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Partial Expression of Endogenous Mouse Mammary Tumor Virus in Mammary Tumors Induced in BALB/c Mice by Chemical, Hormonal, and Physical Agents

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The possible interaction of environmental factors with the endogenous mouse mammary tumor virus (MMTV) genome in the development of mammary tumors in the low-tumor-incidence BALB/c mouse strain was examined. Tumors were induced in virgin female animals by treatment with chemical carcinogen 7,12-dimethylbenz[α]anthracene or urethan, with or without prolonged hormonal stimulation, or by X-irradiation. Concomitant hormonal stimulation resulted in increased tumor incidences compared with those induced by chemical carcinogen treatment alone. The frequency of tumor induction by irradiation alone or in combination with urethan or prolactin stimulation was very low. MMTV expression in the mammary tumors was assayed by nucleic acid hybridization and by immunohistochemical staining. Depending upon the treatment group, 0 to 89% of the tumors contained detectable levels of MMTV RNA ($\geq$0.0005% of the total cellular RNA). Tumors which contained detectable viral transcripts exhibited only low levels of MMTV RNA, which did not appear to represent the accumulation of RNA sequences homologous to the entire MMTV genome; synthesis of MMTV structural proteins was detected in only one tumor. Viral RNA-positive tumors were generally associated with a longer latent period. MMTV RNA expression occurred in tumors classified histologically as adenoacanthomas, as well as in mammary adenocarcinomas, although the cell types in the adenoacanthomas expressing viral RNA were not identified. It does not appear that expression of the endogenous MMTV genome is required for maintenance of all mammary tumors in BALB/c mice, although partial genome expression undetectable by the methods employed cannot be ruled out. Linear regression analyses were performed. The mean time to tumor appearance and the percentage of tumors which were MMTV RNA positive were found to vary linearly as a function of the total dose of 7,12-dimethylbenz[α]anthracene administered. The percentage of tumors which were MMTV RNA positive was also shown to be linearly related to the mean time to tumor appearance. These relationships provide a basis for predictions in the BALB/c system related to these parameters.

BALB/c mice exhibit a low incidence of spontaneous mammary cancer. However, mammary tumors can be induced in the strain by exposure to various agents, including milk-transmitted (exogenous) mouse mammary tumor virus (MMTV), chemical carcinogens, hormones, and X-irradiation (1, 19). Endogenous MMTV sequences, related to those of the exogenous virus, are carried in the BALB/c genome (12, 17, 23). Low levels of MMTV RNA have been detected in BALB/c lactating mammary glands, preneoplastic lesions, and mammary tumors induced by various factors (7, 8, 10, 12, 21, 22, 24, 29).

The role of endogenous MMTV in mammary tumorigenesis in BALB/c animals has not been established. Although it has previously been postulated that MMTV is involved in the formation of all mammary tumors in mice (2), recent studies have suggested that viral genome expression is not required for the maintenance of all mammary tumors (10, 15).

The present study was designed to examine the expression of endogenous MMTV in mammary tumors induced in BALB/c mice by various combinations of chemical, hormonal, and physical agents. Treatments were modified so that the effect of the tumor latency period on virus expression could also be analyzed. The results confirmed our previous observations that not all mammary tumors in BALB/c mice contain detectable levels of MMTV RNA. It was observed that virus expression was more com-

mon in tumors which arose after a longer latent period and that only a portion of the viral genome was expressed in those tumors.

MATERIALS AND METHODS

Viruses and antisera. (C3H)MMTV and (RIII) MMTV, as well as Moloney murine leukemia virus, were obtained from the Biological Carcinogenesis Branch, Division of Cancer Cause and Prevention, National Cancer Institute, as was avian myeloblastosis virus RNA-dependent DNA polymerase. Rabbit antisera directed against (C3H)MMTV [α-(C3H)MMTV] were kindly provided by Robert Cardiff, Larry Arthur, and the Biological Carcinogenesis Branch, Division of Cancer Cause and Prevention, National Cancer Institute; rabbit antiserum against (C3Hf)MMTV [α-(C3Hf)MMTV] was provided by Larry Arthur.

Mice. The BALB/cCrgl/Med mice were bred and maintained in a closed colony in the Department of Cell Biology, Baylor College of Medicine. Mice in this colony have a low mammary tumor incidence (≤1%) in both virgins and retired breeders (21). Experimental animals were housed four to six per cage in a temperature- and light-cycle- (14 h light, 10 h dark) controlled room, fed Wayne Lab Blox, and given water ad libitum.

Carcinogen treatments. 7,12-Dimethylbenz[α]-anthracene (DMBA) was obtained from Calbiochem (Los Angeles, Calif.), dissolved in cottonseed oil (1.0 mg/0.2 ml), and administered intragastrically weekly (1 mg/week), starting with virgin animals 8 weeks of age. The total dosages administered are indicated in the text and table footnotes. Urethan (J. T. Baker Chemical Co., Phillipsburg, N.J.) was dissolved in water (100 mg/ml) and administered intraperitoneally once a week (20 mg) for 10 weeks when virgin mice were between 8 and 17 weeks of age.

When prolonged hormonal stimulation was required, a single pituitary isograft was placed under the left kidney capsule, and it remained in place until the animal was sacrificed. Grafts were implanted when virgin animals were 6 weeks of age unless indicated otherwise in the text.

Tissue processing. Mice were examined biweekly for the development of tumors. The tumors were excised when approximately 0.5 to 1.0 cm in diameter, a segment from each tumor was removed for histological analysis, and the remainder was frozen on dry ice and stored at −70°C until utilized for RNA extraction. The tumor portions reserved for histology were fixed in Tellyesniczky fluid, embedded in Paraplast, and sectioned 4 to 5 μm thick. After staining with hematoxylin and eosin, sections were examined and tumor types were classified by the method of Dunn (9). A tumor was designated an adenoacanthoma if 25% or more of the cells exhibited evidence of keratinization at the light microscope level (13, 18). The remaining cells of the glandular elements resembled those seen in adenocarcinomas.

Acetone-insoluble powders were prepared from mouse livers, BALB/c mouse lactating mammary glands, and fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). The tissue was homogenized in 2 volumes of cold distilled water for 1 min by using a Waring blender, 4 volumes of cold acetone were added, the mixture was incubated on ice for 10 min, and the insoluble material was collected by centrifugation. The precipitate was suspended in saline, homogenized as described above, and recentrifuged. The saline washes were repeated until the supernatant remained clear. The sediment was then suspended in 4 volumes of cold acetone, homogenized, and incubated overnight at 4°C. The sediment was collected by filtration, using a Büchner funnel; each thin layer of sediment was washed repeatedly with acetone until whitish in appearance. The washed sediment was dried at 37°C, ground to a fine powder with a mortar and pestle, and stored at room temperature.

Immunohistochemical staining. Indirect immunological staining, developed by Sternberger (27), employing a peroxidase-antiperoxidase reagent was used to detect MMTV antigens in tissue sections. Details of the staining procedures, including modifications to reduce nonspecific reactivity, have been reported previously by us (14). All slides were read as unknowns by two or three investigators, using either a Zeiss KF2 or an Olympus BH microscope.

To establish the specificity of reactivity of these various MMTV antisera, a series of absorptions was performed. Absorption with virus consisted of mixing an equal volume of diluted antiserum with undiluted virus suspension followed by incubation for 30 min at 37°C. The virus was then removed by centrifugation (15 min, 30,000 lb/in^2; Beckman Airfuge). A similar protocol was followed for absorption with acetone-insoluble powders. Ten milligrams of acetone powder was used for each 100 μl of antiserum absorbed. The powder was first wet with Tris-buffered saline, suspended in antiserum, and incubated for 30 min at 37°C with frequent agitation. The powder was then removed by centrifugation, using either an Eppendorf table centrifuge (5 min) or a Sorvall RC-2B unit (15 min, 15,000 rpm).

Two of the antisera reacted with sections of lactating BALB/c mammary gland (negative for MMTV expression; 22); this reactivity (presumably against casein and other normal mammary cell proteins) was abolished by absorption with acetone-insoluble mouse lactating mammary gland powder (Table 1). Absorptions with mouse liver powder or mucin (Sigma Chemical Co., St. Louis, Mo.) were not successful at abolishing this contaminating reactivity. Subsequently, all antisera were routinely absorbed with mouse lactating mammary gland powder before use. Reactivities directed against viral proteins (in a BALB/cfC3H tumor) were removed by absorption with (C3H)MMTV but not by absorption with heterologous viruses, simian virus 40 and Moloney murine leukemia virus.

Nucleic acid hybridization. Viral RNA levels in tumors were quantitated by the titration hybridization method (30), using previously described procedures (7, 10, 21, 26). S1 nuclease-resistant DNA-RNA hybrids were assayed by adsorption to DEAE-cellulose filters (11) (all tumors induced by DMBA) or by trichloroacetic acid precipitation (all other tumors). A representative MMTV complementary DNA probe was synthesized by avian myeloblastosis virus RNA-dependent DNA polymerase , using heat-denatured, sodium acetate-extracted total MMTV RNA as the template and calf thymus DNA fragments as the random

TABLE 1. *Specificity of reactivity of MMTV antisera established by absorption experiments*

Antiserum[a]	Absorption antigen[b]	Reactivity by PAP test[c]	
		Lactating BALB/c mammary gland	Virus-positive BALB/cfC3H tumor
α-(C3H)MMTV (R.C.)	None	0	++
	LMGP	0	+
	MLP	0	+
	Mucin	0	+
	MuLV	ND	++
	(C3H)MMTV	ND	0
α-(C3H)MMTV (L.A.)	None	0	++
	LMGP	ND	++
	(C3H)MMTV	ND	0
α-(C3Hf)MMTV (L.A.)	None	+	++
	LMGP	0	++
	MLP	+	++
	Mucin	±	++
	MuLV + LMGP	ND	++
	(C3H)MMTV + LMGP	ND	±
	(C3Hf)MMTV	ND	0
	(RIII)MMTV (milk) + LMGP	ND	±
α-(C3H)MMTV (N.C.I.)	None	+	++
	LMGP	0	+
	MLP	++	++
	Mucin	++	++
	FBSP	ND	++
	SV40	ND	++
	MuLV	ND	++
	(C3H)MMTV	+	+
	(C3H)MMTV (NP-40 disrupted)	ND	+
	(RIII)MMTV (milk)	0	0
	(RIII)MMTV supernatant[d]	0	+
	(C3H)MMTV + (RIII)MMTV supernatant	0	0

[a] Rabbit antisera directed against (C3H)MMTV were kindly provided by Robert Cardiff (R.C.), Larry Arthur (L.A.), and the Biological Carcinogenesis Branch, Division of Cancer Cause and Prevention, National Cancer Institute (N.C.I.); antiserum against (C3Hf)MMTV was provided by Larry Arthur (L.A.).

[b] Absorption antigen abbreviations: LMGP, lactating mammary gland powder; MLP, mouse liver powder; FBSP, fetal bovine serum powder; SV40, simian virus 40; MuLV, Moloney murine leukemia virus. The (C3H)MMTV, (RIII)MMTV, and Moloney murine leukemia virus concentrates were obtained from the Biological Carcinogenesis Branch, National Cancer Institute; (C3Hf)MMTV was kindly provided by Larry Arthur. The RIII virus was obtained from milk; the other viruses were prepared from cells in tissue culture. NP-40, Nonidet P-40.

[c] Arbitrary scale to grade extent of peroxidase-antiperoxidase (PAP) reaction: 0, none; ±, trace; +, good; ++, very strong. ND, Not done.

[d] (RIII)MMTV supernatant was obtained by removal of virus from the preparation by centrifugation for 15 to 20 min in a Beckman Airfuge.

primer. The results of assays for MMTV RNA in the different tumor groups were not correlated with peroxidase-antiperoxidase determinations of MMTV antigen expression until the completion of the study.

RESULTS

Mammary tumorigenesis in BALB/c mice treated with chemical carcinogens, hormonal stimulation, or irradiation. The standard dose of DMBA used to induce mammary tumors in mice in the Baylor Mouse Colony is 6 mg. This dose resulted in a tumor incidence of 29%, with a mean latent period before tumor appearance of 5.5 months (Table 2). To induce tumors with longer latent periods, mice were treated with lower doses of DMBA

TABLE 2. *Effect of dose of carcinogen and of pituitary isografts on DMBA-induced mammary carcinogenesis in BALB/c mice*

Dose of DMBA (mg)[a]	Pituitary isograft[b]	No. of tumors/ no. of mice	% Tumors	Mean time of tumor appearance[c] (mo, range)	Mean age of mice dying without mammary tumors (mo, range)
2	None	15/59	25.0	9.6 (3.0–14.5)	14.7 (7.0–17.0)
4	None	20/57	35.0	7.2 (3.0–11.25)	10.9 (6.0–16.5)
6[d]	None	36/125	29.0	5.5 (3.0–9.5)	9.1 (6.0–13.5)
6[d]	4 weeks	29/38	76.3	3.2 (2.7–4.0)	4.0
6[d]	14 weeks	17/40	42.5	6.4 (3.0–9.5)	8.8 (7.0–12.0)

[a] Chemical carcinogen was administered weekly starting at 8 weeks of age at 1 mg per injection for 2, 4, or 6 weeks.
[b] Pituitary glands were implanted under the kidney capsule at either 4 or 14 weeks of host age.
[c] Time refers to months after the initial administration of DMBA.
[d] Data included for comparison purposes from other studies (10; S. Dusing-Swartz, J. S. Butel, D. Medina, and S. H. Socher, submitted for publication).

(2 or 4 mg per animal) (Table 2). The mean times of mammary tumor appearance and of host survival were inversely proportional to the total dose of DMBA administered. Because of the longer survival times, a significant number of mammary tumors which developed late in the DMBA-treated mice were available for analysis of MMTV RNA and protein expression.

Mammotropic hormonal stimulation is known to increase mammary tumor incidence in the BALB/c system (19). The effect of prolonged hormonal stimulation in concert with DMBA was examined by implanting pituitary glands under the kidney capsule of some of the animals treated with 6 mg of DMBA. Continuous prolactin stimulation enhanced DMBA-induced mammary tumorigenesis in BALB/c animals, with an increase in tumor incidence from 29 to 76% (Table 2). If the pituitary isograft was not implanted until after the total dose of DMBA had been administered (14 weeks), the hormonal enhancement of tumor incidence was markedly reduced (from 76% to 42%) even though the animals were examined for 12 months.

To investigate the effect of X-irradiation on mammary tumor incidence and MMTV expression in BALB/c mice, animals were exposed to 225 roentgens of whole body, unfractionated irradiation. Some irradiated animals were subsequently treated with DMBA, urethan, or pituitary isografts (Table 3). The percentage of animals developing tumors induced by these combinations was low (0 to 9%). The combination of irradiation plus DMBA was fatal; since all animals died by 6 months of age owing to a variety of disease states, this group is excluded from the table. The only mice to develop a significant incidence of mammary tumors were those exposed to a combination of urethan and a pituitary isograft (42%). A number of mammary adenocarcinomas from these various treatment groups were examined for the expression of MMTV RNA and viral protein.

TABLE 3. *Mammary tumorigenesis in irradiated or urethan-treated BALB/c female mice*

Treatment group	No. of tumors/ no. of mice	% Tumors	Mean time of tumor appearance[a] (mo, range)	Mean age of mice dying without tumors (mo)
Irradiation[b]	0/80	0	0	10.0
Urethan[c]	1/42	2.5	9.0	12.0
Pituitary isograft[d]	2/70	3.0	10.0	12.0
Irradiation + pituitary isograft	7/76	9.0	7.0	10.0
Irradiation + urethan	5/65	8.0	4.5	10.0
Urethan + pituitary isograft	24/56	42.0	8.2	12.0

[a] Time refers to months after initial treatment with carcinogen or pituitary isograft.
[b] Mice were exposed to 225 roentgens of whole-body, unfractionated irradiation.
[c] Mice were administered 20 mg of urethan intraperitoneally once a week for 10 weeks when the mice were between 8 and 17 weeks of age (total dose, 200 mg).
[d] Mice received a single pituitary isograft under the left kidney capsule at 6 weeks of age which remained in place until the animals were sacrificed.

MMTV expression in tumors induced by different environmental factors. We have previously reported the results of assays for MMTV expression in mammary tumors induced in BALB/c mice by treatment with 6 mg of DMBA (10). Mammary tumors which arose during the 54-week period after treatment with low doses of DMBA (Table 2) were assayed for the presence of MMTV RNA sequences (Table 4). Of the nine tumors assayed that arose in BALB/c animals treated with 2 mg of DMBA, one did not contain detectable levels of MMTV RNA (<0.0005% of the total cellular RNA), whereas the level of MMTV RNA in the other eight tumors ranged from 0.0005 to 0.0036% of the total RNA. Twelve tumors induced in BALB/c mice treated with 4 mg of DMBA were

TABLE 4. *MMTV expression in mammary tumors induced in BALB/c mice by low doses of DMBA*

Dose of DMBA[a] (mg)	Time of tumor biopsy[b] (wk)	Tumor histology[c]	MMTV RNA (% total RNA)	MMTV antigen[d]	
				α-(C3H)MMTV	α-(C3Hf)MMTV
2	13	AAC	<0.0005	ND	ND
	14	MAC	0.0008	–	–
	33	MAC	0.0009	–	–
	38	MAC	0.0022	–	–
	46	MAC	0.0011	–	–
	50	MAC	0.0036	–	–
	51	MAC	0.0009	ND	ND
	51	MAC	0.0011	–	–
	54	MAC	0.0005	–	–
4	15	AAC	0.0009	ND	ND
	15	MAC	<0.0005	–	–
	19	MAC	0.0023	–	–
	21	MAC	<0.0005	ND	ND
	26	MAC	0.0011	–	–
	28	MAC	0.0008	ND	ND
	33	MAC	<0.0005	ND	ND
	36	MAC	<0.0005	ND	ND
	36	MAC	0.0080	–	+
	36	MAC	<0.0005	ND	ND
	36	MAC	0.0007	–	–
	40	MAC	0.0031	–	–

[a] DMBA was administered intragastrically to virgin mice, as indicated in the text and in footnote *a* of Table 2, beginning at 8 weeks of age.

[b] Weeks after initial administration of DMBA when tumor was biopsied (1 to 2 weeks after tumor was first palpated).

[c] AAC, Adenoacanthoma; MAC, mammary adenocarcinoma.

[d] MMTV antigens were detected by immunohistochemical (peroxidase-antiperoxidase) tests, using antisera described in footnote *a* of Table 1. Similar results were obtained with all three antisera directed against (C3H)MMTV. ND, Not done.

assayed; low levels of MMTV RNA (0.0005 to 0.0080%) were detected in seven of the tumors, whereas five contained less than 0.0005% MMTV RNA (Table 4). The extent of hybridization did not exceed 25% in 17 of the 21 tumors tested in these two groups (Fig. 1); this may be a reflection of partial transcription of the MMTV genome. In the remaining four tumors, higher maximum levels of hybridization were detected (i.e., 45, 47, 82, and 93% in tumors containing 0.0009, 0.0011, 0.0080, and 0.0036% MMTV RNA, respectively) (Fig. 1). Interestingly, those four tumors which accumulated RNA homologous to a greater proportion of the MMTV genome developed after long latent periods (36 to 51 weeks after DMBA administration). In fact, three of the four tumors were induced by a low dose of DMBA (2 mg) and had latent periods of 50 to 51 weeks.

The synthesis of virus-specific proteins was monitored in 13 of the same DMBA-induced tumors by the peroxidase-antiperoxidase test (Table 4). We were unable [with one exception, using the α-(C3Hf)MMTV serum] to detect any MMTV proteins in the BALB/c tumors, although the same technique in our previous study (10) had readily detected MMTV antigens in BALB/cfC3H tumors. It is noteworthy that the BALB/c tumor positive for MMTV proteins contained viral RNA which hybridized to a level 82% of that obtained with the MMTV RNA control.

Mammary tumors which were induced in BALB/c mice by whole-body irradiation, in combination with urethan, DMBA, or prolonged hormonal stimulation, were similarly analyzed for the presence of viral RNA and antigens (Table 5). Although only a small number of tumors were assayed for virus expression in each of the treatment groups owing to the low tumor incidence, negligible or very low levels of MMTV RNA (i.e., <0.0020%) were detected. Virus-specific proteins were not detected in any of the 17 tumors examined (Table 5). These data suggest that BALB/c mammary tumors induced by a variety of environmental factors, in addition to DMBA, can be maintained without detectable expression of the endogenous MMTV genome.

Analysis of effect of DMBA dosage and time of tumor appearance on MMTV expression in BALB/c mammary tumors. The data accumulated from these studies were

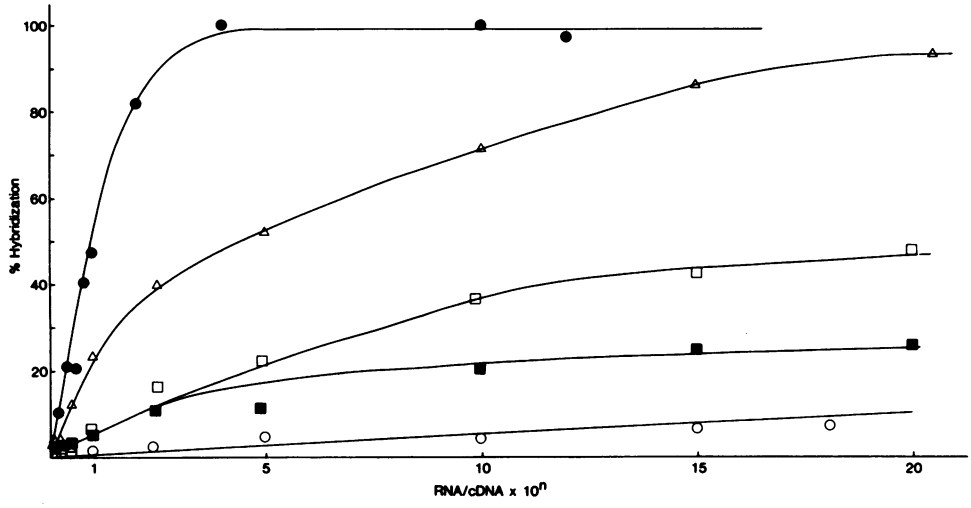

FIG. 1. *Representative hybridization curves obtained with MMTV complementary DNA (cDNA) and RNA extracts from tumors induced by 2 mg of DMBA in virgin BALB/c mice. Hybridization of MMTV complementary DNA with purified MMTV RNA (●), n = 10⁰, and with BALB/c tumor extracts containing <0.0005% MMTV RNA (○), 0.0011% MMTV RNA (■, □), and 0.0036% MMTV RNA (△), n = 10⁻⁴.*

TABLE 5. *MMTV expression in mammary tumors induced in BALB/c mice by irradiation or urethan treatment*[a]

Treatment group	Time of tumor biopsy (wk)	Tumor histology	MMTV RNA (% total RNA)	MMTV antigen	
				α-(C3H)MMTV	α-(C3Hf)MMTV
Irradiation + urethan	14	MAC	<0.0005	–	–
	14	MAC	0.0017	–	–
	23	MAC	<0.0005	–	–
	25	MAC	<0.0005	–	–
Irradiation + DMBA	14	MAC	<0.0005	–	–
	16	MAC	<0.0005	ND	ND
Irradiation + pituitary isograft	22	MAC	0.0018	–	–
	24	AAC	0.0007	ND	ND
	34	MAC	0.0006	–	–
	34	MAC	0.0006	–	–
	34	AAC	<0.0005	ND	ND
	36	MAC	0.0010	ND	ND
Urethan + pituitary isograft	22	AAC	0.0006	ND	ND
	28	MAC	0.0008	–	–
	32	MAC	· ND	–	–
	34	MAC	ND	–	–
	35	MAC	ND	–	–
	36	MAC	ND	–	–
	38	MAC	<0.0005	–	–
	38	MAC	ND	–	–
	43	MAC	ND	–	–
	43	MAC	<0.0005	–	–
	46	MAC	0.0010	ND	ND

[a] Tumors induced as described in the footnotes of Table 3. Other details and abbreviations are given in the footnotes of Tables 1 and 4.

analyzed to ascertain the effect of carcinogen treatment regimens on virus expression in mammary tumors induced in BALB/c mice. For these analyses, RNA levels of ≥0.0005% were used as positive indexes of MMTV expression.

Tumors induced with the lower doses of DMBA (Table 6, groups 2 and 3) more frequently contained detectable levels of MMTV RNA than did tumors induced by the standard high dose of DMBA (Table 6, group 1) (58% versus 89% versus 31%). The number of tumors negative for MMTV RNA decreased in direct proportion to decreasing DMBA dosage (69% versus 42% versus 11%). The differences in the percentages of MMTV-positive tumors between the groups receiving the low and high doses of DMBA were statistically significant (group 3 versus group 1, $P = 0.01$; groups 2 and 3 versus group 1, $P = 0.02$; Fisher's exact test [two-tailed]).

To further examine the observation that decreased levels of carcinogen resulted in increased expression of the endogenous viral genome in the tumors, the relationships among tumor type, tumor latency period, and virus expression were analyzed (Table 7). We found that 29% (2/7) of the adenoacanthomas contained viral RNA, compared with 53% (16/30) of the adenocarcinomas, demonstrating that MMTV expression is not restricted to mammary adenocarcinomas. It must be remembered, however, that there are many glandular elements in the adenoacanthomas which resemble those in mammary adenocarcinomas. We were not able to localize viral expression to a given cell type in the adenoacanthomas since none was positive in the peroxidase-antiperoxidase test; it may well have occurred in the glandular elements. Virus expression was more frequent in tumors which appeared after a long latent period (<30 weeks). The percentage of adenocarcinomas positive for MMTV RNA (≥0.0005%) increased from 31% (4/13) to 71% (12/17) with an increase in the length of the tumor latency period. This difference approached, but did not achieve, statistical significance ($P = 0.06$).

TABLE 6. *Effect of DMBA dose on the expression of MMTV in mammary tumors induced in BALB/c mice*

Group	Dose of DMBA[a] (mg)	Total no. of tumors	No. of tumors with MMTV RNA[b]	
			<0.0005%	≥0.0005%
1	6	16	11 (69)[c]	5 (31)
2	4	12	5 (42)	7 (58)
3	2	9	1 (11)	8 (89)

[a] Carcinogen administered as detailed in footnote *a* of Table 2.

[b] MMTV RNA levels calculated as the percentage of total cellular RNA. The MMTV RNA levels in tumors in group 1 were reported previously by us (10).

[c] Number in parentheses represents percentage of tumors in that group.

TABLE 7. *Relationship between tumor histopathology or tumor latency and MMTV expression in mammary tumors induced in BALB/c mice by DMBA*

Histopathology or latency	Total no. of tumors	No. tumors with MMTV RNA[a]	
		<0.0005%	≥0.0005%
Tumor histopathology			
MAC[b]	30	14 (47)[c]	16 (53)
AAC	7	5 (71)	2 (29)
Tumor latency period[d] (weeks)			
≤30	13	9 (69)	4 (31)
>30	17	5 (29)	12 (71)

[a] MMTV RNA level expressed as percentage of total cellular RNA.

[b] MAC, Mammary adenocarcinoma; AAC, adenoacanthoma. Tumors were classified histologically as described previously (13, 18). The adenoacanthomas contained glandular elements similar to those in adenocarcinomas. Since the viral RNA-positive adenocanthomas were negative for viral antigen expression, it was not possible to localize the expression of MMTV RNA to either the epidermoid (keratinized) or glandular elements of the tumors.

[c] Number in parentheses represents percentage of tumors in that group.

[d] These data include only the adenocarcinomas since that class of tumor developed randomly throughout the course of the experiments, whereas the majority of adenoacanthomas developed by 30 weeks after initial treatment (13; Medina et al., in press).

The correlations between (i) the total dose of DMBA and the mean time to tumor appearance (Fig. 2A), (ii) the total dose of DMBA and the percentage of tumors which were MMTV RNA positive (Fig. 2B), and (iii) the mean time to tumor appearance and the percentage of MMTV RNA-positive tumors (Fig. 2C) were plotted. The data from Tables 2 and 6 were plotted, and the lines were constructed by the methods of least squares. The correlations in each case were highly significant ($r \geq 0.99$, $P < 0.001$). The equations derived from these plots allow certain predictions to be made (see below).

DISCUSSION

The etiology of mammary tumors in inbred strains of mice is very complex, involving factors of genetics, viruses, hormones, chemicals, and irradiation. Low-tumor-incidence strains, such as BALB/c, do not carry an exogenous, milk-transmitted MMTV. However, a few copies of

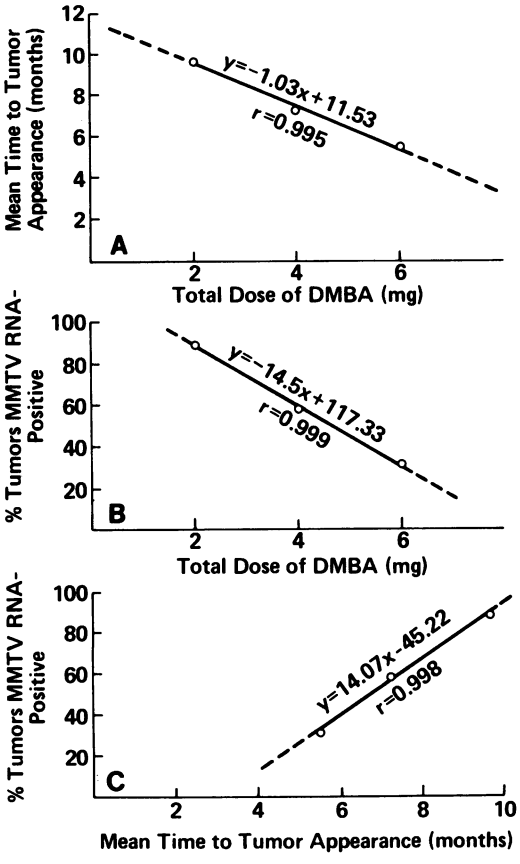

FIG. 2. *Linear regression analyses. (A) Effects of total dose of DMBA (milligrams) on mean time to tumor appearance (months); r = 0.995. (B) Effects of total dose of DMBA (milligrams) on percentage of tumors MMTV RNA positive; r = 0.999. (C) Relationship between mean time to tumor appearance (months) and percentage of tumors MMTV RNA positive; r = 0.998. r = Pearson product-moment correlation coefficient (26a).*

an endogenous MMTV genome are present in all cells in the mice in a proviral form (3, 5, 12, 17, 23). The endogenous sequences can be distinguished from sequences introduced by infection with exogenous MMTV by using restriction endonucleases (4). Physical maps constructed by Cohen et al. (3) revealed that the endogenous MMTV DNA in BALB/c mice is separated into three units located at different positions in the mouse genome, with two of the units (II and III) closely resembling complete proviruses acquired by infection and one unit (I) representing only a portion of the MMTV genome.

Both spontaneous tumors in BALB/c mice and those induced by hormonal, chemical, or physical agents fail to show an increase in the number of MMTV DNA copies per cell, as is observed in mammary tumors induced by exogenous MMTV (4, 12, 15, 16). BALB/c tumors also do not contain the unique "tumor-associated" MMTV sequences found in early-appearing mammary tumors in high-tumor-incidence mouse strains infected with exogenous virus (6). However, this question needs to be reexamined using restriction enzyme analysis of the tumors.

The study reported here addressed the possible interaction of environmental factors with the endogenous MMTV genome in the development of mammary tumors in BALB/c mice. We observed that not all tumors induced by DMBA contained detectable levels of MMTV RNA. Neither was viral RNA transcription apparent in all tumors induced by X-irradiation together with chemical carcinogen treatment or hormonal stimulation. Those tumors which did contain detectable amounts of viral transcripts contained them in only low levels. These results confirm our earlier observations on DMBA-induced tumors in BALB/c animals (10) and are in general agreement with those described by Michalides et al. (15).

The most obvious conclusion that one might be tempted to draw from these results is that the endogenous MMTV is not involved in mammary tumorigenesis in BALB/c mice. However, the experimental approaches employed do not permit such a generalization. Clearly, mature MMTV virions are not produced in detectable quantities in BALB/c tumors; transcription which does occur rarely represents sequences homologous to the entire exogenous (C3H) MMTV genome, confirming our own (7, 8, 10, 21) and others' (15, 16) observations. However, specific integrated viral DNA sequences (e.g., a viral promotor) might be altering the expression of closely associated cellular genes, thereby modifying the growth behavior of the cells. There is also the possibility that a complementary DNA probe made from exogenous viral RNA does not contain sequences homologous to all endogenous virus sequences; transcription in tumors of such nonrepresented sequences would, of course, go undetected. Although Cohen et al. (3) demonstrated appreciable differences in the locations of restriction sites within the genomes of endogenous and exogenous MMTV, those authors concluded that the complementary DNA probe made from milk-borne MMTV was also to detect all restriction fragments generated from the endogenous virus. It would be very informative to know which regions of the endogenous genome are represented in the partial transcripts detected in some BALB/c tumors and which of the "units" described by Cohen et al. (3) are

being transcribed. Dudley et al. (8) noted a preferential expression of MMTV polyadenylate-adjacent sequences in several continuous BALB/c tumor cell lines. Perhaps the subgenomic unit I is derived from the 3′ end of the MMTV genome and is expressed while the two complete units II and III are silent.

It was striking that, with a single exception, no MMTV-specific structural proteins were detected by peroxidase-antiperoxidase immunological staining tests in the virus RNA-possible BALB/c tumors. With the same reagents, virus antigens were readily detectable in many BALB/cfC3H tumors containing comparable levels of viral RNA (10). Michalides et al. (15) obtained similar results in their analysis of BALB/c mammary tumors. Several interpretations of these data are possible. The MMTV RNA detected in the BALB/c tumors may be nonfunctional and not translated in the cells. The existence and nature of such a putative translational block in the tumor cells remain speculative at this time. Alternatively, the RNA may represent noncoding sequences contained in the endogenous viral genome, perhaps possessing regulatory capacities. Finally, the gene product(s) may be synthesized which does not cross-react antigenically with the structural proteins of C3H and C3Hf MMTVs; the antisera currently available are directed against those proteins. A putative transforming or regulatory virus-coded protein would fall into this category. Further research is required to distinguish among these possibilities. It might be noted that noncoordinate expression of MMTV proteins p28 and gp52 in BALB/c and Swiss albino mice has been described (28), so there are no theoretical grounds precluding partial genome expression at the protein level.

An important association was elucidated in this study between detectable MMTV transcription and late appearance of mammary tumors in BALB/c animals. No definitive explanation for this association can be provided at this time. However, one possible suggestion is that different cell types are being affected by the carcinogens. Variability in virus expression might be a reflection of different cell types being transformed initially and proliferating with different rates of growth. Other studies (25; D. Medina, S. H. Socher, G. H. Smith, S. Dusing-Swartz, L. O. Arthur, and J. S. Butel, *in* P. Furmanski and M. Rich, ed., *Biological Carcinogenesis*, in press) have led to the speculation that chemical carcinogens and exogenous MMTV may be affecting different cell populations in the murine mammary gland. A profound need exists for markers to reliably distinguish among various cell types in the intact mammary gland and to identify the origin of the malignant cells recovered from mammary tumors. It is also possible that the appearance of MMTV RNA in the late-appearing tumors is merely the result of relaxation of gene repression reported to occur with increased host age. Increased expression of globin and endogenous murine leukemia virus RNA has been detected in the brains and livers of old mice (21).

The data plots in Fig. 2 illustrate the highly significant linear relationships detected in this study between the total dose of DMBA administered, the mean time to tumor appearance, and the percentage of tumors positive for MMTV RNA. The equations were derived by the least-squares method, and correlation coefficients were calculated by Pearson's product-moment method (26a). The equations allow predictions to be made which will be useful in designing future experiments studying mammary tumorigenesis in BALB/c mice. For example, it can be predicted that a total dose of 3 mg DMBA would induce tumors with a mean latent period of 8.4 months and that 74% of the tumors would express detectable levels of MMTV RNA.

This study has not provided any convincing evidence for a role of endogenous MMTV expression in the maintenance of mammary tumors in BALB/c mice. However, given the limitations of technology and understanding of the system, a role of the viral genome in the formation of at least some tumors has not been excluded. It should be noted that the current study did not address the possible importance of transient MMTV expression during tumor induction. The study has served to raise new questions and define new avenues for future investigation in the complex area of mammary tumorigenesis and has validated the BALB/c mouse strain as being amenable to manipulation of the multiple etiological components of the system.

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