned from mouse plasmacytoma that was inserted at the Bam HI site of pUC18 plasmid DNA, 3) 5.2 kb PyMT DNA inserted at the Bam HI site of pBR322 plasmid DNA, 4) 0.8 kb v-src that was separated by Pvu II cleavage of the Rous sarcome virus. We also used 4.6 kb pSV2neo containing a neomycin resistent gene(12).

*DNA transfection*

The calcium phosphate co-precipitation method was used to transfect the DNAs, and this procedure was prevdously reported(9, 10, 11). In order to confirm the insertion of transfected DNA into BALB3T3, DNAs of every transformant and transfectant were assessed by the Southern blot hybridization technique as previously described(9).

*Anchorage independent growth of cells*

Cells transfected by the oncogenes were assessed for their growth potential in 0.3% agar as previously described(9, 13). Briefly, after inoculation ranging from $10^2$ to $10^3$ cells into 0.3% soft agar, the cell growth was scored microscopically at the 3rd week of cultivation, and the colony formation was expressed as % of plating efficiency = (No. of clusters − No. of original cell aggregates) × 100/ No. of viable nucleated cells plated.

*Antibodies*

In order to detect the cell surface expression of H-2 and endogenous retroviral antigens, we used the following antibodies; monoclonal 1) anti-H-$2K^d$ (code No. 030-210, Meiji Institute of Health Science, Tokyo, Japan), 2) anti-H-$2D^d$ (code No. 030-200, Meiji Institute of Health Science), 3) anti-H-$2L^d$ (30-5-7S, Litoon Bionetics Inc., Charleston, SC.) mouse antibody(14) and ploYclonal guinea pig 4) anti-MuLV serum (No. 5011, Electro-Nucleonics Lab. Inc., Silver Spring, MD.) and 5) anti-MMTV serum (No. 5091, Electro-Nucleonics Lab.

Inc.). We also assessed the expression level of ras gene product p21 and polyoma middle T gene product p55 in some clones of the transfected cells by using anti-p21 mAb rp12(9) and anti-p55 mAb Ab-4 (Oncogene Science, Inc., Manhasset, NY.), respectively.

*FACS analysis of cells stained with antibodies*

The cells were reacted with a saturated amount of 0.1 m*l* of antibodies for 40 min at 4℃ and were washed once with PBS. These cells were then incubated with 0.1 m*l* of 1 : 60-diluted FITC-conjugated goat anti-mouse Ig or 1 : 150-diluted goat anti-guinea pig Ig for 40 min at 4℃. After washing once with PBS, the cells were suspended with 0.5-1.0 m*l* of sheath fluid for FACS analyzer. Samples were then run on a FACS analyzer of Becton Dickinson. For controls of the nonspecific binding of FITC-conjugated antisera to the cells, parallel samples were made by staining with PBS and FITC-conjugated goat antisera. $5\times10^3$ cells per sample were routinely analyzed. We usually set the medium channel number of FACS at approximately 110, and the mean percent of positive immunofluorescence of cells was determined from the data of two assays.

*IFN $\alpha/\beta$ treatment*

Cells at subconfluence were incubated with a culture medium containing $10^2$ U/m*l* mouse IFN $\alpha/\beta$ (Cytimmune Interferons, Lee BioMolecular Research Labo. Inc., San Diego, CA.) for 48 h at 37℃. After the incubation, cells were harvested, washed with PBS, and then analyzed by FACS.

## RESULTS

*Anchorage independent growth of cells transfected with the oncogenes*

By transfecting BALB3T3 with the oncogenes, we obtained a large number of independently-derived transfectants and transformants. In this study, we selected 21 clones as shown in Table 1, and the potential of the anchorage independent growth of each clone in 0.3% soft agar was assessed. These clones were different with one another, since these clones have different flanking sequences for the insertion of respected oncogenes in their genomic DNAs when analyzed by the Southern blot (data not shown). According to the growth potential, these clones were divided into three groups, namely, 'high' with plating efficiency being more than 50%, 'moderate' with 10−50%, 'low' with less than 10%, and 'no' without colony formation. Clones with high anchorage independent growth potential obviously showed the reduced serum requirements and lost contact inhibition on the monolayer cell cultures. These included all the EJ-ras and some of the PyMT-transfected clones. Clones with moderate anchor-

**Table 1** *Anchorage independency of oncogene-transfected BALB3T3 clones*

| clone | oncogenes transfected[a] | anchorage independency[b] | p21[c] | p55[c] |
|---|---|---|---|---|
| BALB3T3 | no | no | | |
| Bneo 4 | pSV2neo | no | − | − |
| Bmyc 7 | c-myc + pSV2neo | no | ± | − |
| Bmyc 10 | c-myc + pSV2neo | no | N. D. | N. D. |
| Bsrc 1 | v-src + pSV2neo | moderate | N. D. | N. D. |
| Bsrc 7 | v-src + pSV2neo | moderate | N. D. | N. D. |
| Bras d | EJ-ras | high | + | − |
| Bras h | EJ-ras | high | + | − |
| Bnr 5 | EJ-ras + pSV2neo | high | + | N. D. |
| Bnr 7 | EJ-ras + pSV2neo | high | + | N. D. |
| Bnr 12 | EJ-ras + pSV2neo | high | + | N. D. |
| BnMT 4 | PyMT + pSV2neo | low | N. D. | + |
| BnMT 9 | PyMT + pSV2neo | low | N. D. | ± |
| BMT 2 | PyMT | moderate | N. D. | + |
| BnMT 1 | PyMT + pSV2neo | moderate | N. D. | + |
| BnMT 5 | PyMT + pSV2neo | moderate | N. D. | N. D. |
| BnMT 8 | PyMT + pSV2neo | moderate | N. D. | + |
| BMT B | PyMT | high | N. D. | + |
| BMT f | PyMT | high | N. D. | + |
| BnMT 11 | PyMT + pSV2neo | high | N. D. | + |
| BnMT 12 | PyMT + pSV2neo | high | N. D. | N. D. |
| BnMT 20 | PyMT + pSV2neo | high | N. D. | + |

a) See materials and methods.

b) Potential of anchorage independent growth was represented by mean plating efficiency as measured from two experiments using 0.3% soft agar; high with more than 50%, moderate with 10 to 50%, low with less than 10%, and no without colony formation.

c) The expression of p21 res product and p55 polyoma virus product was assessed using mAbs rp12 and Ab-4, respectively, as described previously(9). The cytoplasmic expression of these products in the transfected clones was shown for enhanced p21 and p55 level (+) and for unchanged (−) as compared with parental BALB3T3 cells. N. D., not determined.

age independent growth potential remained at a limited level of contact inhibition or serum requirements. These included the v-src and some of the PyMT-transfected clones. Clones with low or no anchorage independent growth potential were difficult to distinguish from BALB3T3 parental cells microscopically.

These included the c-myc and some of the PyMT-transfected clones such as BnMT 4 and BnMT 9. We also studied the cytoplasmic expression of p21 ras product and p55 polyoma virus product by using mAbs rp12 and Ab-4, respectively. Although we did not assess all clones employed in this study, as shown in

Table 1, the expression level of these products was generally comparable with the anchorage independency, indicating the close corelation of the product expression to the tumorigenic phenotype of transfected clones.

*Expression of MHC class I antigens*

To assess the levels of cell surface expression of MHC class I antigens, we used three mAbs, 030-210, 030-200 and 30-5-7S, which detect the gene products of H-2K$^d$, H-2D$^d$ and H-2L$^d$ locus, respectively. The medium channel number of FACS was set at approximately 110, and the percentage of positive immunofluorescence of cells expressing MHC class I antigens was shown in Fig.

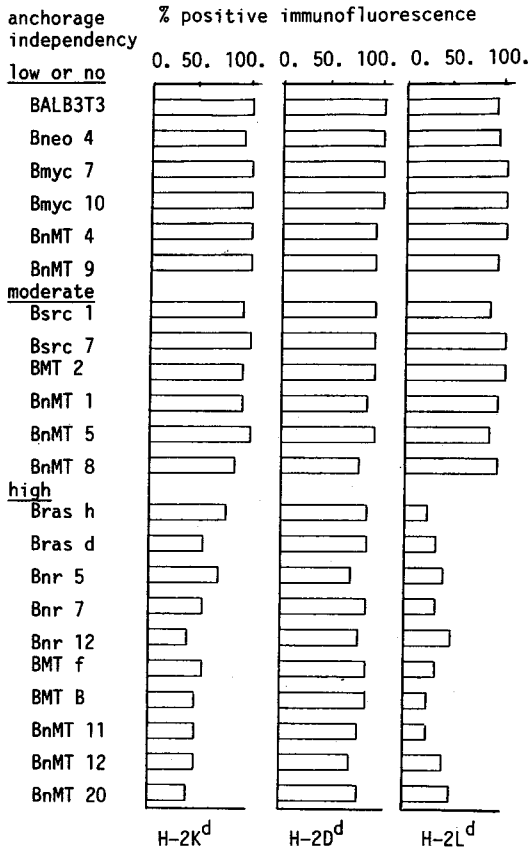


**Fig. 1** Comparison of the percentage of cells expressing MHC class I antigens between parental BALB3T3 and oncogene-transfected clones. The cells were run on a FACS analyzer after indirect immunofluorescence staining with mAbs reacting against H-2K$^d$, H-2D$^d$ and H-2L$^d$ antigens. The clones were divided into three groups by their anchorage independent growth potentials as indicated in the figure.

1. A pSV2neo-transfected clone, Bneo 4, showed no modulation of the expression of each MHC class I antigen. Clones with low or no anchorage independency such as Bmyc 7, Bmyc 10, BnMT 4 and BnMT 9 also expressed these antigens to almost the same degree as parental BALB3T3 and Bneo 4 clone. This seems true for clones with moderate anchorage independent growth potential such as Bsrc 1, Bsrc 7, BMT 2, BnMT 1, BnMT 5 and BnMT 8. In contrast, the reduced expression of H-2L$^d$ antigen was observed in all EJ-ras transformant and PyMT transformants with high anchorage independency. It was noted that the expression of H-2K$^d$ was also moderately reduced. However, H-2D$^d$ antigen was slightly reduced only in a limited number of clones such as Bnr 5, but was for the most part conserved even in the clones with high anchorage

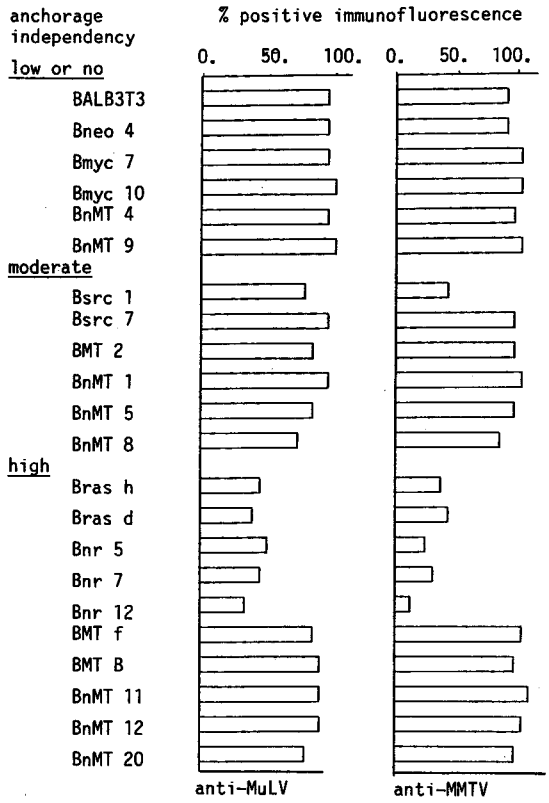


**Fig. 2** Comparison of the percentage of cells expressing MuLV and MMTV antigens between parental BALB3T3 and oncogene-transfected clones. The cells were run on a FACS analyzer after indirect immunofluorescence staining with polyclonal guinea pig anti-MuLV and anti-MMTV serum. The clones were divided into three groups as shown in Fig. 1.

independent growth. There is the possibility that apparent differences in antigen density could be due to difference in the affinity of the antibody rather than differences in the number of antigen molecules. However, this possibility could be denied, since the data was determined for the reactivity between the same mAbs and 21 clones.

*Expression of endogenous retroviral antigens*

Expression of MuLV and MMTV antigens was examined by FACS using xenogenic polyclonal antibodies. We set the medium channel number of FACS at approximately 110, and the percent of positive immunofluorescence of cells expressing these antigens was determined. As shown in Fig. 2, parental BALB3T3 and Bneo 4 showed a high expression of these antigens. Reduced expression was observed in all of five EJ-ras-transformed clones and one of two v-src-transfected clones. In contrast, all the nine PyMT-transfected clones and two c-myc-tyansfected clones showed only a minimum modulation of the expression. These results indicate that there seems no direct relationship in the modulatory mechanisms for the expression of endogenous retroviral antigens with the tumorigenic phenotypes of the cells. The data may rather suggest that the

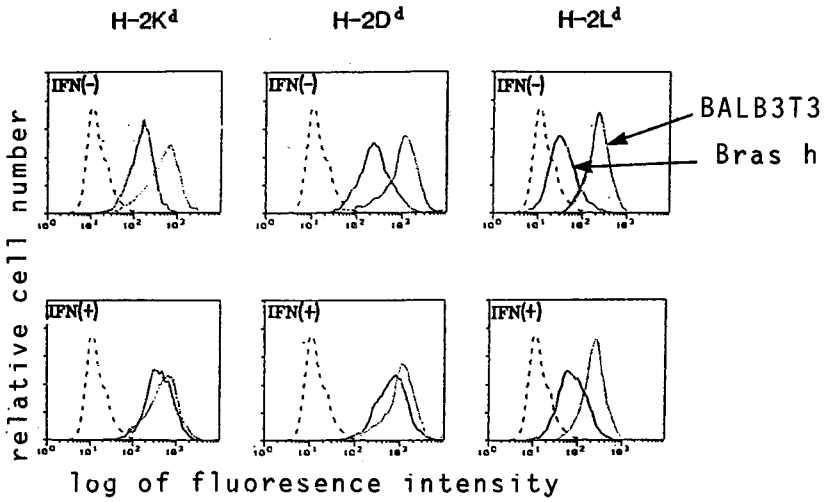


**Fig. 3** FACS profiles of MHC class I antigen expression on parental BALB3T3 (fine dotted line) and Bras h cells (solid line) with or without IFN $\alpha/\beta$ treatment. Bras h cells were incubated in medium with or without $10^2$ $\mu$/m*l* mouse IFN $\alpha/\beta$ for 48h at 37℃. The cells were ramvested, washed with PBS, and then analyzed by indirect immunofluorescence staining with monoclonal anti-H-2K$^d$, H-2D$^d$ and H-2L$^d$ mouse antibody. For controls of the nonspecific staining of FITC-conjugated antiserum to the cells, parallel samples were made by staining with PBS and FITC-conjugated goat antisera (rough dotted line).

expression of endogenous retroviral antigens was dependent on the introduced oncogenes.

*Effect of IFN $\alpha/\beta$ on expression on MHC class I antigens*

IFN $\alpha/\beta$ is known to activate the expression of MHC class I antigens. The effect of mouse recombinant IFN $\alpha/\beta$ on the expression of MHC class I antigens was examined using a EJ-ras-transfected clone, Bras h. In Fig. 3, FACS profiles of MHC class I antigen expression on a Bras h clone with or without IFN treatment are displayed. FACS profiles of BALB3T3 without IFN treatment was also shown as a control. We cultured Bras h cells with a medium containing 10 $\mu/ml$ IFN for 48 h. This condition of IFN treatment has been confirmed previously to give a maximum effect against Bras h. The expression of BALB3T3 with IFN treatment was almost at the same level as that without IFN, suggesting that BALB3T3 cells exhibit perhaps the maximal level of class I antigen expression (data not shown). After the IFN treatment of Bras h, H-2D$^d$ and H-2K$^d$ antigen expression of this clone was restored as much as the level of that of parental BALB3T3. However, this IFN treatment could give only a partial recovery of H-2L$^d$ antigen expression on Bras-h clone.

## DISCUSSION

It is important to analyze the relationship between cellular transformation and expression of MHC class I antigens in tumor immunology, since MHC class I antigens are considered to play a critical role in the immune recognition of tumor cells by CTLs. The clonal analysis of the expression of endogenous retroviral antigens is also a very important issue. These antigens have been considered to be candidates for tumor rejection antigens in some of experimental tumor models (10). Recent studies showed that a variety of tumor cell lines had decreased MHC calss I antigens(15, 16, 17, 18, 19). Adenovirus type 12 (Ad12)-induced transformation of both rodent and human cells is well known to reduce the expression of MHC class I antigens. This may result in escaping from the anti-tumor surveillance by the hosts(4, 5, 20, 21). Furthermore, there are conflicting reports as to the down regulation of class I antigen on Ad12-transfected cell. Friedman et al(22) suggest the transcriptional control, whereas Vaessen et al(23) indicated the post-transcriptional level of the regulation. In contrast, it was also reported from several laboratories that the enhanced expression of MHC class I antigens occurred after the transformation of cells(7, 8). Thus, there seemed to be no definite mechanisms by which the expression of MHC class I antigens on tumor cells could be modulated. These conflicting results appear to be derived from different origins of tumor cells, different methods for the induction of trans-

formation and different levels of neoplastic potential of cells that were used in the experiment(24, 25). Therefore, it is important to compare precisely the cell surface antigens expressed on the transformed cells with those expressed on the non-transformed parental cells.

We transfected several oncogenes into the same parental cell line BALB3T3, and obtained a large number of independently-derived clones. We used 21 different clones in the experiment, and these clones were compared with parental BALB3T3 cells for their cell surface antigen expression. The cells transfected with pSV2neo plasmid DNA alone showed no modulation of the expression of MHC class I antigens and endogenous retroviral antigens. This demonstrates that neither the transfection procedure nor the possible metabolic change induced by a transfected pSV2neo could influence the expression of cell surface MHC calss I and endogenous viral antigens.

The PyMT-transfected cells showed the clonal diversity of growth potential in soft agar. It is interesting to note that the decreased level of MHC class I-H2L$^d$ and H-2K$^d$ antigen expression was observed only in transformed cells with high anchorage independency. EJ-ras-transformed cells, all of which had high anchorage independency, also showed a reduced expression of MHC class I antigens. These results indicate that the modulation of MHC calss I antigen expression might be associated with the transforming process or the neoplastic nature of cells. This is supported by a report of Croce et al(26) and Vasavada et al(27), demonstrating that the reduction of MHC class I antigen expression required the direct involvement of oncogene products in the transforming process. Furthermore, we did not observe an increased expression of MHC class I antigens on any of the clones studied. Our results agree with those of Recioppi et al who reported that the modulation of the expression of MHC class I antigen proceed always in the same direction on the transformants derived from the same parental cell line, regardless of the introduced oncogene(28).

In this paper, we also showed that there were differences in the expressional level among each H-2 product, namely H-2K$^d$, H-2D$^d$ and H-2L$^D$ antigens. H-2L$^d$ expression was the most influenced, while H-2D$^d$ did not show an obvious change even in the clones with high anchorage indepenednecy. Although each H-2 loucs has a highly homologous promotor region(29), the expression of H-2 molecules on the cell surface appears to be idependently regulated on both normal cells(30) and tumor cells, such as AKR leukemia cells(31), T-10 sarcoma(32), Lewis lung carcinoma(33) and MuLV-transformed cells(34). It was reported that the comparison of the expression of transfected H-2L$^d$ gene product on the cell surface with H-2D$^d$ gene product revealed a higher expression of H-2D$^d$ antigen rather than H-2L$^d$, despite the higher amounts of H-2 mRNA and H-2

polypeptide in the H-2L$^d$-transfected cells(35). A recent study revealed that a lower level of cell surface expression of H-2L$^d$ antigen results from its slower processing of N-linked oligosaccharides and its weaker association with beta 2-microglobulin(36). Thus, the expression of H-2L$^d$ antigen is considered to be modulated by post-transcriptional events. This fact may explain why H-2L$^d$ antigen was the most reduced in BALB3T3 transformants. Furthermore, we showed that the reduced expression of MHC class I antigens on Bras-h clone was restored by the treatment of IFN $\alpha/\beta$. This cytokine was known to activate the transcription of MHC class I gene(1), suggesting that the down-modulation of MHC class I antigen expression in the transformed cells of BALB3T3 could be controled at the transcriptional level.

On the other hand, endogenous retroviral antigens expressed on the cell surface were decreased in all of the EJ-ras and one of v-src-transfected clones. Interestingly, all of PyMT-transfected clones displayed almost no alteration of these viral antigen expressions. This suggests that the expression of endogenous retroviral antigens was modulated by certain mechanisms that depend on the introduced oncogenes. It was previously described that adenovirus E1 products, which are located in the nucleus, could activate or repress other viral promotors (37, 38, 39, 40). However, we could not currently explain the mechanism of this particular modulation of endogenous retroviral antigen expression on the oncogene-transfected cells of BALB3T3.

In conclusion, our present study suggests that there might exist two different mechanisms of cell surface antigen expression in the oncogene-induced cell transformation of BALB3T3 cells. The modulation of MHC class I antigen expresion might be associated with the transforming process, and that of endogenous retroviral antigen expression might be associated with the oncogenes that were transfected into the cells. These data will enable us to reveal the detailed mechanism for the association between the cell transformation and expression of some particular cell surface antigens such as tumor rejection antigens in the hosts.

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