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Short- and Long-term Studies on Chemical Carcinogenesis in BALB/Mo Mice*

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ABSTRACT

To study the interactions between chemical carcinogens and oncogenic retroviruses, BALB/Mo mice which carry the Moloney murine leukemia virus (M-MuLV) as an endogenous virus, and conventional (M-MuLV-free) BALB/c mice, as well as their Bc1 (M-MuLV+ or M-MuLV−) hybrids were injected neonatally with a single dose of urethane. BALB/Mo and V+ Bc1 mice showed accelerated lymphoma development; similar results were obtained in BALB/Mo mice receiving one or two doses of urethane transplacentally. Lung adenomas developed with shorter latency and higher incidence in BALB/Mo mice given urethane at birth; however, significant differences in the incidence of lung adenomas in BALB/Mo mice were found only in two experiments.

Additional short-term experiments were carried out to investigate the mechanism of the higher susceptibility to sister chromatid exchange induction observed in BALB/Mo lymphocytes. It was found that BALB/Mo spleen lymphocytes incubated with cordycepin, an antiviral antibiotic, with or without mitomycin C treatment, showed reduction in both M-MuLV synthesis and sister chromatid exchange frequency, and the latter values were similar to those seen in control cultures.

These data suggest that the integration of M-MuLV proviral DNA into the host genome is *per se* not sufficient to increase the susceptibility to carcinogenic stimuli, but that other events, such as viral gene expression and amplification, are most likely required for the chemical-viral synergistic effect to occur.

INTRODUCTION

Despite extensive investigation there is still no clear indication of the mechanisms responsible for the synergistic effect usually observed between viruses and chemical carcinogens in tumor induction.

We have been studying interactions between Moloney murine leukemia virus (M-MuLV) and different chemical compounds in BALB/Mo mice which carry the M-MuLV proviral sequences integrated at a single locus (Mov-1) into the germ line and, consequently, in every somatic cell. Following viral gene expression and amplification within target tissues, BALB/Mo mice develop lymphomas, mostly derived from T lymphocytes (13). The advantages offered by this system are the following: (a) the animals are virus-infected without manipulation; (b) although devoid of a transforming gene, M-MuLV is oncogenic *per se* and, at least before its amplification, may be considered as a pure cloned virus; (c) appropriate M-MuLV-free controls with similar genetic background, e.g., BALB/c mice, are readily available.

Using the sister chromatid exchange (SCE) assay we found that BALB/Mo lymphocytes, compared to BALB/c controls, show a higher base line frequency of SCE and are far more susceptible to SCE induction by mitomycin C, urethane, and potassium dichromate (5, 16, 17). In addition, the clastogenic effect

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induced by urethane evaluated by the micronucleus assay on bone marrow polychromatic erythrocytes is more pronounced in BALB/Mo than in BALB/c cells (1).

In this paper we report long-term results in BALB/Mo and BALB/c mice treated with urethane at perinatal age. Furthermore, preliminary data on the effects of a viral inhibitor on SCE induction are presented.

MATERIALS AND METHODS

BALB/Mo mice were originally obtained through the courtesy of Dr. R. Jaenisch, of the Heinrich-Pette Institute for Experimental Virology and Immunology, University of Hamburg, FRG. Control BALB/c mice were originally purchased from the Jackson Laboratory, Bar Harbor, MN. The animals were maintained by brother x sister inbreeding in plastic cages in temperature-controlled rooms (21–24°C) and fed a standard mouse diet (Piccioni, Brescia) with free access to water.

Urethane Carcinogenesis

Neonatal Treatment. Newborn mice, less than 48 hr old, were injected subcutaneously in the interscapular region with a single dose of 1 mg of urethane (Carlo Erba, Milano) in 0.10 ml of physiologic saline. They were killed at 2, 4, 6, and 8 months of age.

Transplacental Treatment. Pregnant females were injected s.c. in the inguinal region with one or two doses of 35 mg of urethane in 0.35 ml of physiologic saline (approximately 1 mg/g of body weight) on day 3 or days 3 and 1, respectively, before delivery; offspring were killed at 2, 4, 6, and 8 months of age. Older age groups were not studied because of the high incidence of deaths due to "spontaneous" lymphomas which occur in untreated BALB/Mo mice (see also Fig. 1). Some mice receiving neonatal or transplacental urethane treatment died spontaneously or were killed when moribund between death intervals; these were assigned to the nearest experimental group according to the time of death.

All animals underwent complete autopsy. Tumors and all lesions that were suspect for neoplasia were excised and processed for histologic examination. The number of pulmonary tumors was determined by counting surface nodules detected on gross inspection of the lungs. All lung tumors in urethane-treated animals were classified histologically as adenomas (alveologenic carcinoma according to Stewart et al (22)). Lymphomas in untreated and experimental animals were, in the great majority of cases, thymic lymphosarcomas with or without involvement of spleen, lymph nodes, and other organs (9). For statistical analysis, the association χ square was calculated following Cochran's method as described by Fleiss (12).

Short-term Studies on SCE Induction

Cell suspensions obtained from 2-month-old BALB/Mo and BALB/c donors were prepared as already reported (17), and incubated for 68 hr at 37°C in a 5% CO_2 atmosphere. Concanavalin A (5 μg/ml, Con A, Pharmacia, Uppsala, Sweden), and bromodeoxyuridine (10 μg/ml, BUdR, Sigma, St. Louis, MO) were added to the cultures; cordycepin (10 μg/ml, Sigma, St. Louis, MO) and/or mitomycin C (10^{-7} M, MMC, Sigma, St. Louis, MO) were added to the cultures; cordycepin (10 μg/ml, Sigma, St. Louis, MO) and/or mitomycin C (10^{-7} M, MMC, Sigma, St. Louis, MO) were also added to experimental cultures. In the final 4 hr, colchicine (0.4 mg/ml, Merck, Darmstad, FRG) was added, and SCE were detected on chromosome preparations obtained from 2nd generation metaphase cells by staining with Giemsa following the procedures already described (17).

Virus production was determined on end culture supernatants by the UV/XC cell plaque assay (21) as reported in Ref. 7. For statistical analysis, the classical χ square test was used (12).

RESULTS

Long-term studies on Urethane-induced Carcinogenesis

Neonatal Treatment. The results of the first experiments are summarized in Table I. Since no sex differences were observed in these as well as in the other experiments, data concerning male and female animals have been pooled. Urethane injection in newborn BALB/Mo and BALB/c mice induced lung adenomas and lymphomas with an incidence that increased progressively with the age at death. A total of 34 and 15 out of 40 BALB/Mo mice developed lung adenomas and lymphomas, respectively. In the control group, a total of 18 and 4 out of 31 BALB/c mice had lung adenomas and lymphomas, respectively. Thus, the final incidence of both types of tumor was significantly higher ($p <$

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TABLE I—Development of Lung Adenomas and Lymphomas in BALB/Mo and BALB/c Mice Neonatally Injected with Urethane[a]

Strain	Age	No. of Mice Examined	No. of Mice with Lung Adenomas[b]		No. of Mice with Lymphomas	
	mo			%		%
BALB/Mo	2	5	2 (1.0)	40	0	0
	4	11	8 (1.9)	73	3	27
	6	12	12 (2.4)	100	4	33
	8	12	12 (2.6)	100	8	66
Total		40	34	85	15	37
BALB/c	2	5	0	0	0	0
	4	10	6 (2.1)	60	0	0
	6	10	7 (1.7)	70	1	10
	8	6	5 (2.6)	83	3	50
Total		31	18	58	4	13

[a] Mice were injected s.c. with 1 mg of urethane in 0.10 ml of saline within 48 hr of birth.
[b] The mean number of lung adenomas per mouse is given in parentheses.

0.05), and the time of tumor appearance shorter in BALB/Mo than in BALB/c mice. On the other hand, the mean number of lung adenomas per mouse was almost similar in mice of both strains. No other neoplastic lesion was detected either in experimental or control groups.

As already stated, untreated BALB/Mo mice develop lymphomas in high incidence, reaching 70% at 12 months of age; however, the latent period of lymphoma appearance is prolonged, as shown by the cumulative incidence (Fig. 1). Therefore, urethane treatment is effective in considerably accelerating lymphoma development. It should also be added that the incidence of lung adenomas in BALB/Mo and BALB/c mice and of lymphomas in BALB/c untreated mice is less than 1% at the age of 10 months.

In order to further confirm the possible influence of M-MuLV on urethane carcinogenesis, 1st generation back cross (Bc1) mice were produced by mating BALB/c females with (BALB/c × BALB/Mo) F1 males. Since the parental BALB/Mo mice are homozygous regarding the Mov-1 locus (integrated M-MuLV proviral DNA), 50% of Bc1 hybrids should segregate for M-MuLV+ according to Mendelian expectations. Bc1 mice of the same litters were then injected s.c. with 1 mg of urethane when less than 48 hr old. At weaning (4 to 5 weeks of age), the mice were typed for M-MuLV positivity by tail biopsy using the UV/XC cell plaque assay. They were then killed at 4, 6, and 8 months of age. Table II presents the results of this experiment. The final percentage of lung adenomas was 87% in V+, and 63%in V− Bc1 mice; this difference is not statistically significant ($p > 0.10$). On the other hand, the total incidence of lymphomas was significantly higher in V+ (13 out of 23 mice, 57%) than in V− Bc1 mice (4 out of 19, 21%; $p < 0.05$). Moreover, as in the previous experiment, an accelerated appearance of lymphomas in V+ mice was evident when latency was compared to that seen in untreated V+ Bc1 hybrids. Thus, even in a stringently similar genetic host background, M-MuLV appears to facilitate lymphoma development following urethane treatment.

Transplacental Treatment. As shown in Table III, urethane administered transplacentally in BALB/Mo and BALB/c mice produced a tumor pattern essentially similar to that found in previous experiments, and only lung adenomas and lymphomas were detected during the 8-month observation period; tumorigenic activity was moderately less efficient than that observed following neonatal injection, even in offspring of mothers receiving 2 doses of urethane. Again, the incidence of lung adenomas was higher in BALB/Mo than in BALB/c mice at both dose levels, but the difference was significant only in offspring of mothers treated twice ($p < 0.05$).

Lymphomas were not found in BALB/c mice, whereas their total incidence in BALB/Mo mice was 18% ($p < 0.005$) and 42% ($p < 0.001$) in animals receiving one or two ure-

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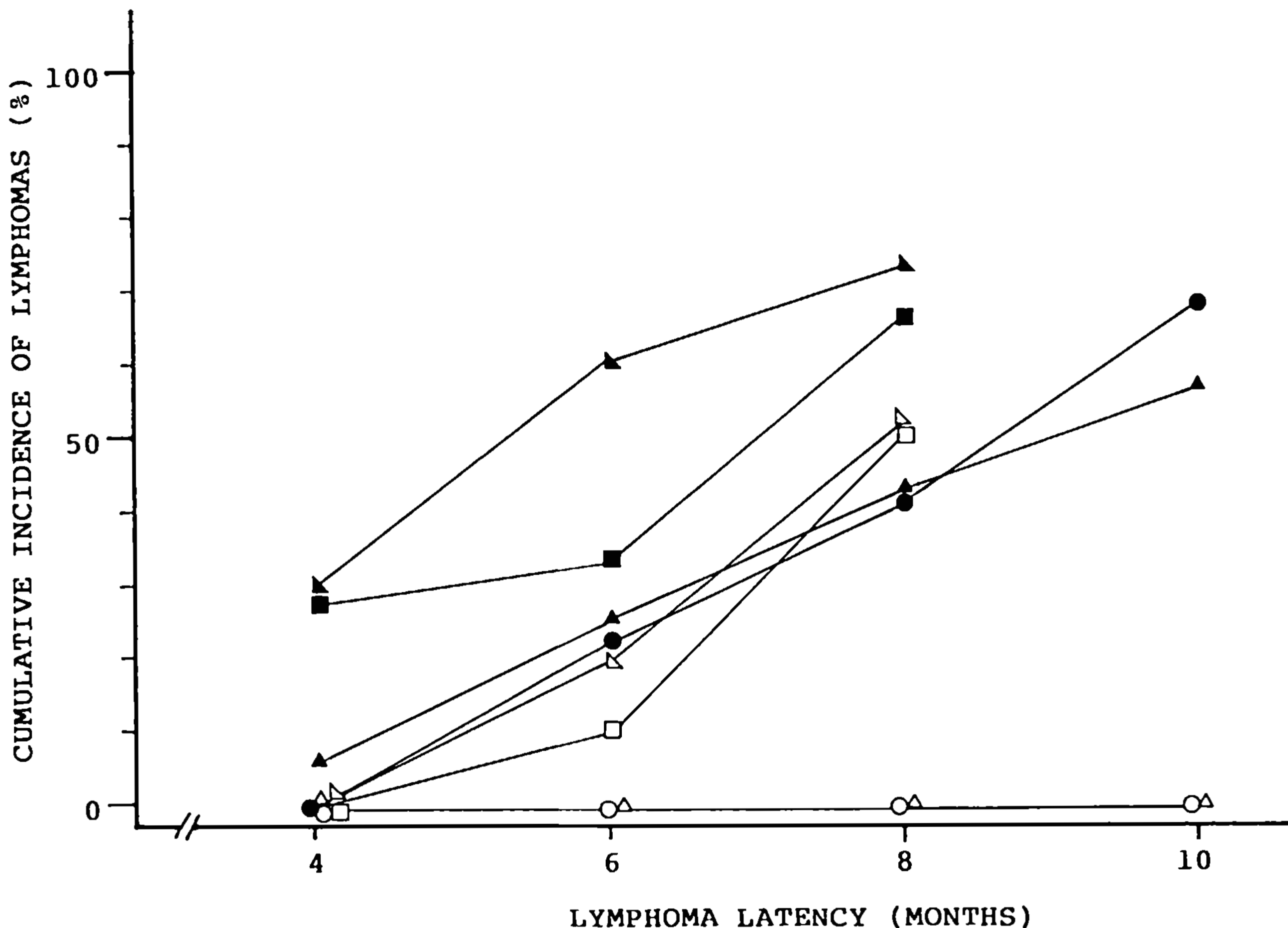

FIG. 1—Incidence of spontaneous and urethane-induced lymphomas in BALB/Mo, BALB/c, and Bc1 mice. ●, BALB/Mo untreated; ○, BALB/c untreated; ▲, V+ Bc1 untreated; △, V− Bc1 untreated; ■, BALB/Mo urethane-treated; □, BALB/c urethane-treated; ◣, V+ Bc1 urethane-treated; ◺, V− Bc1 urethane-treated. Treatment was injection s.c. with 1 mg of urethane in 0.1 ml of saline within 48 hr of birth.

TABLE II—Development of Lung Adenomas and Lymphomas in ♀ BALB/c × ♂ (♀ BALB/c × ♂ BALB/Mo) F1 Mice Neonatally Injected with Urethane[a]

Phenotype of Injected Mice	Age	No. of Mice Examined	No. of Mice with Lung Adenomas[b]		No. of Mice with Lymphomas	
	mo			%		%
V+	4	7	4 (2.0)	57	2	29
(+/−)	6	5	5 (2.4)	100	3	60
	8	11	11 (3.0)	100	8	73
Total		23	20	87	13	57
V−	4	8	3 (1.0)	38	0	0
(−/−)	6	5	4 (2.4)	80	1	20
	8	6	5 (2.1)	83	3	50
Total		19	12	63	4	21

[a] Mice were injected s.c. with 1 mg of urethane in 0.10 ml of saline within 48 hr of birth.
[b] The mean number of lung adenomas per mouse is given in parentheses.

thane doses, respectively. In addition, the cumulative lymphoma incidence observed in both groups of the BALB/Mo mice indicates an accelerating effect when data (not shown in Fig. 1) are compared with those of untreated BALB/Mo controls.

In conclusion, it is evident that perinatal treatment with urethane increases the sus-

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TABLE III—Development of Lung Adenomas and Lymphomas in BALB/Mo and BALB/c Mice Given Urethane Transplacentally

Urethane Treatment to Pregnant Mothers[a] and Strain	Age	No. of Mice Examined	No. of Mice with Lung Adenomas[b]		No. of Mice with Lymphomas	
	mo			%		%
Once (Day − 1)						
BALB/Mo	2	12	1 (1.0)	8	0	0
	4	11	3 (1.7)	27	2	18
	6	14	7 (2.0)	50	5	36
	8	7	6 (2.2)	86	3	43
Total		44	17	39	8	18
BALB/c	2	7	0	0	0	0
	4	10	2 (1.0)	20	0	0
	6	10	3 (1.3)	30	0	0
	8	7	3 (2.0)	43	0	0
Total		34	8	24	0	0
Twice (Days −3 and −1)						
BALB/Mo	2	5	1 (1.0)	20	0	0
	4	6	4 (2.1)	66	2	33
	6	7	6 (2.4)	86	4	57
	8	6	6 (2.8)	100	4	66
Total		24	17	71	10	42
BALB/c	2	6	0	0	0	0
	4	7	2 (1.2)	29	0	0
	6	7	4 (1.7)	57	0	0
	8	6	5 (2.0)	83	0	0
Total		26	11	42	0	0

[a] The mothers were injected s.c. with one dose (35 mg in 0.35 ml of saline) or two doses on day −1 or −3 and −1, respectively, from delivery.
[b] The mean number of lung adenomas per mouse is given in parentheses.

TABLE IV—Effect of *in Vitro* Treatment with BUdR and Cordycepin on Virus Yield from Con A-stimulated Splenic T Lymphocytes of BALB/Mo Mice

Treatment	Yield[a]	Reduction
	pfu/ml	%
BALB/Mo	357.14	
BALB/Mo + Cordycepin	314.29	12.0
BALB/Mo + BUdR	428.57	
BALB/Mo + BUdR + Cordycepin	237.14	44.7

[a] Virus yield evaluated by the UV/XC cell assay on supernatants recovered from 1×10^6 cells after 68 hr in culture with or without cordycepin (10 μg/ml) and BUdR (3×10^{-5} M). pfu, plaque-forming unit.

ceptibility of BALB/Mo mice to lymphoma development, while clear-cut results were not obtained concerning the possibly higher sensitivity of BALB/Mo mice to lung tumor development.

Short-term Studies on SCE Induction

Previous studies demonstrated that spleen lymphocytes obtained from young adult or infant BALB/Mo mice are very susceptible to SCE induction either "spontaneously" or after treatment with carcinogenic compounds (5, 16, 17) (see also Table V). However, since the BUdR treatment necessary to visualize SCE also causes a ready inducibility of retroviruses (14), it remained to be determined whether the integration of M-MuLV *per se*, or its expression, was responsible for SCE increase in M-MuLV-infected lymphocytes. Consequently, experiments were programmed to study the effects of substances known to inhibit the various steps of retrovirus life cycle. Cordycepin (3-deoxyadenosine), which blocks RNA maturation and viral assembly (20), was first investigated. In fact, as reported in Table IV, supernatants of Con A-stimulated BALB/Mo lymphocytes cultured in the presence of cordycepin contained less virus, and the reduction in virus titer (44.7%) was more pronounced in BUdR-treated cultures. The effect of cordycepin on SCE frequency was then assayed. Table V shows that while cordycepin alone did not affect the number of SCE in BALB/c lympho-

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TABLE V—Effect of Cordycepin on SCE Frequency in T Lymphocytes of BALB/c and BALB/Mo Mice Treated *in Vitro* with Mitomycin C

Spleen Cell Donors	Treatment	Total No. of Metaphases	No. of SCE/Methaphase[a]	p
BALB/c	None	180	8.06 ± 0.19	
BALB/Mo	None	180	11.46 ± 0.20	<0.001[b]
BALB/c	Cordycepin, 10 μg/ml	200	8.18 ± 0.22	
BALB/Mo	Cordycepin, 10 μg/ml	200	8.25 ± 0.21	>0.5[b], <0.001[c]
BALB/c	MMC, 10^{-7} M	100	50.19 ± 1.12	
BALB/Mo	MMC, 10^{-7} M	100	74.33 ± 1.58	<0.001
BALB/c	MMC, 10^{-7} M, + Cordycepin	100	51.87 ± 1.20	
BALB/Mo	MMC, 10^{-7} M, + Cordycepin	100	50.30 ± 1.60	>0.6[b], <0.001[c]

[a] Mean ± standard error.
[b] Refer to similarly treated BALB/c lymphocytes.
[c] Refer to respective BALB/Mo lymphocytes not treated with cordycepin.

cytes, it decreased the BALB/Mo SCE frequency to base line BALB/c values. Moreover, when a potent mutagenic chemical, such as MMC, was added, cordycepin treatment again lowered the SCE frequency of BALB/Mo lymphocytes to the levels observed in BALB/c cultures. Thus, inhibition of virus synthesis by cordycepin led to a "normalization" of the SCE frequency in M-MuLV-infected BALB/Mo lymphocytes.

DISCUSSION

It has long been known that fetal and neonatal periods of life are highly susceptible to chemical carcinogenesis, and indeed theoretical and practical proposals to incorporate perinatal exposure into routine toxicology assays for carcinogen detection have been presented (6). The main reasons for this particular sensitivity are (a) the enzymatic activity, which becomes fully expressed only a few days after parturition, may slow down the metabolic inactivation of carcinogens, (b) the high number of structurally and functionally immature cells may provide an extra amount of critical targets for carcinogenic stimuli, and (c) the still poorly developed immune system may facilitate tumor development and progression through an inefficient surveillance mechanisms.

Urethane was one of the first chemicals to undergo extensive investigation for its transplacental and neonatal carcinogenic activity, and an increased tumorigenic response was observed, especially for lung, liver, skin, and lymphoid organs, (3, 8, 10). It was also demonstrated that urethane injected into newborn mice is elminated at only a tenth of the adult rate and that the long retention time is due to the lack of liver esterase which metabolize urethane to CO_2 (18). Moreover, the thymus and other lymphopoietic organs are dramatically affected by urethane treatment (11) as are different parameters of immune function (15, 19).

In the present studies, urethane perinatal carcinogenesis was utilized to investigate interactions between chemical carcinogens and retroviruses. The slow transforming leukemogenic M-MuLV, carried as an endogenous virus on the BALB/c genetic background, was the viral companion for the chemical. Both neonatal and transplacental urethane treatments induced accelerated lymphoma appearance in BALB/Mo (v+/v+) and in Bc1 mice segregating for M-MuLV positivity (v+/−), thus confirming previous data on the synergistic leukemogenic effect exerted by the drug and MuLV (4). However, the results on lung tumor induction were less clear, because a significant difference between BALB/Mo and BALB/c animals was observed only in two experiments.

No other tumors, and particularly no hepatomas, were detected in urethane-treated mice; this could be due either to the relative short observation periods in relation to the more prolonged latency required for liver tumor appearance (3), or to the relatively low susceptibility of BALB/c mice to develop hepatomas (23). In any case, from the present results it appears that the synergistic tumorigenic effect involves preferentially the lymphatic tissue, which is a target specific for M-

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MuLV-induced neoplastic transformation. The sole fact that the M-MuLV provirus is carried in every somatic cell is perhaps not sufficient to confer a higher sensitivity to carcinogenic stimuli, and other events such as expression of viral genes or integration of multiple viral copies through repeated cell infection cycles are probably required.

This possibility is also supported by the finding that cordycepin treatment reduced SCE frequency in M-MuLV-infected lymphocytes. As reported by Price et al (20), this antiviral antibiotic is able to inhibit induction by 5-iodo-2′-deoxyuridine of endogenous retrovirus by interfering with poly(A) synthesis. In the present experiments, which attempted to elucidate the synergism between M-MuLV and chemical carcinogens observed in the induction of short-term mutagenic effects (1, 5, 16, 17), we found that incubation of BALB/Mo spleen lymphocytes in the presence of cordycepin produced a reduction in both M-MuLV yield and SCE frequency and that the decrease was even more marked after MMC treatment. Since no variation in SCE values was evident in both untreated and MMC-treated BALB/c lymphocytes after cordycepin incubation, it may be concluded that this SCE reducing effect is peculiar to BALB/Mo lymphocytes and most likely depends upon a reduction in virus synthesis. Although other experiments are programmed to investigate whether "normalization" of SCE values correlates with inhibition at other specific steps in the M-MuLV life cycle, these preliminary findings indicate that the mutagenicity-enhancing capacity of M-MuLV may well fit in with the idea that retroviruses act as insertional mutagens by interrupting or activating host cell genes (2). In our assay system, however, proviral integration in a single copy into the cell DNA does not seem sufficient *per se* to bring about detectable genetic effects for the induction of which viral expression seems to play a more crucial role.

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