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High-frequency cotransfer of the transformed phenotype and a tumor-specific transplantation antigen by DNA from the 3-methylcholanthrene-induced Meth A sarcoma of BALB/c mice

(transfection/tumor DNA)

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ABSTRACT **We transformed BALB/3T3 mouse cells with cellular DNA extracted from the Meth A sarcoma, a 3-methylcholanthrene-induced tumor of BALB/c mice, and asked whether foci arising in the transfection possess the previously defined Meth A tumor-specific transplantation antigen (TSTA). Five of eight foci selected from one experiment possessed Meth A TSTA. DNA extracted from one of the five TSTA-positive clones was used in secondary rounds of transfection transformation. Four out of five foci tested from the secondary transfections possessed Meth A TSTA. These results suggest that in the Meth A sarcoma a transforming gene and a genetic determinant of TSTA are intimately related: they may be identical or very closely linked; alternatively, a particular transforming gene might induce the expression of a particular TSTA. Another possible explanation for these results is that the cotransfer of certain cellular genes by DNA transfection is considerably higher than predicted from the limited studies presently available.**

An intriguing class of tumor antigens is the tumor-specific transplantation antigens (TSTAs) of carcinogen-induced murine sarcomas. TSTAs are defined by the ability of tumor cells and tumor cell particulates or solubilized antigens to immunize a syngeneic host against subsequent challenge by live cells of the same tumor (1–6). Each chemically induced sarcoma appears to possess a unique TSTA even if the tumors were induced by the same agent in the same animal. The genetic basis of TSTAs of carcinogen-induced tumors is unknown, as is the relationship of the antigens to the transforming events that induced the tumor.

To investigate the relationship between the genetic determinants of TSTAs and the transformed phenotype, we transformed BALB/3T3 mouse cells with DNA from the 3-methylcholanthrene-induced Meth A sarcoma (5), using the calcium phosphate precipitation technique (7–10), and asked whether the transformants possess the previously defined Meth A TSTA (5, 6, 11, 12). Because the stable expression of mammalian cell genes after DNA transfection is very inefficient, one would expect the frequency of cotransfer of the two phenotypes, transformation and TSTA, to be only 0.01–0.001 if they are specified by unlinked genes (13, 14). However, if the two phenotypes have common or linked determinants, one would expect a high frequency of cotransfer.

The Meth A sarcoma was selected for these experiments because its TSTA has been well characterized immunologically and biochemically and shown to be distinct from TSTAs of all other syngeneic neoplasms assayed to date (5, 6, 11, 12). The tumor was induced in a BALB/c mouse in 1960 (5). Within a year of its isolation an ascites form of the tumor was established, and both the solid and ascites forms possess the Meth A TSTA. Cell lines have been established from both forms of the tumor, and these lines possess Meth A TSTA. The ascites form of Meth A and its derivative cell line appear to be negative for expression of type C viruses or viral antigens (15).

Here we report that the two phenotypes transformation and Meth A TSTA show a high frequency of cotransfer through two successive rounds of DNA transfection.

MATERIALS AND METHODS

DNA Transfection. Cellular DNA was extracted (16) from the following tissue culture cell lines: mouse NIH/3T3 (17); BALB/3T3 clone 7 (ref. 18; see below); mouse 64F3 clone 7, a nonproducer mink cell transformed by the replication-defective Gardner–Arnstein feline sarcoma virus (FeSV) (19); and a tissue culture line derived from the ascites form of the Meth A sarcoma Meth A (a) (15).

Recipient cells were a subclone of BALB/3T3 clone A31 (18) designated BALB/3T3 clone 7 and were grown in Dulbecco's modified Eagle's medium with 10% calf serum.

DNA was precipitated with calcium phosphate essentially as described (7, 20). Specifically, recipient cells were seeded at 1–1.5 × 10^6 per 10-cm dish for some experiments or 4 × 10^5 per 6-cm dish for others, and 40 μg of high molecular weight DNA was sheared by two passages through a 20 gauge hypodermic needle and resuspended in 1.25 ml of precipitation buffer, or 20 μg of DNA in 0.625 ml of buffer was added to 6.25 ml or 3.5 ml of medium in 10-cm or 6-cm dishes, respectively. In each experiment, 60–160 μg of each DNA was used. Five hours after addition of the DNA, the plates were washed and the cells were glycerol shocked to enhance transfection efficiency (21): 17% (vol/vol) glycerol containing, per liter, 8 g of NaCl, 5 g of Hepes, 0.37 g of KCl, and 1 g of dextrose (pH 7.0) was applied for 1 min and 40–45 sec, the shock was quenched by addition of medium, the plates were washed, and the medium was replaced. Six to 10 hr later, the cells were treated with trypsin and reseeded at 2–3 × 10^5 per 10-cm dish or 10^5 per 6-cm dish. Foci were observed at 14 days on live cells, 17 days–6 weeks on fixed cells. Cells were fixed with Formalin/phosphate-buffered saline and stained with cresyl violet acetate (1% aqueous). Transformed foci were usually picked at 3–4 weeks by using glass cloning cylinders with an inner diameter of 5.5

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Abbreviations: TSTA, tumor-specific transplantation antigen; FeSV, feline sarcoma virus.

Table 1. Transformation frequencies obtained by transfection with cellular DNA from the Meth A sarcoma, a Meth A transfectant, and a FeSV-transformed nonproducer mink cell line

	Foci per μg of donor DNA						
Exp.	MA-I	MA-II	MA-III	BMA-I	BMA-II	FeSV	3T3
1	0.15					0.23	0
2	0.11						+
3	0.09					0.06	+
4				0.03			+
5	0.11	0.05		0.07		0.13	0
6	0.20		0.21				0
7			0.62				0
8			0.50				0
9					0.08		0

MA-I, -II, and -III, three independent DNA preparations from the Meth A (a) cell line. BMA-I and -II, two DNA preparations from the Meth A transfectant designated BMA-II-7a. FeSV, DNA from 64F3 clone 7, a FeSV-transformed nonproducer mink cell line. 3T3, NIH/3T3 DNA was used in Exp. 1. One preparation of BALB/3T3 DNA was used in Exps. 2–9. Between 60 and 120 μg of each DNA was used in each experiment. The average number of foci obtained per experiment with the three Meth A DNA preparations was 19. The results of all experiments performed are shown. A + indicates that foci were observed. As discussed in the text, these are believed to be spontaneously arising foci.

mm. Subclones were selected in 0.3% agar plated on a 0.7% agar base layer.

TSTA Assays. Meth A TSTA is known to be distinct from TSTAs of all other syngeneic neoplasms assayed to date, including CI-4 and CII-10, two 3-methylcholanthrene-induced sarcomas of BALB/c (22), and mKSA, a simian virus 40-induced sarcoma of BALB/c (23). These three tumors were used in the present study as specificity controls.

Tissue culture-propagated transfectants and control cell lines were used to immunize BALB/c females 8–12 weeks of age. Usually 2–3 × 10^6 x-irradiated cells [10,000 roentgens (2.6 coulombs/kg)] were inoculated subcutaneously, three times, at weekly intervals, and challenge with 1–2 × 10^4 Meth A cells, or other appropriate control cells, was carried out 7 days after the last immunization. BALB/3T3 clone 7 cells were always included as controls in the immunization procedure. Mean tumor volumes and the immunogenic index were used to determine tumor inhibition and were calculated as described (11, 12). The mean tumor volume and immunogenic index were calculated at 20 days after challenge because all tumors grew progessively and killed the host in 25–35 days in the absence of induced immune protection.

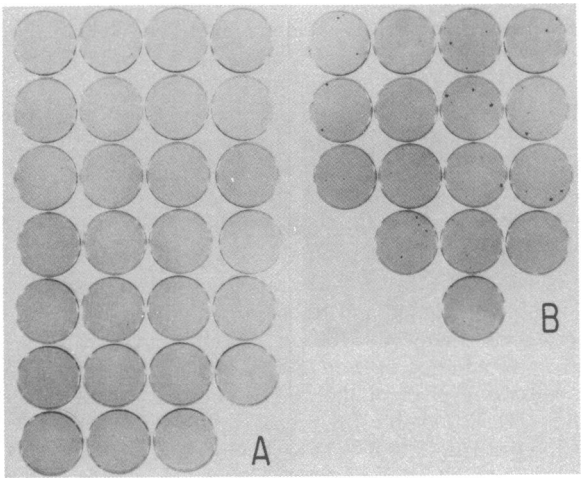

FIG. 1. Transformation of BALB/3T3 cells with DNA from the Meth A sarcoma. Sister 6-cm dishes seeded with BALB/3T3 cells were transfected with 20 μg each BALB/3T3 DNA (four dishes) or Meth A DNA (four dishes). Approximately 8 hr after transfection, each plate was trypsinized and the cells were reseeded at 10^5 per 6-cm dish. These plates were fixed and stained 17 days later. Vertical columns in the figure are descendants of each of four plates transfected with BALB/3T3 DNA (*A*) or Meth A DNA (*B*). Differences in the number of plates obtained from a single transfected plate presumably result from differences in the extent of cell killing during glycerol shocking of the transfected cells. The result shown is from transfection experiment 6 (MA III DNA, see Table 1).

RESULTS

Transformation of BALB/3T3 Cells by Cellular DNA from the Meth A Sarcoma and Biological Properties of the Transformants. DNA extracted from a cell line derived from the ascites form of the Meth A sarcoma was used to induce morphological transformation of BALB/3T3 cells by the calcium phosphate precipitation technique. The results of seven transfection experiments using three preparations of Meth A DNA are summarized in Table 1, and the results of one experiment (experiment no. 6, using MA III DNA, see Table 1) are shown in Fig. 1. Meth A DNA induced transformed foci at frequencies of 0.05–0.6 focus per μg. High molecular weight DNA from NIH/3T3 or BALB/3T3 cells failed to induce foci of transformation. However, it must be noted that in three of nine experiments foci appeared on BALB DNA-transfected control plates at frequencies of 0.01–0.1 focus per μg. In these cases,

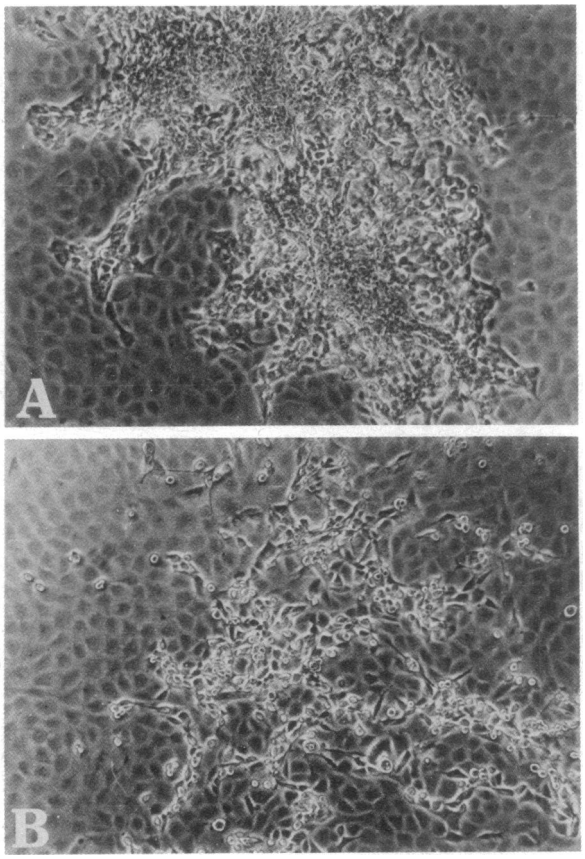

FIG. 2. Foci induced in BALB/3T3 cells by Meth A DNA and FeSV-transformed nonproducer mink cell DNA. Microscopic views of live cells. (*A*) Focus induced by Meth A DNA. This particular focus was cloned to yield the cell line designated BMA-5. (*B*) Focus induced by FeSV-transformed nonproducer mink cell DNA. (×40.)

Table 2. Immunogenic properties of Meth A-transfected sublines

Group	Immunization* Cells	Immunization* No.	Challenge Cells	Challenge No.	No. tumors/ no. challenged	Mean tumor volume, mm^3	Immunogenic index	Inhibition, %
I	BMA-11	$(2 \times 10^6) \times 2$	Meth A	10^4	4/8	10.6	10†	90
	BMA-12	$(2 \times 10^6) \times 2$	Meth A	10^4	8/8	100	1	None
	BALB/3T3 control	$(2 \times 10^6) \times 2$	Meth A	10^4	8/8	123.6	—	—
II	BMA-11-7a	$(2 \times 10^6) \times 3$	Meth A	10^4	3/7	10.5	13.5	> 90
	BALB/3T3 control	$(2 \times 10^6) \times 2$	Meth A	10^4	8/8	141.4	—	—
III	BMA-11	$(3 \times 10^6) \times 3$	mKSA	5×10^4	8/8	400	—	None
	None (controls)		mKSA	5×10^4	7/7	400	—	—
	BMA-11-7a	$(3 \times 10^6) \times 3$	CI-4	10^4	9/14	88.4	—	None
	None (controls)		CI-4	10^4	5/8	87.5	—	—
	CI-4‡	$(3 \times 10^6) \times 2$	BMA-11-7a	3×10^5	7/7	435.2	—	None
	None (controls)		BMA-11-7a	3×10^5	7/7	410	—	—
	CII-10‡	$(3 \times 10^6) \times 2$	BMA-11-7a	3×10^5	6/8	617.0	—	None
	CI-4	$(2 \times 10^6) \times 2$	BMA-11-7a	3×10^5	8/8	1010	—	None
	None (controls)		BMA-11-7a	3×10^5	8/8	1014	—	—

* BMA-11 and BMA-12 are primary transfectants induced with Meth A DNA. BMA-11-7a is a subclone of BMA-11. BMA-11-7a was established as a progressively growing sarcoma *in vivo* in syngeneic BALB/c mice and its immunogenicity and immunosensitivity were assayed at various transfers (see Table 3). Inhibition of Meth A sarcoma growth with BMA-11-7-a was significant at $P < 0.02$. The immunogenicities of two other subclones, BMA-11-5a and BMA-11-9a, were also assayed against Meth A challenge; BMA-11-5a was immunogenically comparable to BMA-11-7a, whereas BMA-11-9a did not immunize.

† Inhibition with BMA-11 was significant at $P < 0.02$. BMA-11-immunized mice did not resist challenge with 5×10^4 mKSA sarcoma cells.

‡ Complete inhibition of growth with these immunizations was achieved against 10^5 CI-4 tumor cell challenge and 10^4 CII-10 cell challenge, respectively.

however, sister plates that were not transfected at all also possessed foci, and thus we assume that these foci arose spontaneously. The structure of the spontaneous foci was variable but distinct from that of typical foci induced by Meth A DNA (Fig. 2). To induce foci on BALB/3T3 cells with a known transforming agent we used DNA extracted from 64F3 clone 7 cells, a nonproducer mink cell transformed by the replication-defective Gardner–Arnstein FeSV (19). A typical focus induced by this DNA is shown in Fig. 2. Three foci induced by the mink FeSV nonproducer cell DNA and an agar-selected subclone of one of them were tested for rescue of FeSV, and all yielded transforming virus after infection with Moloney leukemia virus.

A Meth A-induced transformant, BMA-11, that proved to be positive for the Meth A TSTA (see below) was subcloned in agar. Two preparations of DNA extracted from a subclone designated BMA-11-7a induced foci in BALB/3T3 at frequencies of 0.03–0.08 focus per μg in three experiments (Table 1). Morphologically these foci resembled those induced by Meth A DNA.

BALB/3T3 cells transformed by DNA from the Meth A sarcoma grew to high saturation densities. Six foci tested had plating efficiencies in 0.3% agar of approximately 100% relative to their plating efficiency on tissue culture dishes. BALB/3T3 cells failed to form colonies in agar (frequency $<10^{-3}$).

BALB/3T3 cells consistently failed to form tumors when 1–3 $\times 10^6$ cells were inoculated subcutaneously into adult BALB/c recipient mice. All Meth A-induced transformants tested were tumorigenic. Several transformants have been passaged *in vivo* by subcutaneous inoculation for more than 15 consecutive transfers. The phenotypic expression of Meth A TSTA in one of them, BMA-11-7a, is discussed below.

Presence of Meth A TSTA on BALB/3T3 Cells Transformed by Meth A DNA. Eight transformants induced by Meth A DNA and picked from experiment 1 (see Table 1), and several subclones of some of these transformants, were coded and sent to L.W.L. to be tested for the presence of Meth A TSTA. Negative controls, also coded, consisted of the BALB/3T3 cell line, foci arising in BALB/3T3 cells transfected with BALB/3T3 DNA and believed to be spontaneously arising foci (see above), and foci induced in BALB/3T3 by DNA from the FeSV-transformed cell line. The identity of the samples was revealed only after the results of the assays had been tabulated. Five of eight transformants induced by transfection with Meth A DNA were found to immunize mice against tumor growth by the Meth A sarcoma. Representative tumor growth inhibition data for TSTA-positive and -negative transfectants are shown in Table 2 and graphically in Fig. 3, and the results for all the transfectants tested are summarized in Table 4. That the protection against tumor induction

Table 3. Immunogenicity and immunosensitivity of the *in vivo* adapted transfectant, BMA-11-7a

In vivo passaged generation	Immunization*	Tumor challenge†	% of control tumor volume‡
4	Meth A	BMA-11-7a	7
5	BMA-11-7a§	Meth A	5
7	Meth A	BMA-11-7a	16
9	BMA-11-7a	BMA-11-7a	10
10	Meth A	BMA-11-7a	50
	BMA-11-7a	BMA-11-7a	<1
14	Meth A	BMA-11-7a	<1
	BMA-11-7a	BMA-11-7a	2
15	BMA-11-7a	Meth A	10

* Immunizations as follows: 2–3 $\times 10^6$ irradiated BMA-11-7a sarcoma cells were inoculated subcutaneously once a week for 3 weeks; 10^6 Meth A ascitic cells (irradiated) once a week for 2 weeks or immunization with soluble Meth A antigen followed by challenge of 10^4 Meth A cells.

† Tumor cell challenges: BMA-11-7a, 1–3 $\times 10^5$ cells (trypsinized from solid tumor) or 10^4 Meth A cells (ascites). Both tumor cell challenges represent an 80–100% lethal dose. Challenges were done 7 days after last immunization.

‡ Statistically significant tumor inhibition ($P < 0.01$) at 75% inhibition or greater of tumor growth.

§ Immunizations at 5th and 15th *in vivo* passages were done by inoculations of live tumor cells into the planar region of a hind footpad, followed by surgical excision of 10 × 20 mm tumors; tumor cell challenges were done 10–14 days after excision.

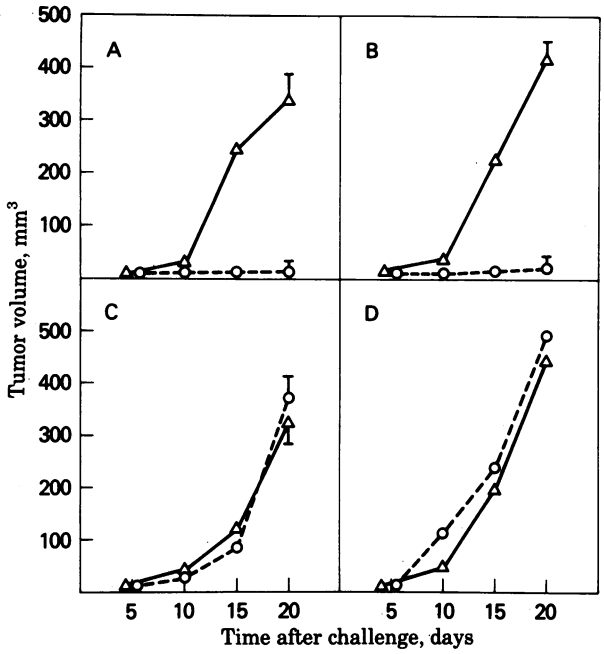

FIG. 3. Representative tumor growth inhibition studies. (*A*) BALB/c mice immunized with 10^6 irradiated Meth A cells (○) and nonimmunized control mice (△) challenged with 10^4 Meth A cells. (*B*) Mice immunized with BMA-11-7a tissue culture-propagated cells (irradiated with 10,000 roentgens) (○) and mice inoculated with BALB/3T3 clone 7 control cells (△), challenged with 10^4 Meth A cells. BMA-11-7a, a subclone of the primary transfectant BMA-11 induced with Meth A DNA, is positive for Meth A TSTA. (*C*) Mice inoculated with MF-12 tissue culture-propagated cells (irradiated with 10,000 roentgens) (○) and control mice inoculated with irradiated BALB/3T3 clone 7 cells (△), challenged with 10^4 Meth A cells. MF-12 is a line transformed by DNA from the FeSV-transformed nonproducer mink cell and is negative for Meth A TSTA. (*D*) Specificity control. Mice immunized with irradiated CII-10 sarcoma cells (○) and nonimmunized BALB/c mice (△) challenged with *in vivo* passaged BMA-11-7a cells. There were eight mice per group. Differences between immunized and control groups in *A* and *B*, $P < 0.01$. Bars at 20-day readings indicate SEM.

afforded by immunization with Meth A TSTA-positive transfectants was specific for the Meth A sarcoma is indicated by the fact that immunization with the Meth A transfectant designated BMA-11 and its subclone BMA-11-7a failed to protect mice against tumor challenge by other tumors syngeneic for BALB/c, including the CI-4 and CII-10 sarcomas, each having its own distinct TSTA, and MKSA, a simian virus 40-induced sarcoma (Table 2, group III). All are known from previous assays to be noncrossreactive with Meth A TSTA (22).

Because the transformants arising after transfection with Meth A DNA are tumorigenic it was possible to determine whether immunization of BALB/c mice with irradiated Meth A sarcoma cells protected them from the progressively growing sarcomas induced by Meth A transfectants. As shown in Table 3, immunization of BALB/c mice with Meth A sarcoma cells inhibited tumor growth by the transformant designated BMA-11-7a. Furthermore, the mutual cross-protection conferred between the Meth A sarcoma and the transfectant BMA-11-7a was stable after 15 passages *in vivo* of the BMA-11-7a line (Table 3).

Four of five secondary transformants induced in BALB/3T3 with DNA from the Meth A transformant designated BMA-11-7a were found to possess the Meth A TSTA (Table 4).

Table 4. Immunogenicity of primary and secondary transfectants against Meth A sarcoma

Clone	Donor DNA	Exp.*	Meth A TSTA†
Primary transfectants‡			
BMA-2	Meth A	1	–
BMA-3	Meth A	1	+
BMA-4	Meth A	1	–
BMA-5	Meth A	1	+
BMA-6	Meth A	1	+
BMA-6-9-a	Meth A		+
BMA-9	Meth A	1	+
BMA-9-1a	Meth A		–
BMA-11	Meth A	1	+
BMA-11-5a	Meth A		+
BMA-11-7a	Meth A		+
BMA-11-9a	Meth A		–
BMA-12	Meth A	1	–
Secondary transfectants§			
SBMA-41	BMA-11-7a	4	+
SBMA-61	BMA-11-7a	5	+
SBMA-62	BMA-11-7a	5	+
SBMA-64	BMA-11-7a	5	+
SBMA-66	BMA-11-7a	5	–
Negative controls¶			
BALB/3T3 control	(NIH/3T3)	1	–
B-3	(BALB/3T3)	2	–
B-10	(BALB/3T3)	3	–
B-11	(BALB/3T3)	3	–
MF-1	64F3 (FeSV)	1	–
MF-1-3a	64F3 (FeSV)		–
MF-3	64F3 (FeSV)	1	–
MF-12	64F3 (FeSV)	5	–

* Refers to the transfection experiment from which the indicated cell lines were derived (see Table 1). In Exp. 1, cells from two plates transfected with a total of 80 μg of Meth A DNA were pooled and reseeded at 2×10^5 on 25 10-cm dishes. Twelve foci appeared on 10 of the 25 plates.

† Mice were immunized subcutaneously with x-irradiated (10,000 roentgens) cells ($2–3 \times 10^6$ cells) three times and challenged with 10^4 Meth A sarcoma cells 7 days after the last immunization. Transfectants designated + gave at least 80% inhibition of Meth A tumor growth.

‡ Foci induced by Meth A DNA were picked and the resulting cell lines were designated BMA-. Subclones of these lines are given the name of the parent line followed by the subclone number and an "a" to indicate that the subclone was selected in agar.

§ Foci induced by BMA-11-7a DNA were cloned and the resulting cell lines were designated SBMA-. In transfection Exp. 5, six 6-cm dishes were transfected with 20 μg each of DNA from the primary transfectant BMA-11-7a. Each transfected plate was trypsinized separately and the cells were reseeded at 10^5 per 6-cm dish to yield a total of 38 dishes. Eight foci appeared on these plates. The four SBMA foci from Exp. 5 that were analyzed for Meth A TSTA were obtained from different transfected plates and therefore must have arisen independently.

¶ BALB/3T3 control: BALB/3T3 cells were transfected with NIH/3T3 DNA but no foci were observed. B-3, B-10, and B-11: foci arising on plates transfected with BALB/3T3 DNA. These are believed to be spontaneously arising foci (see text). MF-: MF-1, MF-3, and MF-12 all derived from foci induced by DNA from FeSV-transformed nonproducer mink cell. MF-1-3a is a subclone of MF-1 and was selected in agar.

DISCUSSION

Limited studies to determine the frequency of cotransfer of mammalian cell genes by DNA transfection have indicated that when one phenotype is selected, a second unselected marker will be present in the transfectants at a frequency of approximately 0.01–0.001 (13, 14). Thus the studies reported here, in which we selected for the transformed phenotype and found

that Meth A TSTA appeared on more than 50% of the transfectants arising in primary transfection with Meth A DNA and 80% (four of five) of the foci obtained from secondary rounds of transfection, would seem to indicate an intimate association between the genetic determinants of transformation and the unique TSTA of the Meth A sarcoma. These two phenotypes might be specified by the same gene, by very closely linked genes, or alternatively, a particular transforming gene might induce the expression of the Meth A TSTA. It must be noted, however, that these simple interpretations are all based on the assumption that cotransfer of mammalian cell genes by DNA transfection occurs at frequencies of 0.01–0.001. Given the very limited data available, we cannot exclude the possibility that certain genes can be cotransferred at much higher frequencies, in which case, the genetic determinants of transformation and TSTA in the Meth A sarcoma might have no relationship to one another.

The fact that not all Meth A transfectants possessed the Meth A TSTA may argue against the identity of the genes specifying the transformed phenotype and Meth A TSTA and argue in favor of the other alternatives. However, it also seems possible that variability in the expression of the antigen in different transfectants might render the TSTA undetectable in some clones, in which case the frequency of cotransfer might actually be higher than what we detected. Purification, possibly molecular cloning, of a determinant of transformation in the Meth A sarcoma would probably be necessary, although not necessarily sufficient, to determine if a transforming gene in this tumor also specifies the Meth A TSTA. A recent finding that Meth A DNA cleaved with appropriate restriction enzymes retains transforming activity and that this activity resides in a DNA fragment of a size appropriate for molecular cloning (unpublished results) suggests the feasibility of this approach.

In vivo, recognition of TSTAs is mediated by the cellular immune system, and in general it has proved difficult to elicit a serological response to a TSTA or to demonstrate that anti-tumor sera recognize the same determinants that elicit tumor rejection (5, 24). However, DeLeo *et al.* (15) succeeded in raising an antiserum that is specifically cytotoxic for the Meth A sarcoma, and recent evidence suggests that the serologically defined Meth A antigen (TSSA) and Meth A TSTA may be the same or closely related (25). So far it has not been possible to immunoprecipitate the Meth A TSSA. It should be noted that this antigen is thus distinct, by serological definition, from the p53 protein that is precipitated from many transformed cells by the serum that also specifically recognizes Meth A TSSA in a cytotoxic test (26). The Meth A transfectants described in this report have been assayed for the presence of the Meth A TSSA, and it appears that some possess the serologically defined Meth A antigen, although the number of positive clones is lower than the number positive for Meth A TSTA (unpublished results).

The simple interpretation of our findings, namely that genes specifying TSTAs may in some cases be intimately associated with or identical to transforming genes, seems surprising in light of recent findings suggesting that the number of genes responsible for oncogenic transformation may be quite small, whereas TSTAs are distinct for each tumor. Clearly it will be of great interest to determine whether the transformed phenotype and TSTAs cotransfer when DNA from carcinogen-induced murine tumors other than Meth A are used. Negative results in such studies might, however, be difficult to interpret. TSTAs are a functionally defined group of antigens, and many different types of molecules that might appear on the surface of tumor cells might serve as rejection antigens *in vivo*: for example, differentiation antigens, endogenous type C viral antigens, etc. Thus, one might not expect high-frequency cotransfer of the transformed phenotype and TSTA to be a general phenomenon even if transforming genes can frequently be associated with specific rejection antigens.

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