Jpn. J. Cancer Res. (Gann), 79, 718–725 ; June, 1988

Isolation of Genomic DNA Controlling Mouse Melanoma Antigen Defined by Monoclonal Antibody

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We have isolated the genomic DNA controlling the expression of murine specific melanoma antigen by employing cosmid shuttle vector and monoclonal antibody. Transfection of the cosmid library derived from mouse melanoma cells into human melanomas and repeated cell sortings of the fluorescence-bright population enabled us to enrich the antigen-positive transfectants. We rescued a 34.8 kb DNA fragment from the transfectants by *in vitro* packaging and showed it to be responsible for the antigen expression. However, we noticed instability of the antigen expression when the selection pressure imposed by the cell sorting was removed. This seemed to be due to the fact that the insert DNA was preferentially deleted from this cosmid vector without loss of the vector sequence itself.

Key words: Melanoma — Shuttle vector — Transfection — Cell sorter

A number of tumor-associated antigens have been reported, most of which seem to be expressed only in the malignant state. The host immune system exerts itself to eliminate them through their recognition. However, tumor cells manage to escape from this surveillance system and grow indefinitely. In order to understand how this occurs, we have been working on B16 mouse melanoma cells as a model system. Our previous studies have shown that the mouse melanoma antigen defined by monoclonal antibody is responsible for the generation of specific suppressor T cells which inhibit the induction of cytotoxic T lymphocytes (CTL)[*5] against melanomas.[1] This fact can explain the escape of tumor cells from the host defence system.

We have also demonstrated that the melanoma antigen playing a critical role in the immune system possesses both cross-species and mouse-specific determinants. One of the monoclonal antibodies we developed by syngeneic immunization (M 2590) recognizes GM3 ganglioside with cross-species reactivity to various melanomas of different species origin, whereas M562 and M622 antibodies recognize protein determinants with mouse-specific melanoma reactivity.[2,3] Sequential precipitation analysis using two kinds of antibodies has confirmed the biochemical properties of the melanoma antigen, showing that it is composed of GM3 ganglioside and protein molecules.[4] Recent biochemical analyses have demonstrated that the M562 epitope is present on the 80k molecule (Sakiyama *et al.*, manuscript in preparation). Moreover, M562 antibody could inhibit anti-melanoma CTL activity in the effector phase, suggesting that the M562 epitope is involved in the structure recognized by anti-melanoma CTL. Concerning other possible biological properties of the M562 molecule, it might be involved in the growth regulation or metastasis of B16 melanoma cells. This is suggested by the fact that

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[*5] Abbreviations: CTL, cytotoxic T lymphocytes; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; kb, kilobase(s); PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

intravenous injection of M562 antibody could inhibit lung colonization of B16 cells, while isotype control antibody (M2590 and M622) did not.[5]

In order to further understand the melanoma antigen at a molecular level we attempted to isolate genomic DNA controlling the expression of this antigen by employing cosmid library transfection and monoclonal anti-melanoma antibody. We successfully cloned a 34.8 kb genomic DNA fragment. The expression of melanoma antigen in transfectants was unstable, however, unless we continued to select antigen-bright transfectants by cell sorting.

Materials and Methods

Cells and Medium Mouse melanoma cells (B16) and human melanoma cells (SK-Mel 28) were maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 4% fetal calf serum (GIBCO Laboratories). Before the DNA transfection, human melanoma cells, which grow rapidly in the medium, were cloned by limiting dilution and cultured in RPMI 1640 with 10% fetal calf serum. The selection medium contained mycophenolic acid (10 μg/ml), xanthine (250 μg/ml), hypoxanthine (13 μg/ml) and thymidine (3.87 μg/ml) in RPMI 1640 with 10% fetal calf serum.[6]

Construction of B16 Genomic Library with Shuttle Vector High-molecular-weight DNA from B16 cells was isolated as described[7] and was partially digested with *Mbo* I restriction enzyme. DNA fragments (30–45 kb) were purified after ultracentrifugation at 36,000 rpm (Hitachi RPS40T, Hitachi Co. Ltd., Tokyo) for 12 hr at 20° in a 10–40% neutral sucrose density gradient. The vector DNA, pCV103,[8] was completely digested with *Bam*HI and the 5′ end of the DNA was dephosphorylated with calf intestinal alkaline phosphatase (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD). The dephosphorylation was monitored by self ligation of the vectors. About 0.5 μg of the fractionated DNA was ligated with 1 μg of linearized pCV103 vector using T4 ligase (Bethesda Research Laboratories). Ligated DNA (0.6 μg) was packaged *in vitro* as described.[9] *Escherichia coli* ED8767, grown overnight in L broth with 0.2% maltose, was suspended in 10m*M* $MgSO_4$ and incubated with packaging mixture at 37° for 15 min to allow phage absorption. We examined colonies randomly to evaluate our library based on the average length of inserts and colony numbers. This library was estimated to contain 1.7×10^7 kb insert DNA of B16 origin covering 560% of the total mouse genome. We confirmed that there was no preferential amplification of special DNA fragments in this library. Transformed ED8767 was cultured with constant vigorous shaking in L broth with ampicillin (40 μg/ml) for 5 hr. An aliquot was stocked at −80° in 50% glycerol.

Transfection by Protoplast Fusion The basic procedure of protoplast fusion was described previously.[10] Briefly, ED8767 harboring the B16 cosmid library was grown in 200 ml of L broth with ampicillin (40 μg/ml) up to an absorbance of 0.7 at 600 nm. The bacteria were collected and suspended in 10 ml of cold 20% sucrose/50m*M* Tris-HCl, pH 8.0. They were then incubated with 2 ml of lysozyme (freshly prepared solution of 5 mg/ml in 0.25 *M* Tris-HCl, pH 8.0) on ice for 5 min followed by another incubation with 4 ml of 0.25*M* EDTA, pH 8.0, on ice for 5 min. Then 4 ml of 50m*M* Tris-HCl, pH 8.0, was added and the cells were incubated at 37° for 5 min with gentle shaking. At the end of incubation the bacteria were examined under a microscope to ascertain that more than 95% of them were converted to protoplasts. The bacteria were then diluted at least three-fold with MEM medium containing 10% sucrose and 10 m*M* $MgCl_2$. At this point the protoplasts were ready for fusion.

The procedure for fusion of protoplasts and SK-Mel 28 cells is essentially the same as that for making hybridomas.[11, 12] Equal volumes of protoplasts and SK-Mel 28 cells (1×10^7) were mixed and centrifuged at 500*g* for 5 min. The pellet was suspended gently in 1 ml of 45% polyethylene glycol (molecular weight 1,500, BDH Chemical Ltd., Poole, UK) in DMEM, with slow stirring for 2 min, then 10 ml of DMEM was added gradually to dilute the polyethylene glycol, and the mixture was centrifuged at 150*g* for 5 min. The cells were carefully suspended in RPMI 1640 with 10% fetal calf serum and kanamycin (200 μg/ml) and plated in a 24-well multiplate (NUNC, Roskilde, Denmark). On the following day the selection medium containing mycophenolic acid was added to the culture and medium change was performed as required. About 2 weeks later, colonies of transfectants became visible. In this experiment the transfection efficiency was 1×10^{-5}.

Cell Surface Staining and Cell Sorting Transfectants (1×10^7) grown in the selection medium were washed with Hanks' balanced salt solution (HBSS) and incubated with M562 antibody (100 μg/ml) on ice for 1 hr. They were then washed three times with HBSS and incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin (20 μg/ml) on ice for 30 min. Fluorescence intensity was analyzed by the use of a FACS IV (Becton Dickinson,

Mountain View, CA) with a logarithmic amplifier. Cell sorting with FACS IV was performed at the flow rate of 1,500–2,000 cells/min.[13, 14]

Indirect Immunofluorescence Staining Transfectants were cultured on glass cover slips (18 mm × 18 mm) overnight. The next day they were washed three times with phosphate-buffered saline (PBS) followed by fixation with 3.7% formalin for 30 min. They were then washed three times with PBS, incubated with M562 antibody (100 μg/ml) at room temperature for 1 hr, again washed three times with PBS, and further incubated with FITC-conjugated rabbit anti-mouse immunoglobulin (20 μg/ml) at room temperature for 30 min. They were examined by fluorescence microscopy (VANOX AHBS-RFL, Olympus Kogaku, Tokyo).

Southern Blot Analysis High-molecular-weight DNA (10 μg) was digested with *Eco* RI restriction enzyme and subjected to electrophoresis on 0.8% agarose gel. DNA fragments were transferred to a nitrocellulose filter (BA85, Schleicher & Schuell, Inc., Keene, NH) and hybridized with an [α-^{32}P]-dCTP labeled probe in 50% formamide/5X SSPE (0.75*M* NaCl, 50m*M* $Na_2PO_4 \cdot H_2O$ and 0.5m*M* EDTA)/1X Denhart's solution/0.1% SDS (sodium dodecyl sulfate)/100 μg/ml denatured salmon sperm DNA/10% dextran sulfate at 42° overnight. The filter was washed with 0.1X SSC (15m*M* NaCl and 1.5m*M* sodium citrate)/0.1% SDS at 65°. Kodak X-ray film (XAR-5, Eastman Kodak Co., Rochester, NY) and an intensifier screen were used for autoradiography.

Results

Unstable Expression of Melanoma Antigen on Transfectants We transfected the cosmid library of B16 melamona cells into human melanoma cells, SK-Mel 28, which were negative for the antigen detected by the monoclonal antibody (M562). Transfectants, which acquired the *Ecogpt* gene from pCV103, could grow in the selection medium containing mycophenolic acid. They were examined for M562 antigen expression by means of the cell sorter (Fig. 1). At the initial staining we could hardly observe any antigen-positive transfectants. However, repeated sortings of less than 5% fluorescence-bright population at 10- and 13-day intervals enabled us to enrich the antigen-positive cells (Fig. 1 A-B, B-C). On the other hand, if the sorting interval was 20 days, the antigen expression was only three-fifths of that of the original population (Fig. 1 B-D, E-F). If we sorted transfectants at a 51-day interval or 62-day interval, the expression was only one-tenth (Fig. 1 F-G) or had disappeared (Fig. 1 F-H).

Rescue of Genomic DNA by *in vitro* Packaging This shuttle vector has the advantage of enabling the rescue of the library DNA by *in vitro* packaging, if at least two *cos* sequences are integrated at suitable intervals. Therefore, the *in vitro* packaging technique allows only the donor DNA with *cos* sequence to be rescued from the transfectant DNA. After 7 cycles of cell sorting, we rescued 26 colonies from 10 μg total DNA of M562-positive transfectants with Amersham's *in vitro* packaging kit (Amersham International plc, Buckinghamshire, UK). They were classified into three groups (pD2-1, pD2-2, pD2-7) based on the restriction patterns with the *Eco* RI restriction enzyme. However, 2,400 colonies were rescued from 10 μg total DNA of transfectants by Gigapack packaging extract (Vector Cloning Systems, San Diego, CA), with selection by 11 cycles of cell sorting, and all of those examined (24 clones) were pD2-7. Although it is known that the latter packaging system is generally 5 times more efficient than the former one, we were able to obtain about 100 times more colonies by the latter in our experiments. Based on the above calculation, the 11 cycles of sorting was about 18 times more efficient than the 7 cycles.

In order to determine the DNA fragment for antigen expression precisely, three kinds of DNA were individually transfected into human melanoma cells. The results of the cloned DNA transfection showed that pD2-7 alone was sufficient for the antigen expression (Fig. 2). We also confirmed this result by cell surface staining with indirect immunofluorescence (Fig. 3). We detected bright fluorescence on the membrane of transfectants. Therefore, it is concluded that the 34.8 kb DNA (pD2-7) with 5 *Eco* RI sites encompasses the gene for melanoma antigen expression.

Analysis of Instability of Antigen Expression in Transfectants We noticed unstable antigen expression even in the cloned DNA transfectants, as observed in the genomic library transfectants. When we reexamined the pD2-7 transfectants three weeks after the immunofluorescence staining experiments (as shown in Fig. 3), the antigen was scarcely detectable by the same assay.

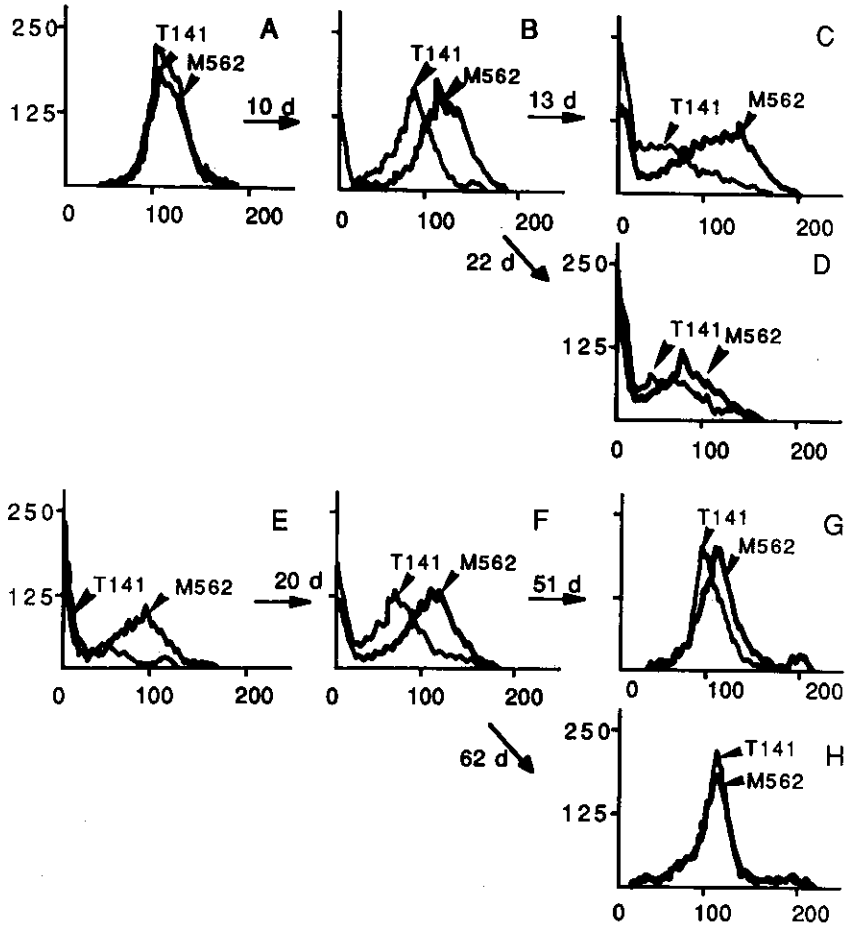

Fig. 1. Cell surface staining profiles of cosmid library transfectants after sequential cell sortings. The vertical axis shows relative cell number and the horizontal axis indicates fluorescence intensity. A, Initial staining pattern of transfectants; B, 10 days after A; C, 13 days after B; D, 22 days after B; E, 10 days after C; F, 20 days after E; G, 51 days after F; H, 62 days after F. T141 is the control antibody with the same isotype as anti-mouse melanoma antibody M562 (IgM, κ). See "Materials and Methods."

To assess the reason for this, we examined the integration of the vector and insert DNA by Southern blot analysis. *Eco* RI-digested DNAs from the host, SK-Mel 28, and pD2-7 transfectants with diminished antigen expression, were hybridized with either 2.8 kb *Eco* RI fragment from pD2-7 or pCV103 DNA (7.6 kb) as probes. The results showed that the pCV103 probe detected a strong signal on DNA from the transfectants, while a very weak signal was obtained when hybridized with the 2.8 kb probe (Fig. 4A). No signals were detected on DNA from human melanoma cells with the pCV103 vector probe. Since we observed background signals with the 2.8 kb probe because of the repetitive sequences within this probe, cDNA probe mapped in pD2-7 genomic DNA (manuscript in preparation) was used for a more precise examination. We compared signals on DNA from pD2-7 transfectants with different amounts of antigen; one expressed antigen highly and

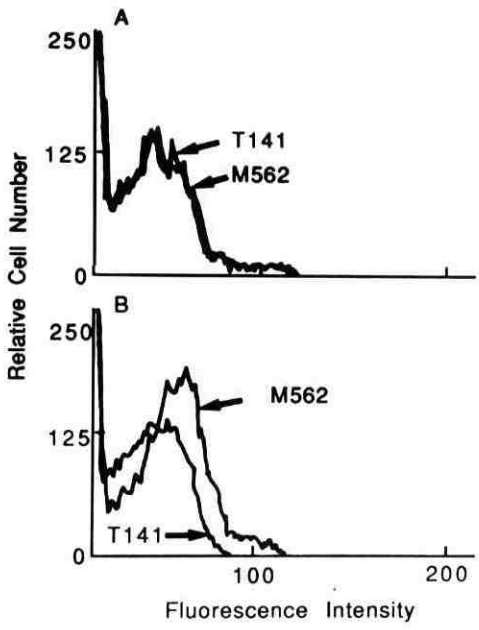

Fig. 2. FACS analysis of melanoma antigen expression of pD2-7 transfectants. A, SK-Mel 28; B, pD2-7 transfectants of SK-Mel 28. T141 is the control antibody of M562. See "Materials and Methods."

the other (originally highly expressed) showed diminished expression due to longer sorting intervals. We detected strong signals of B16 origin on pD2-7 transfectants with high antigen expression (Fig. 4B, lane 3) but weak signals on transfectants with low expression (Fig. 4B, lane 4) based on the comparison with standard signals from B16 cells (Fig. 4B, lanes 1 and 5) with one copy per haploid.

Densitometric analysis showed that the signal detected by the pCV103 probe was 4 times stronger than that by the 2.8 kb probe on DNA from pD2-7 transfectants (as there are background signals, the actual value is more than 4 times), adjusting the density according to the lengths of labeled probes. In this experiment the specific activities of these probes were almost the same (pCV103 probe, 5.1×10^8 cpm/μg; 2.8 kb probe, 4×10^8 cpm/μg). In addition, the doses of DNA on the filter and the conditions of hybridization and washing were identical. This indicates that the insert DNA is preferentially deleted compared with the vector DNA.

Discussion

We have isolated the genomic DNA controlling the expression of the melanoma anti-

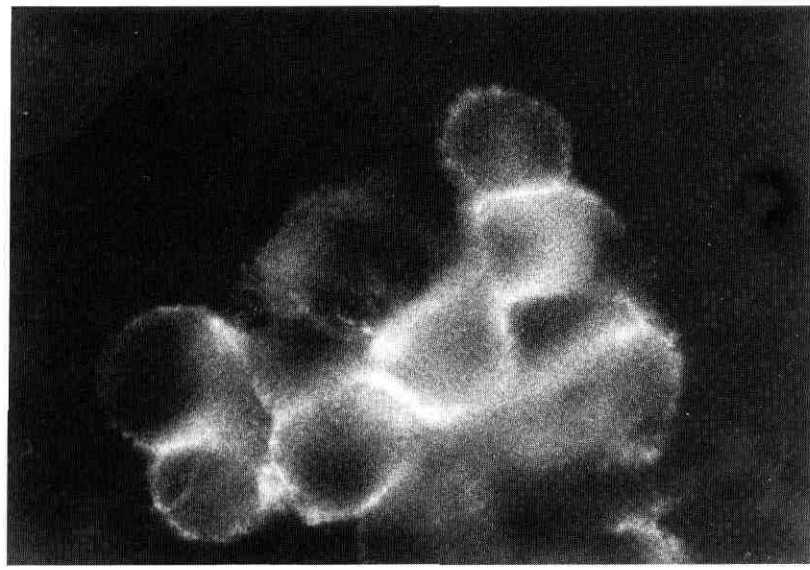

Fig. 3. Immunofluorescence staining of pD2-7 transfectants of SK-Mel 28. See "Materials and Methods."

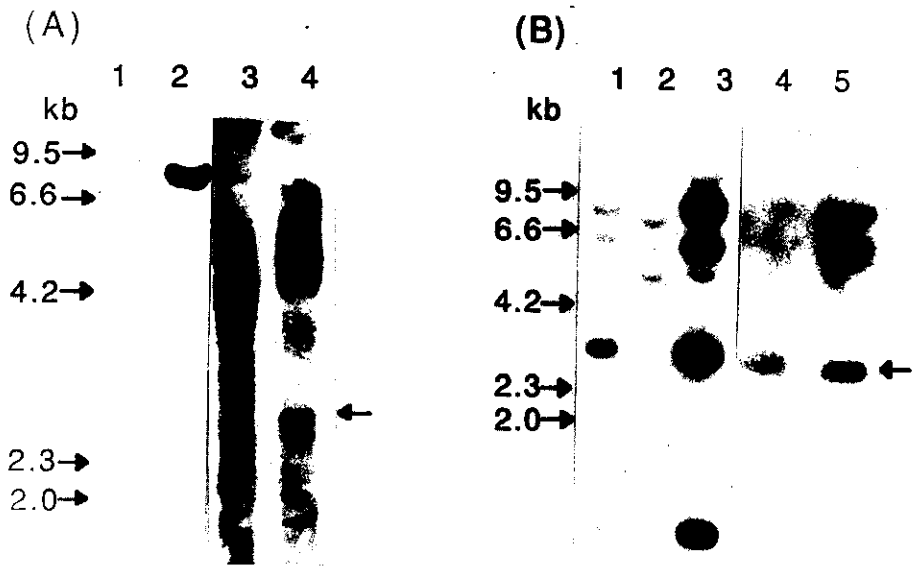

Fig. 4. Southern blot analysis on DNA from SK-Mel 28, its pD2-7 transfectants and B16 cells. (A) DNA: lanes 1, 3, SK-Mel 28; lanes 2, 4, pD2-7 transfectants of SK-Mel 28 (diminished antigen expression). Probes: lanes 1, 2, pCV103; lanes 3, 4, 2.8 kb *Eco* RI fragment from pD2-7. Size markers were *Hin*d III-digested lambda DNA. (B) DNA: lanes 1, 5, B16; lane 2, SK-Mel 28; lanes 3, 4, pD2-7 transfectants of SK-Mel 28 (lane 3, high antigen expression; lane 4, diminished antigen expression). Probes: cDNA mapped to pD2-7 genomic DNA. We detected cross-hybridized bands on human melanoma cell SK-Mel 28 (lane 2), which was overlaid in transfectants (lane 3) in addition to B16 origin signals. See "Materials and Methods."

gen. This 34.8 kb DNA possesses genes encoding the melanoma antigen or controlling its expression in either a *trans*-acting or *cis*-acting manner. Since our biochemical study shows that M562 antibody reacts with the 80k molecule, the cloning of structural genes in this region would obviously be helpful for clarifying this issue. We are currently working on the sequencing and analysis of cDNA mapped within this area to determine whether cDNA transfectants are stained by M562 antibody.

Continuous sorting of antigen-positive transfectants at short intervals seems to be necessary to keep them fluorescence-bright. As reported here, sorting at 10-day intervals is necessary to maintain antigen-positive cells. If we sort cells at more than 20-day intervals, the antigen expression becomes diminished or disappears. Moreover, frequent cell sorting at short intervals not only maintains the antigen expression but seems to amplify the genes.[15,16] In fact, we rescued 18 times more colonies from 11 cycle transfectants than from 7 cycle ones, taking into consideration the packaging efficiency. This might be partly due to the amplification of this cosmid DNA, but we must also consider the integrated physical states of the *cos* sequences. However, in spite of the gene amplification, there was no significant difference in the antigen expression between the 11-times and 7-times sorted cells. This might be explained by the saturation of antigen molecules on the cell surface, as has been discussed.[17]

These are many factors involved in the negative regulation of exogenous DNA expression. In our case we speculated that the deletion of insert DNA was the major reason, as shown in the cases of Ltk^- revertants from Ltk^+ cells.[18,19] We examined this assumption by Southern blot analysis and demonstrated that the insert DNA was preferentially deleted compared with the vector DNA (Fig. 4). In addition, the *Ecogpt* gene should be kept

intact in the host because transfectants grew in the selection medium. Since gene amplification is usually accompanied with instability of genes,[20] it is easy to understand the deletion of exogenous DNA in this case. Concerning this point, Lau and Kan have reported that this cosmid might exist as extrachromosomal elements rather than integrated forms in the host chromosome.[21] We have not yet characterized the physical structure or the localization of this vector. However, if this vector exists in episomal forms, it would be interesting to examine whether the cosmid DNA can constitute a double minute, which would be direct evidence of gene amplification.[17, 22]

Since the epitope recognized by M562 antibody is not detected in human melanomas, the identification of the human homologue of this molecule is of particular interest. We could not analyze the structure of human melanoma antigen precisely, but we believe that a similar immune regulation to that observed in the murine system operates in the human system, and that this kind of melanoma antigen would be a keystone of immune surveillance. Identification of human counterpart DNA as shown in Southern blot analysis (Fig. 4) would be a pivotal point for characterizing the structure of human melanoma antigen and analyzing human immune responses against melanomas. It should also prove to be a useful tool for diagnostic and therapeutic applications.

Acknowledgments

This work was supported by Grants-in-Aid for Cancer Research and for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture, Japan, and by Grants from the Princess Takamatsu Cancer Research Fund and the Uehara Memorial Foundation, Japan. We express our gratitude to Chimi Saitoh for her secretarial assistance.

(Received Feb. 22, 1988/Accepted April 27, 1988)

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