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Generation of oncogenic mouse type C viruses: *In vitro* selection of carcinoma-inducing variants

(retroviruses/leukemia viruses/cell transformation)

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ABSTRACT **Type C retroviruses are endogenous in most vertebrate species. These viruses generally are of low pathogenicity in their natural hosts. Variants that contain cell-derived transforming genes have been isolated infrequently in the past upon continued passage *in vivo*. We report here a procedure that allows the isolation of new mouse leukemia-, sarcoma-, and carcinoma-inducing type C viruses entirely in cell culture. The viruses generated after passage in chemically transformed mouse cells and selection in epithelial mink lung cells produced pulmonary adenomas and adenocarcinomas and also ovarian carcinomas. Viruses with cell-transforming capacity, as determined by their ability to induce normal murine or mink cells to form progressively growing colonies in soft agar, appeared only transiently (2–4 days) after acute infection of "spontaneously" or chemically transformed mouse cells by nontransforming helper viruses. These transiently appearing transforming viruses can be "captured" by selecting the cells that respond to their newly acquired growth-stimulating ability. This system may lend itself to the systematic isolation of tissue-specific transforming functions from any cell that can be efficiently infected by retroviruses.**

Type C viruses and their progenitors (1) are part of the normal genetic makeup of many, and possibly all, vertebrate species (2, 3). Although most of these endogenous retroviruses are poorly infectious and have low oncogenicity, variants arising at low frequency upon continued passage *in vivo* and causing malignant transformation of various cell types (4–8) have been isolated. The endogenous type C viruses thus have the capacity, in rare instances, to acquire information that greatly increases their ability to transform cells. The experiments presented here were designed to test the suitability of an endogenous type C virus from C3H mouse cells for the systematic isolation of new transforming functions from other mouse cells in culture. The characterization of a C3H murine leukemia virus (MuLV) as a vector for biological cloning of cellular genes associated with malignant transformation, as well as elucidation of the mechanism by which this occurs, might provide the tools necessary to obtain genetic material related to specific cellular functions from any cell type in which retroviruses can replicate.

Our results show that endogenous C3H MuLV can indeed act *in vitro* as a vector for cell-derived transforming functions. The critical steps in this process are: (*i*) a chronic, persistent infection, especially of spontaneously or chemically transformed cells, during which the variant virus forms accumulate; (*ii*) "conversion" or unmasking of the novel virus forms during a few rounds of acute infection in permissive cells; (*iii*) selection of cells containing viruses with transforming potential by plating in soft agar. Using this procedure, we have been able to select rapid leukemia- and sarcoma-inducing viruses (9, 10). In this presentation, we extend these findings to include the isolation of carcinoma-inducing viruses.

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MATERIALS AND METHODS

Animals. The inbred mouse strain NSF/N, derived from National Institutes of Health Swiss mice, was obtained from the National Institutes of Health animal colony. New-born mice were injected intraperitoneally with freshly cloned viruses grown in NIH/3T3 cells (11); a dose of 2000–5000 infectious units (see below) was used per mouse. The animals were weaned at 4 weeks and examined twice weekly for development of disease. Animals that showed signs of disease were autopsied. The tumor tissue was processed for histological and tissue culture studies, portions were taken for transplantation in animals, and cell-free extracts were prepared for detection of viruses *in vivo* and in cell culture.

Preparation of Viral Extracts from Tumors. Extracts were prepared either by forcing minced tissue through a wire mesh and washing the filtrate with phosphate-buffered saline or as described by Moloney (7). The buffered saline wash was filtered and used as an inoculum as described in the legend to Table 2.

Transplantation of Tumors. Tumor tissue was minced with scissors and small pieces were inserted, via trocar, subcutaneously into weanling NSF/N mice.

Cells. All cell lines were maintained in Eagle's minimal essential medium with 10% heat-inactivated fetal calf serum (GIBCO). The origin and characteristics of the cell lines used for virus growth and assays have been described (12, 13). The C3H/10T1/2 CL8 line was developed from a C3H/Heston mouse embryo (14). This strain has a low "spontaneous" leukemia incidence. Ecotropic viruses were assayed by using SC-1 feral mouse cells (15) or NIH/3T3 cells (11). Xenotropic or recombinant amphotropic virus was titered on the mink lung cell line Mv-1-Lu (16). Cells were cloned from transformed foci by using cloning cylinders or by dislodging and aspirating cells with a pasteur pipette.

Viral Assays. The DNA polymerase induction assay was performed as described (9). Prior to infection, cells were seeded at 1×10^5 per 60-mm dish. One day later, the cells were treated with Polybrene (20 μg/ml) for 1 hr and then infected with 0.2 ml of virus suspension. After incubation for 2 hr, the virus inoculum was removed and 5 ml of complete medium was added. Culture fluids were collected at early confluency for determination of particle-bound DNA polymerase activity (13). At this time, cells were lethally UV-irradiated and overlayered with 1×10^6 rat XC cells per 60-mm dish (17). XC-plaque-forming virus was cloned from large plaques that developed in cultures infected at limiting dilutions. Cultures were overlayered with 0.5% agar subsequent to the addition of XC cells. Two days later, agar stabs were obtained from the plaques that formed and were transferred to uninfected NIH/3T3 cell cultures (11) and grown in medium containing Polybrene at μg/ml until the

Abbreviation: MuLV, murine leukemia virus.

culture fluid became positive for viral DNA polymerase activity.

Growth in Soft Agar. To test the growth potential of infected and control cells in soft agar, cells were seeded into 0.3% agar (Difco, Agar Noble) made up in complete medium containing 10% fetal calf serum; 2 ml was plated on top of 5 ml of 0.5% basal agar. Each week, 2 ml of complete medium with 0.3% agar was added to the top. The fraction of cells able to form colonies in soft agar was determined (examination with a stereoscopic microscope) after 1, 2, and 3 weeks of incubation at 37°C. Rapidly growing colonies were selected 7–10 days after seeding with a pasteur pipette. The agar stab was transferred to a 24-well microtiter plate (Linbro 76-033-05), broken up with needles, grown to confluency in the microtiter wells, and then transferred to T75 culture flasks (Falcon 3023).

RESULTS

Selection of Rapid Leukemia-Inducing Viruses. BrdUrd-induced mouse-tropic viruses derived from C3H/10T1/2 cells in culture were used as the starting material for the isolation of transforming viruses. During the logarithmic phase of growth, C3H/10T1/2 cells were treated with BrdUrd for 24 hr and then subcultured until they were homogeneously virus-positive. The virus produced by this chronically infected culture does not form plaques in the XC cell assay (11) and is replication deficient (12, 13). SC-1, a continuous line of feral mouse cells (15), C3H/MCA5, a chemically transformed line of C3H/10T1/2 cells (12), or spontaneously transformed NIH/3T3 cells were infected with this virus such that progeny from the first cycle of infection could go through additional rounds of replication before the culture was homogeneously virus-positive. At this time, or after several successive acute infections (9), culture fluids were tested for the presence of viruses with novel biological traits such as rapid replication, XC plaque-formation, altered host range, or the ability to stimulate cells to grow in soft agar. The most effective selection scheme for the isolation of competent rapid leukemia-inducing viruses involved cloning the virus released from the extra large XC plaques. This yielded rapidly replicating, XC-positive, mouse-tropic MuLV.

Isolation of Solid Tumor-Inducing Viruses. The basic protocol was similar to that described above with one major modification. Rather than selecting and isolating virus, we selected the virus-producing cells that were altered as the result of viral infection. C3H/10T1/2 indicator cells that respond to the virus stocks described above by acquiring abnormal growth properties, either the ability to grow in soft agar or the overgrowth of cell monolayers, were selected. The transmission of virus from the selected cells generally yielded low titers of transforming viruses which often decreased during subsequent viral passages. Cloning of transformed cells was considered to be a means to stably "capture" and to yield a continuous source of transforming genomes. This approach is presented schematically in Fig. 1. To increase further the incidence of tumor formation, we established cell lines from tumors induced in the animals and used them as a potentially more efficient source of transforming viruses. The experiment described below was initiated to determine whether *in vitro* transforming properties of the various virus stocks corresponded to their ability to produce tumors in susceptible animal hosts.

Sarcoma- and Carcinoma-Inducing Viruses. Virus was obtained by selecting cells from transient foci appearing in monolayers or from soft agar colonies of infected C3H/10T1/2 cells as outlined in Fig. 1 and described above. Virus produced by these cells was filtered and inoculated into newborn NSF/N mice. This procedure yielded sarcomas at a moderate incidence (Table 1). Virus clones selected for rapid replication also induced some sarcomas, as reported (9, 10). The tumor-producing capability of a given stock could be significantly enhanced by selecting clones of transformed cells from the induced tumors and using those cells as a source of transforming virus; 21 of 53 animals inoculated developed sarcomas with a latent period of 2–6 months.

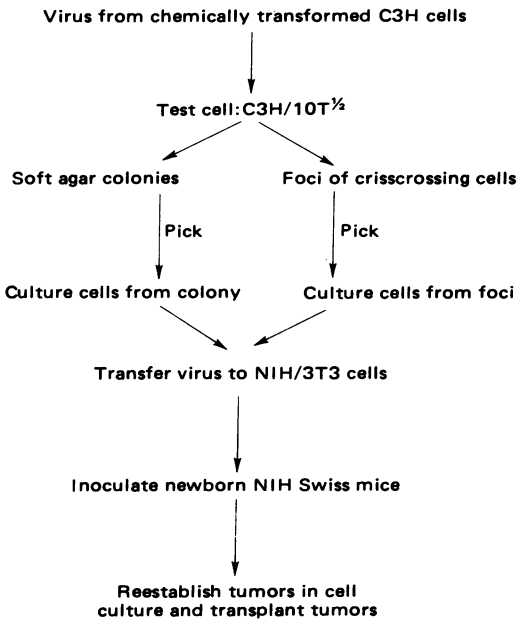

FIG. 1. Derivation of tumor-inducing viruses from C3H virus grown in chemically transformed cells. Cells were isolated from foci by using cloning cylinders. Single cells were seeded in 0.3% Noble agar on 5 ml of 0.5% basal agar to determine their capacity for anchorage-independent growth. Growing colonies were selected with a pasteur pipette, disrupted with needles, and cultured in 24-well Linbro plates. Virus production was tested by assaying culture fluids for particle-bound reverse transcriptase (9). Cell lines were established by mincing and disrupting tumor tissue into single cells with trypsin or collagenase and were grown in Dulbecco's modification of Eagle's medium containing 10% fetal calf serum and antibiotics. Tumor fragments were transplanted via trocar subcutaneously into weanling NSF/N mice.

The same selection scheme that gave rise to sarcoma-inducing virus stocks also yielded carcinoma-inducing viruses (Table 2). Again, establishing cell strains from induced tumors provided a source of tumor-inducing virus. This procedure was not as effective in increasing the titer of carcinoma virus as it had been in the isolation of sarcoma virus. A possible reason for this is that the cell cultures established from the carcinoma tissues generally were not homogeneous and contained variable proportions of fibroblastic cells in addition to the tumor cells. The main tissues in which carcinomas arose were the lung and

Table 1. Development of sarcomas after inoculation with different virus stocks

Virus	Incidence No.	Incidence %	Latency, months
BrdUdr-induced C3H MuLV	0/120	0	≥18
Rapid replicator clones *in vitro*	4/86	4.6	3–6
Focus/agar selected clones	78/530	14.7	3–9
Tumor cell derived	21/53	39.5	2–6

NSF/N mice were inoculated (100 μl) intraperitoneally at birth with culture fluids from selected virus clones. The fluid was filtered through Millipore filters (pore size, 0.22 μm). The animals were weaned at 4 weeks and, if overtly sick, were autopsied after death. Diagnosis was based on histological examination of all major tissues.

Table 2. Development of carcinomas after inoculation with different virus stocks

Virus	Ovarian Incidence	Ovarian Latency, months	Lung Incidence	Lung Latency, months
BrdUrd-induced C3H MuLV	0/120	>18	0/120	>18
Focus/agar selected clones	18/530	2–6	58/530	4–12
Tumor cell derived	3/64	2–6	ND	

Newborn NSF/N mice were inoculated intraperitoneally and observed for development of overt disease as described in the legend to Table 1. ND, not done.

the ovary. Lung tumors were uniformly diagnosed as alveologenic adenomas and adenocarcinomas. These tumors developed only after a relatively long latent period (4–12 months). The adenomas as well as the adenocarcinomas were found to be readily transplantable in syngeneic NSF/N hosts.

A representative ovarian tumor, identified as an adenocarcinoma, is illustrated in Fig. 2. In most cases, tumor formation was restricted to one ovary, but we did observe some animals in which both ovaries were affected. These tumors have been successfully maintained by transplantation from animal to animal as well as by establishing cell strains that provide a continuous source of tumor-inducing viruses. Other malignancies that have occasionally been seen include hemangioendotheliomas (two animals) and a carcinoid tumor (one animal). In addition, some of the tumor-inducing stocks caused disorders of the immune system with tubular degeneration in the kidneys and other lesions apparently caused by the deposition of immune complexes.

Selection of Carcinoma Viruses. The isolation of variants from C3H MuLV based on their ability to specifically infect

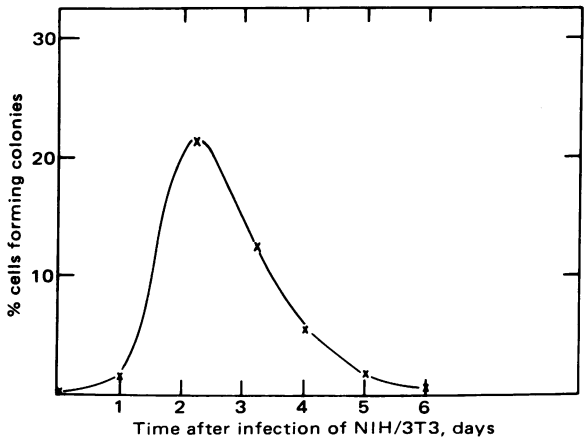

FIG. 3. Emergence of soft agar-growth stimulating activity for mink cells after acute infection of NIH/3T3 cells with rapidly replicating C3H MuLV. Spontaneously transformed NIH/3T3 cells were infected in the logarithmic phase of growth with C3H MuLV at a multiplicity of infection of 3–5. Culture fluids were filtered and transferred to mink lung cells at the indicated times. After one subculture, the infected mink cells were seeded into 0.3% soft agar at densities of 10^6, 10^5, 10^4, and 10^3 cells per 60-mm dish. Colony growth was monitored every 4 days. Plates that received 10^5 cells showed optimal colony growth and were used for the subsequent isolation of the large, progressively growing colonies.

and transform epithelial mink lung cells, Mv-1-Lu (CCL 64, American Type Culture Collection), instead of C3H/10T1/2 fibroblasts, significantly improved the yield of carcinoma-inducing viruses. The general scheme followed for this selection was described above as "basic protocol".

Fig. 3 shows the kinetics with which soft agar-growth stimulating viruses with the ability to infect Mv-1-Lu emerge in the

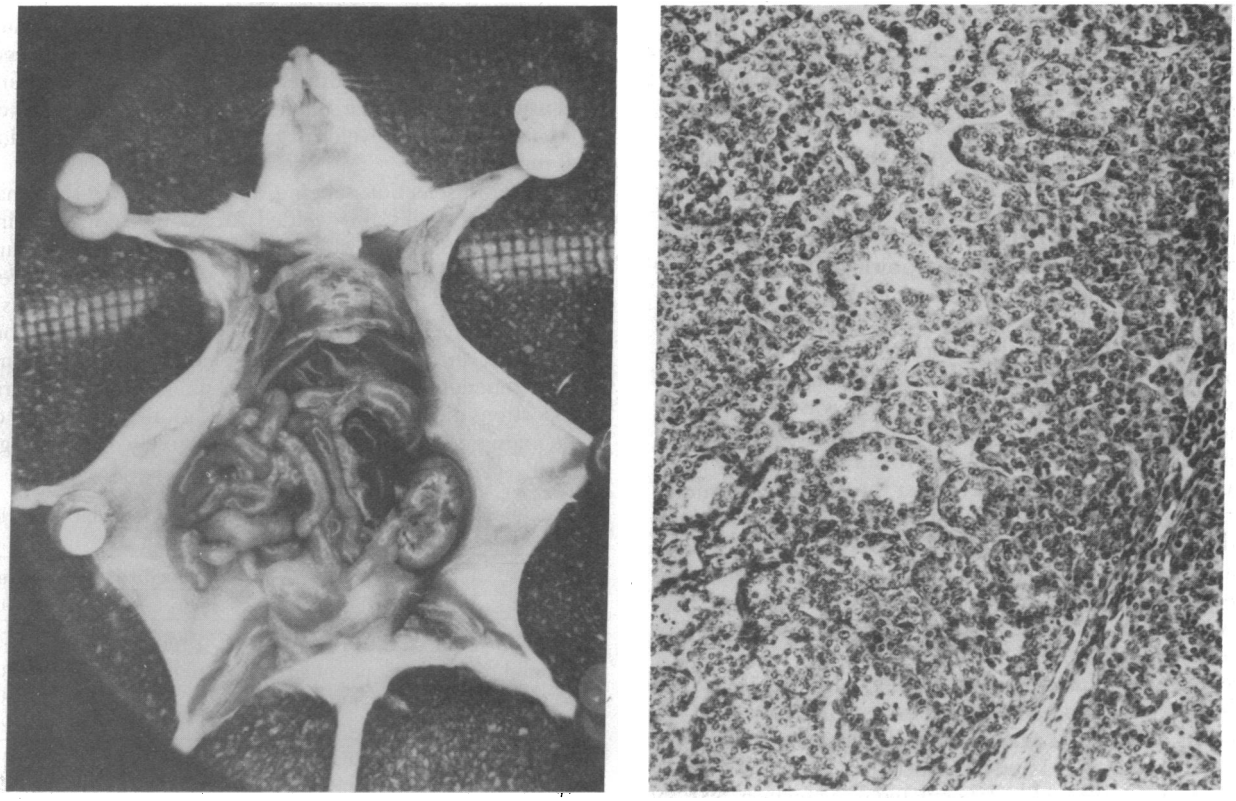

FIG. 2. Ovarian tumor induced in a NSF/N mouse. (*Left*) Macroscopic view of the tumor tissue. (*Right*) Section through the tumor. (Hematoxylin and eosin; ×150.)

Table 3. Agar growth stimulating activity of selected virus stocks

Virus	Distribution by colony size, %			
	<0.13 mm	0.13–0.26 mm	0.26–0.52 mm	>0.52 mm
BrdUrd-induced C3H MuLV	100	<0.01	<0.01	<0.01
Rapid replicator clones *in vitro*	96	3.8	<0.01	<0.01
Focus/agar selected clones	92	7.2	0.56	0.027
None	100	<0.01	<0.01	<0.01

C3H/10T1/2 cells were infected at a multiplicity of infection of 3–5, grown to confluency, and seeded into soft agar. Growth was monitored at weekly intervals. The data were obtained 10 days after seeding. BrdUrd-induced C3H MuLV was obtained from C3H/10T1/2 cells in phase III of growth (12). Diameter of 0.1 mm corresponds to four to eight cell diameters.

progeny of the acute infection of spontaneously transformed NIH/3T3 cells. The transformed NIH/3T3 cells were infected with virus produced by NIH/3T3 cells chronically infected with rapidly replicating C3H MuLV at a multiplicity of infection of 3–5. Culture fluid was tested daily for the presence of virus with the ability to stimulate mink lung cells to grow in soft agar. This is a double selection for variant MuLVs with the capacity both to infect and to transform the epithelial mink lung cell line. At the same time this procedure selects against the potentially interfering mouse-tropic parental virus. Mink cells inoculated with culture fluid were grown to confluency and subcultured once before being seeded into soft agar. This protocol resulted in the appearance of mink cell agar-growth-stimulating virus with an optimal yield at 2–4 days after infection of the spontaneously transformed NIH/3T3 cells. The frequency of infected mink cells growing in agar was very high (close to 20%) but many of the cells were found to go through only a few cell divisions before stopping. Others showed various toxic effects such as vacuolization or extreme volume increase through vesicle formation, followed by lysis. Table 3 shows the size heterogeneity of soft agar colonies induced by infection of mink cells with selected virus stocks.

A dozen of the larger mink cell colonies that were progressively growing in agar after viral infection were selected. Transforming viruses could be rescued from these clones either with baboon type C virus helper M7 (18) or with 1504A amphotropic MuLV helper (19). Inoculation of the rescued viruses from clones 41 and 67 (2 of 12 cell clones) into newborn NSF/N mice produced a low incidence of ovarian adenocarcinomas (Table 4). Neither rescuing virus alone caused solid tumor formation in NSF/N mice.

The nonproducer transformed mink cells that were isolated from the experiment described in Fig. 2 and that yielded tumor-inducing virus as presented in Table 4 had a pronounced

Table 4. Ovarian carcinomas induced by transforming virus rescued from transformed mink nonproducer cells

Cell clone	Helper virus	Carcinoma	
		Incidence	Latency, months
Virus control	Baboon virus (M7)	0/12	>9
Virus control	Mouse virus (1504A)	0/10	>9
Agar clone 67	Baboon virus (M7)	2/7	4–5
Agar clone 41	Mouse virus (1504A)	2/9	4–5

Rescues were performed by infection of nonproducer mink cells, derived from soft agar colonies, with baboon endogenous virus (M7) or 1504A amphotropic MuLV (18, 19). The soft agar colonies were selected from the experiment described in Fig. 3.

Table 5. Lung adenomas induced by mink lung cell selected transforming virus

Virus	Lung adenoma		Leukemia	
	Incidence	Latency, months	Incidence	Latency, months
XC plaque-positive cloned C3H MuLV	2/48	9–12	19/48	9–12
Mink cell soft agar selected clone 15	16/20	6–12	0/20	>12

Virus was selected as described in *Results* and tested for pathogenicity as detailed in the legend to Table 1.

tendency to revert when continuously subcultured. In order to decrease this tendency, we modified the experiment described in Fig. 2 to allow for additional consecutive rounds of infection of the mink cells. Instead of acutely infecting spontaneously transformed NIH/3T3 cells and then transferring progeny virus to the mink lung cell line Mv-1-Lu, we cocultivated cells chronically producing C3H MuLV with virus-free transformed NIH/3T3 cells for 2–4 weeks (as the permissive "converter" cells) in ratios of 5:95, 10:90, 20:80, and 40:60. That mixture then was cocultivated with the test cell, Mv-1-Lu, in a ratio of 1:1 and grown in medium containing Polybrene at 4 μg/ml to facilitate virus spread. This cocultivation procedure allowed for various periods of virus spread in the "converter" cells and continually provided the opportunity for selection of mink cell-transforming virus. Culture fluid was transferred to uninfected mink cells when transformation of mink cells became apparent. The focus-inducing capacity of the culture fluid could be diluted out; foci developing near the end point of the titration were selected. These transformed cells were found to be much more stable; most did not revert to normal morphology. The tumor-inducing capacity of one such virus stock is shown in Table 5. This isolate (clone 15) induced slow-growing soft agar colonies of Mv-1-Lu cells in culture and a high incidence of alveologenic lung adenoma in newborn NSF/N mice; 80% of the infected mice developed tumors within 12 months. The virus clone selected by the procedure outlined above, however, had decreased or no capacity to induce leukemia compared to the parent stock.

DISCUSSION

We report here a general procedure for systematic isolation of new transforming type C viruses. Specific applications of this scheme have yielded rapid leumia-inducing viruses (9), sarcoma viruses (10), and, as described here, adenoma- and adenocarcinoma-inducing viruses. This technique is simple and reproducible and thus should lend itself to similar experimentation with retroviruses of other morphological classes (type B, type D, etc.) and from species other than the mouse.

Briefly, the first step of our procedure involves the adaptation of endogenous type C viruses to high-titered growth on a given cell type *in vitro*. This gives rise to replication-competent viruses that can spread readily in cell culture and spread to various tissues when inoculated into the animal. In a second step, such replicating viruses can then be used to select for variants that have the ability to transmit transforming functions to recipient cells. These can be identified by screening for virus stocks capable of stimulating target cells to form colonies that grow in soft agar. The target cell specificity for transformation *in vivo* may be directed by the choice of host cells used for the detection of transforming viruses *in vitro*. Thus, selection for transformation of mink lung epithelial (Mv-1-Lu) cells gave rise predominantly to carcinoma-inducing variants, whereas selection

for fibroblast transformation, using C3H/10T1/2 cells, yielded mostly sarcoma and acute leukemia-inducing isolates. It is not clear by what mechanism the epithelial cell transforming genes are transmitted by the helper virus genomes or whether the new viruses represent deletion or substitution recombinants with the parent "helper" virus.

Viruses that may be analogous have been identified in the avian (20, 21), murine (22–26), and feline (27) systems. Among the rapidly transforming avian type C viruses, isolates that primarily induce carcinomas in the chicken have been described (28); these have had a complicated history of passage from animal to animal so that their precise origin is obscure. It is interesting to note, however, that one isolate, MH2, was originally obtained from a "spontaneous" ovarian tumor in the chicken (29). The most rapidly oncogenic viruses traditionally have been selected by passaging and reisolating virus from those animals whose tumors appear earliest (30, 31). Although the explanation for the change in latency period is poorly understood, the selection of more efficiently transforming variant viruses is probably a contributing factor. The present report shows that similar events can be recapitulated by the systematic selection of variant viruses with transforming properties in better-defined cell culture systems.

Type C viruses with transforming genes that have some cell-type specificity have been isolated from laboratory strains of transforming avian as well as mouse type C viruses (20, 21). The cells transformed by these viruses can contain new transformation-specific, virus-coded polypeptides. In some instances these are phosphorylated and have an associated protein kinase activity (32–35). A phosphorylated polyprotein has also been identified in some of the new transforming virus isolates we have described (36) but whether these new poteins have any direct role in the transformation process remains to be determined. A limitation to working with the long-established laboratory strains of transforming type C viruses is that these viruses allow only a retrospective analysis of their transforming genes. The genetic differences observed between presumptive parent viruses and their oncogenic relatives may well exceed the minimal genetic changes required for transformation. The ability to generate new, specific tumor viruses in a systematic fashion under defined *in vitro* conditions now makes it possible to undertake a prospective study of the origins of such type C viral oncogenes.

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