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The Expression of Tumor Rejection Antigen on Rat Fetus Fibroblasts Transformed by the *ras* Oncogene

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The expression of tumor rejection antigens (TRA) was analyzed on clones of rat fetus-derived fibroblasts, WFB, transformed or transfected by oncogenes. It was shown that a tumorigenic W14 clone, which is an activated H-*ras* transformant of parental WFB, expressed TRA in transplantation experiments using syngeneic WKA rats. The data also showed that W14 TRA was acquired in the event of cell transformation, since it was not detected on parental non-transformed WFB cells or Wmyc-4 clone which is a transfectant of WFB by mouse plasmacytoma-derived c-*myc* DNA. However, TRA was not expressed or at least was not detected on highly tumorigenic W31, another clone of H-*ras* transformants of parental WFB, in the transplantation experiments. We also assessed the level of expression of major histocompatibility antigens (MHC) class I molecules on these cells by using R4-8B1 Mab that reacts specifically with rat class I antigen. The data indicated that it was decreased on W14, W31, and Wmyc-4, but not on parental WFB. Although this molecule was weakly positive on W14 cells, W31 and Wmyc-4 showed even greater decreases. These data may indicate that the TRA expression and its recognition by syngeneic hosts are dependent upon the transformed clones, although their parental cell is the same. We discuss in detail this difference of expression and recognition of TRA in the context of the cell transforming process.

Key words: Tumor rejection antigen — Transformation — Class I — *ras* — *myc*

The molecular nature and the expression mechanism of tumor rejection antigens (TRA) remain unknown, although several successful analyses of their biochemical characteristics have been reported.[1-3] Recently, Srivastava *et al.*[4] have demonstrated the molecular cloning of the genomic 5′ end of the TRA gene of chemically induced BALB/c mouse Meth A fibrosarcoma. This is the first report on a genomic DNA that encodes a TRA molecule of a murine tumor. They indicated the presence of a nucleotide sequence homology between Meth A and another fibrosarcoma CMS5, although these tumors could not cross-react with one another in transplantation experiments. However, the data raised several fundamental questions. How could each TRA of these tumors gain the immunological specificity of an antigen? What is the molecular mechanism to determine the specificity? The nucleotide sequence encoding Meth A TRA was also detected in the syngeneic adult liver DNA. Furthermore, there was no enhancement at the transcriptional level of the gene between Meth A and adult liver. It is not known whether the clonal expression of TRA is really related to the cell transformation. It seemed desirable to investigate whether this antigen could be acquired during the cell transforming process or after complete cell transformation.

In this study, we examined the clonal expression of TRA on oncogene transformants or transfectants by using the same non-transformed recipient cells for the oncogene transfection. The WKA rat fetus-derived parental clone, WFB, has been shown to maintain strictly several phenotypes as the non-transformant and to be sensitive to oncogene transfection.[5,6] The data indicated that the expression of TRA of WFB transformants was dependent upon the transformed clones. This was suggested to be linked to the cell

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transformation, since TRA was not detected on parental WFB cells or WFB transfectants of c-*myc* of mouse plasmacytoma.

Materials and Methods

Animals Inbred Wistar-King-Aptekman (WKA)-H rats were obtained from CLEA Japan Inc., Shizuoka. In the transplantation experiment, 6- to 10-week-old male animals were used.

Cells, Oncogenes and DNA Transfection WFB, a WKA rat fetus-derived cell line, was previously reported,[5,6] and was used as the parental and recipient cells for oncogene transfection in this study. It was shown that WFB had a doubling time of approximately 37 hr *in vitro*. Strict phenotypes were observed for the non-transformant, such as anchorage dependency of cell growth in 0.3% agar, contact inhibition and high serum dependency of monolayer cell growth in Eagle's modified culture medium supplemented with 6% fetal calf serum and 292 μg/ml of L-glutamine. The culture of WFB *in vitro* has passed 200 passage generations.

Oncogenes used in this study were 6.6 kb EJras,[7] recombinant mouse c-*myc* cloned from mouse plasmacytoma inserted at the *Bam*HI site of pUC18 plasmid DNA.[8] These oncogenes was previously described.[5] The calcium phosphate co-precipitation method was used to transfect several oncogenes into cells,[9] and a part of this procedure has already been reported.[5] In order to gain WFB-c-*myc* transfectants, WFB was cotransfected with a mixture of DNAs at a ratio of 1 mol of pSV2neo containing neomycin resistance gene[10] and 10 mol of c-*myc*. At 48 hr after transfection, the cells were trypsinized, and the contents of each dish (Falcon #3002, Oxnard, CA) were divided into four dishes. After an additional 48 hr, a medium containing 400 μg/ml of G418 (Gibco Laboratories, Grand Island, NY) was fed every 3 days. After 21 days of culture, G418 resistant colonies were picked up from the dishes.

Cells transfected by the oncogenes were assessed for growth potential in 0.3% soft agar. The procedure has been reported in our previous paper.[11] In this study, we used the following transfected cell clones; W14 and W31, WFB transformants by EJras, and Wmyc-4, a WFB transfectant by c-*myc* and pSV2neo. Southern blot analysis of these clones demonstrated the insertion of each transfected DNA into the cells, as reported previously.[12] W14 and W31 showed different flanking sequences of DNA in Southern blot analysis when the cellular DNA digested by *Sac*I was hybridized with the *Bam*HI digested-6.6 kb EJras probe, indicating that these two EJras-transformants were different clones derived from the same parental WFB.

Determination of TRA Activity For the determination of the expression of TRA on these tumor cells, we injected subcutaneously $5–10\times10^5$ W14 or W31 cells into five to seven rats per group on the left side of the back. One or two weeks after the injection a W14 or W31 tumor appeared at the injection site. The tumors were surgically resected, and a week later these primed animals were challenged subcutaneously with $1–2\times10^5$ W14 or W31 cells in the right side of the back. We also immunized subcutaneously another group of rats with 10^6 WFB or Wmyc-4 cells. A week later these rats were challenged with W14 or W31 transformants as described above. In the transplantation experiments, it was difficult to assess TRA activity against these transformants in terms of tumor incidence. It seemed to us that this could be due to the highly tumorigenic nature of W14 and W31 cells. In the present paper, therefore, TRA activity was indicated and analyzed in terms of the tumor diameter. The *P* values for differences of tumor growth were calculated by means of Student's *t*-test.

FACS Analysis of Cells Stained with Mab Reacting against Rat MHC Class I Products WFB, W14, W31 and Wmyc-4 were washed twice with PBS, and $1–2\times10^6$ cells in 0.2 ml of PBS were incubated with a saturating amount of Mab R4-8B1 that reacts with rat major histocompatibility antigens (MHC) class I products for 30 min at 4°. Cells were washed twice with PBS and incubated with 0.1 ml of FITC-conjugated goat anti-mouse Ig diluted 1:40 in PBS for 30 min at 4°. Cells were then washed twice with PBS and fixed in 1–2% paraformaldehyde-PBS. Samples were run on a Coulter Epics V FACS analyzer. As a control for nonspecific binding of mouse Ig or FITC-conjugated goat antimouse serum to the cells, parallel samples were made by staining with normal mouse serum diluted 1:5 and/or FITC-conjugated goat antimouse Ig alone diluted 1:40. We routinely analyzed $1–2\times10^5$ cells per sample.

Results

Anchorage-independent Growth of the Oncogene Transfectants in Soft Agar The potential to grow anchorage-independently in 0.3% agar was assessed for each of the transfectants. Table I showed the anchorage-independent growth of cells and tumorigenicity *in vivo* of parental WFB, W14, W31 and Wmyc-4 cells. When 10^3 cells were seeded into 0.3% agar, W31 showed a very high ability to grow in the agar. W14 showed moderate growth. However, WFB and Wmyc-4 could

Table I. Anchorage-independent Growth of the Transfectants of WFB by Oncogenes in Soft Agar

Cell	Oncogene[a]	% Plating efficiency[b] at the 3rd week after seeding into 0.3% agar
WFB	no	0
W14	EJras	52.0±8.8
W31	EJras	91.5±4.1
Wmyc-4	c-*myc*	0

a) See "Materials and Methods."
b) Anchorage-independent cell growth in 0.3% soft agar was determined for inocula of 10^3 cells. The cell growth was scored microscopically at the 3rd week after plating, and the colony formation was expressed as % plating efficiency = (No. of clusters − No. of original cell aggregates) × 100/(No. of viable nucleated cells plated). The data are given as mean ± SE.

not form any clusters or colonies of cells even after 6 to 10 weeks of cultivation following seeding of the cells into agar. Furthermore, both W14 and W31 showed a 100% incidence of tumor growth in nude mice and syngeneic WKA adult rats when injected subcutaneously (10^6 cells) into three to five animals per group. However, parental WFB and Wmyc-4 cells failed to develop any tumors during 15 weeks after injection of cells even if 5×10^7 cells were injected (data not shown).

Tumor Rejection Profiles of W14 and W31 Transformants To test the possibility that the expression of TRA is associated with cell transformation, we examined the expression of TRA on W14 and W31 transformants based on a precise comparison with their parental WFB nontransformed cells. As shown in Table IIA, W14 cells clearly express TRA that could induce strong rejection of the challenging W14 tumor cells. On the other hand, the mice immunized with parental WFB cells could not reject W14 tumor challenge, suggesting that the TRA of W14 was conferred during the transformation of WFB cells. The mice immunized with W31 transformed cells could not withstand the challenge of W14 tumor cells, although these transformants were derived from the same parental WFB. Table IIB shows that the data in the transplantation experiments were highly reproducible. It was demonstrated that the mice immunized with Wmyc-4 cells could not reject the challenge with W14 tumor cells. The fact that Wmyc-4 could not grow in an anchorage-independent manner in the soft agar shows that this clone is not transformed,

Table II. The Tumor Rejection Profiles of Challenging W14 Cells in Rats Immunized with Transfectants of WFB by Oncogenes

Exp.	Immune[a]	Mean tumor diameter (mm) at the 3rd week	
A			
I	no	32.9±5.6	
	W14	1.7±3.4	($P<0.001$)
II	no	46.8±6.2	
	WFB	48.4±8.2	
	W31	42.5±5.5	
B			
III	no	21.0±4.8	
	WFB	21.6±6.2	
	Wmyc-4	24.4±3.8	
	W14	10.7±5.2	($P<0.05$)
	W31	37.2±7.4	
IV	no	17.2±6.2	
	WFB	12.5±7.8	
	Wmyc-4	10.5±8.6	
	W14	0	($P<0.004$)
	W31	30.1±9.2	

a) Rats were injected with $5\text{–}10\times10^5$ W14 or W13 cells (five to seven rats per group) on the left side of the back. One or two weeks after injection, W14 and W31 tumors appeared at the injection site. The tumors were surgically resected, and a week later these primed animals were challenged subcutaneously with 2×10^5 (exp. AI, AII and BIII) or 1×10^5 (BIV) of W14 cells in the right side of the back. Some rats were also immunized subcutaneously with 10^6 WFB or Wmyc-4 cells, and a week later these rats were challenged with W14 cells as described above.

Table III. The Tumor Rejection Profiles of Challenging W31 Cells in Rats Immunized with Parental WFB and W14 Cells

Exp.	Immune[a]	Mean tumor diameter (mm) at the 3rd week	
I	no	33.7±6.2	
	W31	22.2±7.4	(NS)[b]
II	no	29.3±8.6	
	WFB	38.0±6.8	
	W14	37.3±5.4	

a) Rats were immunized as described in Table II. These animals were challenged with 2×10^5 W31 cells.
b) Statistically not significant.

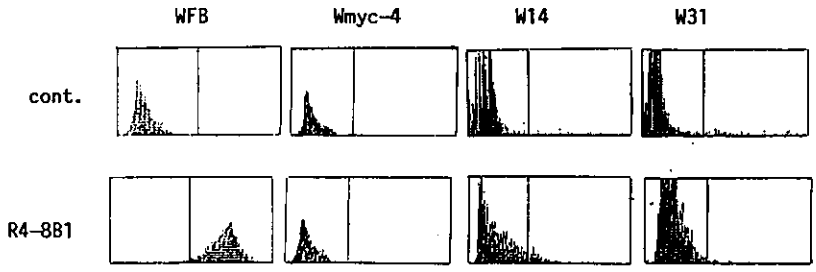

Fig. 1. FACS analysis of cells stained with monoclonal antibody R4-8B1 reacting against rat MHC class I products. The cells (WFB, Wmyc-4, W14 and W31) were washed with PBS, and 1–2×10^6 cells in 0.2 ml of PBS were incubated with a saturating amount of R4-8B1 monoclonal antibody (lower column) for 30 min at 4°. Cells were washed with PBS and incubated with 0.1 ml of FITC-conjugated goat anti-mouse serum diluted 1:40 in PBS for 30 min at 4°. Cells were then washed with PBS and fixed in 1–2% paraformaldehyde-PBS. Samples were run on a Coulter Epics V FACS. As a control for nonspecific reaction of the FITC-conjugated antiserum to the cells, parallel samples were run that were stained with this antiserum alone (upper column). Abscissa: log of fluorescence intensity. Ordinate: relative cell number.

although the saturation density in the monolayer culture of Wmyc-4 clone is relatively high compared to that of parental WFB. The data of these tranplantation experiments may indicate that TRA of W14 was clonospecifically acquired, and was associated with the transformation of WFB. Table III showed the rejection profiles of challenging W31 transformed cells in mice immunized with W31, parental WFB and W14 cells. The mice immunized with W31 cells could not reject the challenge with homologous W31 cells, although these mice showed a slightly reduced growth rate when compared to nonimmune mice. This indicates that the TRA might not be conferred on the W31 clone. The parental WFB as well as W14 cells could not induce the tumor rejection of W31 in the transplantation experiments.

Expression of MHC Class I Antigen We studied the expression of class I molecules of rat MHC on the cells. It is generally accepted that the expression of MHC class I antigen directly affects the cytotoxicity of cytotoxic T lymphocytes. This implies that the expression of class I antigen as well as TRA on tumor cells is necessary for the effective rejection of the tumor by the host. The data in Table II suggest that W31 but not W14 might be devoid of TRA expression. These data also suggest that there was a difference as to the expression of MHC class I molecule between W14 and W31 EJras transformants. Using R4-8B1 monoclonal antibody that reacts with rat class I antigen, we assessed the level of expression of this antigen molecule by using the FACS analyzer. Figure 1 indicates that the level of expression of class I molecules was reduced on W14, W31 and Wmyc-4 cells. The expression on W31 and Wmyc-4 was almost undetectable, but the expression on W14 was weakly positive. In contrast, the parental WFB showed a very high expression of this antigen. These data may support the notions that (1) W14 TRA is not expressed on parental WFB cells and that (2) it is directly associated with the transformation of WFB cells.

Discussion

TRA is a very important antigen, because it provides protection from tumor growth in the hosts. Investigations of the expression mechanism of TRA may lead to an application for the manipulation of host immune responses against tumors. There have been numerous reports on the immunological profiles of TRA. It has been generally accepted that TRA of chemically induced tumors is individually specific for the tumor and could induce rejection of a challenging tumor of homologous origin.[13] However, several studies have

shown that some of these chemically induced tumors may have TRA that is commonly expressed among the tumors.[14-18] This is particularly important for analysis of the expression mechanism of TRA on the tumor cells, since there is a possibility that the expression of TRA is associated with the cell transformation and activation of the highly conserved genomic DNA that codes TRA molecules.

It is considered that the process of cell transformation is not a single event but rather one having multiple steps.[19, 20] These steps might be associated with or result in new or enhanced expression of cell surface antigens.[21, 22] These antigens are oncogene products,[23, 24] heat shock proteins,[25] and the product of an activated endogenous provirus.[21] It is also possible that the product of specific genomic DNA of the cells could be expressed as a result of the gene activation dependent on the specific site of oncogene insertion or the activation of other metabolic processes. It is obviously very important to investigate whether the tumor antigens on the cell surface of neoplastic cells are directly associated with the cell transformation or linked with the mechanism responsible for the transformation process, since these approaches could lead to an understanding of the molecular genetics of TRA expression.

In this paper, we have analyzed the expression of TRA in transfectants by oncogenes on a WKA rat fetus-derived fibroblast, WFB. We also assessed the level of expression of MHC class I molecules on these transfectants. Our main purpose in the experiment was to test whether TRA could be expressed during the transforming process of cells, since there is no definitive report that compares the TRA expression between parental non-transformed cells and their transfectants or transformants. Our data indicated that W14 but not W31 clone expresses TRA, although these cells were derived from the same parental WFB that maintains the characteristics of non-transformed cells. In this study, it was shown that W14 TRA was acquired in the event of cells transformation, because this TRA was not detected on parental WFB or on Wmyc-4 clone that was a transfectant of WFB by mouse plasmacytoma-derived c-*myc* gene. Furthermore, the TRA of WFB was not the product of an activated endogenous provirus on the cell surface associated with the transformation of this parental cell, since all of WFB, Wmyc-4, W14 and W31 showed almost the same density of expression of murine endogenous leukemia virus or mammary tumor virus products when antisera to these products were used (data not shown).

We should, however, be careful in interpreting the TRA expression on these cells, since the expression of TRA is decided only on the basis of growth *in vivo* of the challenging tumor cells. It has been noted that there are complex host immune responses against tumors. One of the important factors that directly affects the challenging tumor growth against the host tumor resistance is the expression of MHC class I molecules on the cell surface. In our study, W14 weakly expressed this molecule. In contrast, W31 and Wmyc-4 were almost devoid of class I expression. However, parental WFB showed a high expression of class I molecules. We can conclude from our data that W14 TRA was acquired on the transformation of WFB, and was recognized by the syngeneic hosts. The level of expression of class I antigen on W14 was reduced, but might still be enough for recognition by cytotoxic T lymphocytes. The possibility exists that W31 also may possess TRA during the transformation. However, this might not be recognized by the hosts due to a remarkably reduced level of class I antigen expression. We could not determine whether TRA is conferred on the Wmyc-4 clone, since this clone was not tumorigenic, and lacked class I antigen expression.

Recent studies have indicated that there should be some rules for the expression of tumor antigens, although a high polymorphism of these antigens has also been demonstrated.[13] In fact, it was suggested that there is a limited extent of enhanced transcription of mRNA in the cells between the resting NIH3T3 and the growth-stimulated or transformed cells.[26] This suggests that one of these products may be a candidate for TRA of NIH3T3 cells. W14 and W31 clones are the transformants derived from the same parental WFB clone by using EJras oncogene. Hence there is a possibility that the TRA is commonly expressed on these transformants, although we do not have evidence indicating that the TRA of W14 is conferred on W31

clone. We are further studying the relationship between TRA of W14 and W31.

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REFERENCES

1) DuBois, G. C., Appella, E., Law, L. W., DeLeo, A. B. and Old, L. J. The soluble antigen of BALB/c sarcoma Meth A: a relationship between a serologically defined tumor specific surface antigen (TSSA) and the tumor associated transplantation antigens (TATA). *Transplant. Proc.*, **13**, 1765–1773 (1981).
2) Sato, N., Yagihashi, A., Okubo, M., Torigoe, T., Takahashi, S., Sato, T. and Kikuchi, K. Characterization of tumor rejection antigen molecules of chemically induced murine colon tumor C-C26. *Cancer Res.*, **47**, 3147–3151 (1987).
3) LeGrue, S. J., Pellis, N. R., Riley, L. B. and Kahan, B. D. Biochemical characterization of 1-butanol-extracted murine tumor-specific transplantation antigens. *Cancer Res.*, **45**, 3164–3172 (1985).
4) Srivastava, P. K., Chen, Y. T. and Old, L. J. 5′-Structural analysis of genes encoding polymorphic antigens of chemically induced tumors. *Proc. Natl. Acad. Sci. USA*, **84**, 3807–3811 (1987).
5) Sato, N., Torigoe, T., Yagihashi, A., Okubo, M., Takahashi, S., Takahashi, N., Enomoto, K., Yamashita, T., Fujinaga, K. and Kikuchi, K. Assessment and establishment of a WKA rat fetus derived cell for the oncogene transfection. *Tumor Res.*, **22**, 15–26 (1988).
6) Yagihashi, A., Sato, N., Torigoe, T., Okubo, M., Konno, A., Takahashi, N., Yamashita, T., Fujinaga, K., Kuzumaki, N. and Kikuchi, K. Identification of the transformation-associated cell surface antigen expressed on the rat fetus-derived fibroblasts. *Cancer Res.*, in press.
7) Krontiris, T. G. and Cooper, G. M. Transforming activity of human tumor DNAs. *Proc. Natl. Acad. Sci. USA*, **78**, 1181–1184 (1981).
8) Taub, R., Kirsch, I., Morton, C., Lenoir, G., Swan, D., Tronick, S., Aaronson, S. and Leder, P. Translocation of the c-*myc* gene into the immunoglobulin heavy chain locus of Burkitt lymphoma and murine plasmacytoma cells. *Proc. Natl. Acad. Sci. USA*, **79**, 7837–7841 (1982).
9) Loyter, A., Scangos, G. A. and Ruddle, F. H. Mechanism of DNA uptake by mammalian cells: fate of exogenously added DNA monitored by the use of fluorescent dyes. *Proc. Natl. Acad. Sci. USA*, **79**, 422–426 (1982).
10) Southern, P. J. and Berg, P. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.*, **1**, 327–341 (1982).
11) Sato, N., Sato, T., Takahashi, S. and Kikuchi, K. Establishment of murine endothelial cell lines that develop angiosarcomas *in vivo*: brief demonstration of a proposed animal model for Kaposi's sarcoma. *Cancer Res.*, **46**, 362–366 (1986).
12) Fujinaga, K., Sawada, Y. and Sekikawa, K. Three different classes of human adenovirus transforming DNA sequences; highly oncogenic subgroup A, weakly oncogenic subgroup B, and subgroup C specific transforming DNA sequences. *Virology*, **93**, 578–581 (1979).
13) Old, L. J. Cancer immunology: the search for specificity — G.H.A. Clowes Memorial Lecture. *Cancer Res.*, **41**, 361–375 (1981).
14) Hellström, K. E., Hellström, I. and Brown, J. P. Unique and common tumor-specific transplantation antigens of chemically induced mouse sarcomas. *Int. J. Cancer*, **21**, 317–322 (1978).
15) Leffel, M. S. and Coggin, J. H., Jr. Common transplantation antigens on methylcholanthrene-induced murine sarcomas detected by three assays of tumor rejection. *Cancer Res.*, **37**, 4112–4119 (1977).
16) Reiner, J. and Southern, C. M. Further evidence of common antigenic properties in chemically induced sarcomas of mice. *Cancer Res.*, **29**, 1814–1820 (1969).
17) Rogers, M. J. Tumor-associated transplantation antigens of chemically-induced tumors: new complexities. *Immunol. Today*, **5**, 167–168 (1984).
18) Sato, N., Sato, T., Takahashi, S., Fujimoto, N., Koshiba, H., Ishii, Y. and Kikuchi, K. Partial purification of C-C36 tumor rejection antigen molecules commonly expressed on syngeneic murine colon tumors, C-C36 and C-C26. *Jpn. J. Cancer Res. (Gann)*, **77**, 473–479 (1986).
19) Newbold, R. F. and Overell, R. W. Fibroblast immortality is a prerequisite for transformation by EJ c-H-*ras* oncogene. *Natur*, **304**, 648–651 (1983).
20) O'Brien, W., Stenman, G. and Sager, R. Suppression of tumor growth by senescence in virally transformed human fibroblasts.

Proc. Natl. Acad. Sci. USA, **83,** 8659–8663 (1986).

21) Becker, D., Lane, M. A. and Cooper, G. M. Transformation of NIH3T3 cells by DNA of the MCF-7 human mammary carcinoma cell line induces expression of an endogenous murine leukemia provirus. *Mol. Cell. Biol.,* **4,** 2247–2252 (1984).

22) Sato, N., Sato, T., Takahashi, S., Okubo, M., Yagihashi, A., Koshiba, H. and Kikuchi, K. Identification of transformation-related antigen by monoclonal antibody on Swiss3T3 cells induced by transfection with murine cultured colon 36 tumor DNA. *J. Natl. Cancer Inst.,* **78,** 307–313 (1987).

23) Drebin, J. A., Stern, D. F., Link, V. C., Weinberg, R. A. and Greene, M. I. Monoclonal antibodies identify a cell surface antigen associated with an activated cellular oncogene. *Nature,* **312,** 545–548 (1984).

24) Drebin, J. A., Link, V. C., Stern, D. F., Weinberg, R. A. and Greene, M. I. Downmodulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. *Cell,* **41,** 695–706 (1985).

25) Ullrich, S. J., Robinson, E. A., Law, L. W., Willingham, M. and Appella, E. A mouse tumor-specific transplantation antigen is a heat-shock protein. *Proc. Natl. Acad. Sci. USA,* **83,** 3121–3125 (1986).

26) Marshall, C. J. Oncogenes and growth control 1987. *Cell,* **49,** 723–725 (1987).