Effect of Helper Virus on the Number of Murine Sarcoma Virus DNA Copies in Infected Mammalian Cells

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Cell lines of four mammalian species were each examined for the number of Moloney murine sarcoma virus (M-MSV) DNA copies in total cellular DNA after M-MSV transformation. Sarcoma-positive, leukemia-negative (S+L-) M-MSV-transformed cells were compared to M-MSV-transformed cells infected with a replicating leukemia virus. Both unfractionated M-MSV complementary DNA and complementary DNA representing the MSV-specific and the MSV-murine leukemia virus-common regions of the M-MSV genome were hybridized to total cellular DNA of various species. DNAs of mouse, cat, dog, and human S+L- cells contained from less than one to a few proviral M-MSV DNA copies per haploid genome. In contrast, helper virus-coinfected, M-MSV-producing cells of each species showed a 3- to 10-fold increase in M-MSV proviral DNA over that found in corresponding S+L- cells. MSV-specific and MSV-murine leukemia virus-common nucleotide sequences were each increased to a similar degree. A corresponding examination of cellular DNA of leukemia virus-infected normal or S+L- mammalian cells was performed to establish the resulting number of leukemia proviral DNA copies. The infection of normal or S+L- mammalian cells with several leukemia-type viruses that did not have nucleotide sequences closely related to the cell before infection resulted in the appearance of one to three corresponding leukemia proviral DNA copies.

Avian and mammalian species contain within their DNA multiple proviral copies of RNA tumor viruses, which can often be induced by various means (1, 2, 7, 9, 25, 35, 37, 38). Infection of a cell with an RNA tumor virus (either ecotropic and related to endogenous proviral sequences or heterologous and unrelated to endogenous proviral sequences) results in the insertion into DNA of the appropriate provirus (6, 7, 9, 18, 22, 35, 40).

In avian RNA tumor virus systems, various estimates of endogenous proviral DNA copies in chicken cells ranged from 1 to 10, and the number of copies added after infection with ecotropic avian RNA tumor viruses was reported to vary from 1 to 20 (23, 34, 35, 38, 40). In duck cells, which do not contain avian leukosis virus sequences, an infection with several strains of avian sarcoma virus gave rise to a maximum of six DNA copies (39). Recently, a detailed study on the temporal relationships of avian leukosis DNA in chicken cells infected with an avian RNA tumor virus showed that initially after infection there were about 100 copies of viral DNA per cell, which, after several cell passages, stabilized at about 1 to 3 copies per haploid genome (23).

Mammalian cells also contain multiple cop-

ies of endogenous RNA tumor virus sequences in their DNA; mouse cells were reported containing 5 to 10 DNA copies, and baboon cells were reported containing an approximately equal number (1, 6, 7, 9, 37). If cells of a given species were infected with an ecotropic virus sharing sequences with the endogenous virus, or if an RNA tumor virus infected unrelated mammalian cells, about 1 to 3 copies of the newly introduced sequences were generally found in cell DNA (6, 7, 22, 37).

Little is known about mammalian sarcoma virus-specific nucleotide sequences in mammalian DNA. Moloney murine sarcoma virus (M-MSV)-specific nucleotide sequences are found in normal mouse DNA, and transformation by M-MSV resulted in the addition of, at most, one to two M-MSV proviral copies per cell (18). Infection of cat cells with M-MSV gave rise to detectable M-MSV DNA, but there was a significantly higher M-MSV DNA copy number in productively infected cat cells when compared to M-MSV-transformed, sarcoma-positive, leukemia-negative (S+L-) cat cells (20). Because M-MSV-producing cat cells were more transformed and had more M-MSV-specific viral RNA than their S+L- counterparts, it was of interest to determine whether this was a gen-

eral phenomenon in M-MSV-transformed virus-producing cells (20). A number of mammalian cell lines were examined, each after many passages in culture, and it was found that significant increases in M-MSV proviral copy number were of regular occurrence in M-MSV-producing cells. These MSV-transformed mammalian cells infected with a heterologous helper virus contained only one to a few copies of the helper-type proviral DNA. Infection of mammalian cells with only helper viruses yielded a few helper DNA proviral copies if the viral sequences were unrelated to any in the host cell and, possibly, additional copies if there were related endogenous viral sequences.

MATERIALS AND METHODS

Cells and viruses. Most of the cells were from continuous mammalian cell lines; these cells have been cloned at least several times, and their properties were described previously. Briefly, all the cat cells were derived from a single CCC (Crandell's cat cells) clone. Single-hit infection with the S+L- strain of M-MSV gave rise to transformed cells from which M-MSV could be rescued with cat cell-tropic helper RNA tumor viruses. After three cycles of cloning, an S+L- clone, 81, was selected for the fact that viruses such as feline leukemia (FeLV) and murine xenotropic viruses (MuX) could be readily assayed in 81 cells as focus-inducing units. The 81 S+L- line could be superinfected with the Rickard strain of FeLV propagated in this laboratory in a single strain of diploid feline embryo fibroblasts for 9 years, and an MSV(FeLV)-FeLV producer culture could be maintained. Alternatively, CCC cells were infected directly with the same strain of MSV(FeLV)-FeLV, and a virus-producing cloned culture, P521, was regularly maintained for about 5 years (13, 15-17, 20, 21, 28, 30, 31). Mouse cells were also described in detail: these consisted of normal cloned 3T3FL (outbred Swiss origin), 3T3FL infected with the IC isolate of Moloney murine leukemia virus (M-MuLV), "IC-3T3"; 3T3FL cell clones transformed in a single-hit fashion by the same strain of S+L- M-MSV, "FG-10" and "3B11"; and an S+L- mouse cell clone superinfected with M-MuLV and producing high titers (10^6 focus-forming units per ml) of M-MSV and 10^6 focus-inducing units of M-MuLV per ml, "3B11-IC" (3, 4, 14, 20, 28). Human cells were derived from a human amnion continuous cell line; a clone, F49-1, was then infected in a single-hit fashion by the S+L- type of M-MSV, and, several cloning cycles later, a clone "HUT" was chosen (30). HUT was also infected with simian sarcoma-associated virus (SSAV), which was passed only in primate and, in this laboratory, only in human diploid embryonic muscle-skin cells. The resulting culture, S-HUT, produced 10^6 focus-forming units of M-MSV continually (15, 30, 36). RD cells producing the RD-114 virus and human diploid embryonic lung cells (HEL) producing FeLV were both described (15, 26). Dog cells were the continuous dog kidney epithelioid line (DK), cloned several times.

A single-hit infection with the S+L- strain of M-MSV(CCC) gave rise to partially transformed cells (clone H-6) from which M-MSV could be rescued. Alternatively, a productive infection was initiated by the M-MSV(CCC)-CCC virus complex, which yielded $>10^5$ focus-forming units of M-MSV per ml and 10-fold less CCC in a cell line termed 6D-DK (31). Rat cells were the standard NRK cell line; they were infected with the AKR strain of ecotropic MuLV, two BALB/c cell-derived MuX strains (BALB-2 and B-MuX), and with SSAV (1, 9, 12, 13).

M-MSV assays were performed in 3T3FL cells or diploid feline embryo fibroblasts with appropriate helper virus: 2×10^4 focus-inducing units of M-MuLV or FeLV, respectively (3, 15). MuLVs were assayed in the S+L- assay FG-10 line, and MuX and FeLV were assayed in 81 cat S+L- as focus-inducing units. SSAV or CCC were quantitated by the terminal dilution that induced reverse transcriptase activity in human diploid cells.

Immunological assays were performed by indirect immunofluorescence with specific anti-Friend MuLV p30 and gp71 sera as described (32). Each assay was twice confirmed, both by specific radioimmunoassay for MuLV p30 and gp71 antigens and by complement fixation; the latter was done through the courtesy of D. Bolognesi and R. Huebner.

Cellular DNA and cDNA's. DNA was prepared from 10 g of cells from each cell line. The DNA was extracted by a modified Thomas procedure, sonically treated to 4S size, suspended at 10 to 12 mg/ml in $0.1 \times$ SSC ($1 \times$ SSC = 0.15 M sodium chloride, 0.015 M sodium citrate), and stored at -20°C until used in hybridization reactions (19).

To detect M-MSV sequences in the cellular DNA of the various mammalian cell lines, we prepared a radioactive DNA complementary to nucleotide sequences in the M-MSV-M-MuLV virus complex by the endogenous reverse transcriptase reaction. The virus stocks used contained a threefold excess of M-MSV over M-MuLV, as measured by biological assays. The complementary DNA (cDNA) was made in the presence of 100 μg of actinomycin D per ml, as described (21). This M-MSV-MuLV cDNA hybridized 90% with homologous high-molecular-weight viral RNA and protected 90% of iodinated M-MSV-MuLV subunit RNA from RNase T₁ digestion at a nucleotide ratio of cDNA/RNA of 3:1 to 4:1 mol. This M-MSV-MuLV cDNA detected MSV sequences in the cat and dog S+L- and producer cells because there were no M-MuLV viral sequences in normal cat and dog cells or in the helper viruses used to make producer cell lines from the S+L- cat and dog cell lines (20; see Table 2).

However, in normal mouse cells there are multiple copies of M-MuLV-related sequences (7, 9, 19). The M-MSV-MuLV cDNA hybridized 75% with a $C_{0,t_{1/2}}$ of 100 to 200 mol $\cdot$ s/liter (indicating about 5 to 10 copies per haploid genome) with DNA of uninfected mouse cells. Consequently, changes in the DNA of mouse cells secondary to infection with M-MSV cannot be easily documented with M-MSV-MuLV cDNA. Recently, we described the fractionation of the MSV-MuLV cDNA by sequential absorptions with MuLV and the FeLV pseudotype of MSV

[MSV(FeLV)] RNA (21). In this manner, three sets of nucleotide sequences, which represent different portions of the M-MSV viral complex, were obtained: a sarcoma virus-specific fraction (MSV-specific cDNA) with sequences that had no homology to M-MuLV RNA but which hybridized to MSV(FeLV) RNA, a sarcoma-leukemia fraction (MSV-MuLV-common cDNA) with sequences common to MSV as well as M-MuLV viral RNA, and a leukemia-specific cDNA (MuLV specific) representing those nucleotide sequences found only in M-MuLV. The MSV-specific cDNA represented about 25% of the total M-MSV sequences, whereas the MSV-MuLV-common cDNA represented the remainder of the M-MSV viral genome. The MSV-MuLV-common cDNA and the MuLV-specific cDNA represented about 55 and 45%, respectively, of the M-MuLV viral genome. Using the MSV-MuLV-common cDNA again, multiple copies per cell of M-MuLV-related proviral DNA were found in normal, uninfected mouse cells (19). When the MSV-specific cDNA was hybridized with normal mouse cells, the majority of cDNA sequences hybridized, but with a $C_0t_{1/2}$ of $\sim 2,000$ mol·s/liter, indicating one or a few copies per cell (18). Thus, MSV-specific cDNA offered an appropriate tool for investigating changes in MSV proviral copy numbers in MSV-infected mouse cells. Furthermore, as no changes were noted in the endogenous MSV-specific sequence concentration after infection of mouse cells with M-MuLV alone (27), the MSV-specific cDNA should be able to detect changes related to the infecting MSV. The MSV-specific cDNA hybridized 70 to 80% with M-MSV(FeLV) viral RNA but only 5 to 7% with M-MuLV high-molecular-weight RNA with a 6% background. The MSV-specific cDNA protected 20 to 25% of iodinated M-MSV-MuLV subunit RNA and M-MSV(FeLV) high-molecular-weight RNA at high molar cDNA/RNA ratios (21).

The human S+L- and producer cell lines were also analyzed by using the MSV-specific cDNA because the helper virus, SSAV, was found to have significant homology with M-MuLV (5, 7; see also Results, Table 4).

The FeLV, BALB-2, SSAV, M-MuLV, and RD-114 viruses were obtained from Electro-Nucleonics Laboratories, Inc., and cDNA was prepared as for MSV-MuLV cDNA. AKR cDNA was a gift of S. Chattopadhyay.

cDNA-DNA hybridization. Hybridization of cDNA's with cellular DNA were carried out at 70°C in 0.75 M NaCl with 5 to 7 mg of DNA per ml and 1,000 cpm (5×10^{-8} μ g) of 3 H-labeled cDNA per 60 μ g of cellular DNA. Samples were taken at various times (2 min to 48 h) and analyzed on hydroxyapatite at 50°C with 0.14 and 0.3 M phosphate buffer (PB) washes. Hybridized cDNA and cellular DNA were eluted by the 0.3 M PB wash. The ratio at the 0.3 M PB wash of counts per minute or optical density to the total counts per minute or optical density yielded the fraction of cDNA hybridized or cellular DNA reassociated, respectively.

As an internal standard in every DNA-cDNA hybridization, the self-reannealing of unique-sequence DNA was measured by optical density measure-

ments on the 0.14 and 0.3 M PB washes. The $C_0t_{1/2}$ for cellular unique sequences was calculated for each reaction. By dividing the cellular unique sequence $C_0t_{1/2}$ by the cDNA $C_0t_{1/2}$ we computed the proviral copy number per haploid genome. These sets of controls were performed to test the validity of the internal standardization. 3 H-labeled mouse cellular DNA was added to unlabeled mouse cellular DNA, and the rate of reannealing was measured by assaying both trichloroacetic acid-precipitable counts per minute and optical density. Labeled mouse globin cDNA was reacted with mouse cellular DNA again under conditions identical to those of the other hybridization reactions, and the globin gene copy number was calculated. Finally, cDNA's complementary to various RNA tumor viruses were reacted with helper virus-infected mammalian cell DNAs with known proviral copy numbers and with the DNA of the M-MSV-transformed cells infected with the corresponding helper type C virus.

Thermal denaturation profiles. Samples of the FeLV cDNA-DNA hybridization reactions at C_0t values of 10,000 mol·s/liter were removed and dissolved in 0.12 M PB. The material was then loaded on a hydroxyapatite column at 50°C, and the column was washed with 0.12 M PB at 5°C intervals. One thousand counts per minute of MuLV 32 P-labeled cDNA hybridized to MuLV high-molecular-weight RNA was added in each thermal denaturation experiment. The optical density and trichloroacetic acid-precipitable 3 H and 32 P counts per minute were assayed on each wash. The internal standard consisted of the T_m of unique-sequence cellular reannealed DNA measured by optical density and the T_m of the 32 P-labeled MuLV cDNA-MuLV RNA hybrid.

RESULTS

Virus-producing and S+L-, M-MSV-transformed mammalian cell systems. Various well-characterized, M-MSV-transformed mammalian cells were used. Cells of all species tested, both S+L- and virus producing, were generally cloned at least once. Each clone was in continuous culture for more than 6 months. Mouse, cat, and dog S+L- cells were only partially hyperrefractile but were clearly altered in morphology, grew in soft agar (2 to 10%), formed solid monolayers, and grew to cell densities 1.5- to 4-fold greater than their parental normal cell clones. Human S+L- cells were more hyperrefractile and formed only a loosely adherent layer. Virus production and antigenic properties are detailed in Table 1. S+L- cells were generally free of virus; however, in mouse S+L- cells a low level (50 focus-forming units per ml) of spontaneously appearing focus-forming virus with unusual envelope properties (27) was detected. The cat S+L- cell clone 81 also occasionally released up to 10^3 focus-forming units of MSV, which was coated with the endogenous feline CCC-RD-114-type virus (17).

Each S+L- cell was readily rescuable with

TABLE 1. Properties of M-MSV-transformed S+L- or virus-producing mammalian cells

Cloned cell line	Rescuable ^a				
	Free M-MSV (FFU/ml)	Leukemia virus (FIU/ml)	M-MSV helper virus (MOI = 1) (FFU/ml)	M-MSV-co- ded ^b MuLV gs-1	MuLV ^c gp71
Mouse FG-10 S+L-	≤50	0	≥10 ⁶	+	±
Mouse 3B11-IC producer, M-MuLV helper	2.5 × 10 ⁶	1 × 10 ⁶	-	+	+
Cat S+L/ 81	0-10 ³ (CCC) ^d	0-10 ² (CCC)	≥10 ⁵	+	0
Cat P521 producer, FeLV helper	4 × 10 ⁴	10 ⁴	-	+	0
Dog H-6 S+L-	0	0	≥10 ⁴	+	0
Dog 6D-DK producer, CCC helper	2 × 10 ⁵	~10 ⁴ ^d	-	+	0
Human S+L- HUT	0	0	≥10 ⁵	+	0
Human S-HUT producer, SSAV helper	1 × 10 ⁵	≥10 ⁵ ^d	-	+	0

^a A multiplicity of infection (MOI) of 1 infectious unit of helper virus was added and the resulting rescued MSV was harvested at optimal times for each system and assayed in the most susceptible system known (3T3FL for mouse, feline embryo fibroblasts for the others) with appropriately optimal (2 × 10⁴ focus-inducing units (FIU) per dish) helper virus. FFU, Focus-forming units.

^b Determined by complement fixation, fluorescent antibody, and radioimmunoassay with anti-MuLV p30 sera.

^c Determined by fluorescent antibody and radioimmunoassay with anti-MuLV gp71 sera.

^d As assayed by terminal dilutions inducing gs-antigen or reverse transcriptase activity in human cells.

any mammalian leukemia helper virus capable of growing in that cell. Considerable variations in rescue efficiency were seen with various helper viruses capable of growth in each cell system. Virus-producing cell systems were derived by either superinfecting the S+L- cell with helper virus, as in mouse or human or cat cells, or, alternatively, coinfecting normal cat or dog cells with virus mixtures where M-MSV was in excess. In each virus producer cell system, M-MSV generally exceeded the attending helper viruses, as previously observed (21, 29, 30). All S+L- producer cells were positive for the M-MSV-coded, MuLV-derived gs-1 determinant of p30, as assayed by immunofluorescence radioimmunoassay and complement fixation with anti-MuLV p30 sera (17, 30, 32). As detailed previously, the MuLV gs-1 determinant was found on the intracellular p60 precursor protein coded by MSV (30). None of the cells examined had any expression of MuLV gp71 determinants when checked with anti-Friend MuLV gp71 sera in the above assays (32).

Amplification of M-MSV nucleotide sequences in M-MSV producer cells. It was originally observed that in cat P521 cells producing M-MSV(FeLV) and FeLV there was more MSV-specific RNA and more MSV-specific DNA than in the corresponding S+L- cat cells (20). To determine whether an increase of M-MSV DNA was a common feature of virus-producing cell systems, normal, S+L-, M-

MSV producer, and leukemia virus-infected mouse, cat, dog, and human cells were each tested for M-MSV DNA. Both total M-MSV-MuLV cDNA and MSV-specific fractionated cDNA were used as required.

In normal mouse cells, fractionated, MSV-specific cDNA had to be used because there are multiple copies of M-MuLV-related sequences per cell (18, 19). The human S+L- and producer cell lines obtained after SSAV infection were also analyzed by using MSV-specific cDNA because the helper virus, SSAV, was found to have significant homology with MuLV. Unfractionated M-MSV-MuLV cDNA hybridized 25% with SSAV-infected human cell DNA and 32% with SSAV virus RNA (Table 2). The nucleotide sequences of SSAV which did hybridize with MSV were those representing the "common" region, possibly coding for "gag" (group-specific antigen) proteins. Neither MSV-specific nucleotide sequences nor the MuLV sequences not in common with M-MSV were found in SSAV. The hybrid of MSV-MuLV cDNA-SSAV viral RNA melted at 68°C, as opposed to M-MSV-derived cDNA common MSV(FeLV) viral RNA hybrids, which melted at 78°C.

For each cellular DNA tested, the unique-sequence DNA reassociated with a $C_0t_{1/2}$ of 1,000 to 3,000 mol·s/liter. When ³H-labeled AKR mouse cellular DNA was added to BALB/c mouse DNA, the optical density internal stan-

TABLE 2. Homology of Moloney sarcoma and leukemia viral sequences to SSAV nucleotide sequences^a

cDNA ^b	Percent hybridization to:	
	SSAV-infected human cell DNA	SSAV viral RNA
MSV-MuLV, unfractionated	25	32
MSV specific	12	4
MSV-MuLV common	NT	26 ^c
MuLV specific	NT	4

^a DNA hybridization was performed with SSAV-human embryonic muscle and skin cell DNA and the various cDNA's by using the methods described in the text. The internal standard unique-sequence cellular DNA reannealed 80% with a $C_0t_{1/2}$ of 1,200 mol·s/liter. RNA hybridizations were performed with 1,000 cpm (5×10^{-5} μ g) of cDNA and 1 μ g of SSAV viral RNA in a 50- μ l reaction for 24 h at 62°C in 0.22 M PB until reaching a $C_0t_{1/2}$ of 5 to 10 mol·s/liter. The hybrid was then analyzed identically to the DNA hybrid assays on hydroxyapatite at 50°C with 0.14 and 0.3 M PB washes as described in the text.

^b The cDNA's had a background of 5 to 7% and hybridized maximally with homologous viral RNA at 70 to 80% (20). The MSV-MuLV cDNA hybridized maximally at 9% with uninfected human cell DNA (F49-1 human amnion cell line), and the MSV-specific cDNA hybridized 12 to 15% with uninfected human cell DNA.

^c The 26% hybridization seen between MSV-MuLV-common and SSAV viral RNA had a T_m of 68°C, versus 78°C for the T_m of MSV-MuLV-common with M-MSV(FeLV) viral RNA.

dard measurement yielded a $C_0t_{1/2}$ of 1,500 mol·s/liter. The ³H-labeled AKR DNA hybridized with a unique-sequence $C_0t_{1/2}$ of 1,800 mol·s/liter. When ³H-labeled mouse globin cDNA, a gift of P. Leder, was hybridized with BALB/c mouse DNA, the unique-sequence cellular $C_0t_{1/2}$ was 800 mol·s/liter, indicating 1.6 globin gene copies per haploid genome, within expected values (24). Hybridization of AKR cDNA, BMuX cDNA, and RD-114 cDNA with infected mammalian cells yielded proviral copy numbers (per haploid genome) of 0.5, 2 to 3, and 2, respectively (see Table 5). These results compare with 1, 1 to 2, and 1 to 2, respectively, observed by Benveniste and Todaro and by Chattopadhyay et al. (7, 9, 10). Since the cellular DNA reassociated with the expected $C_0t_{1/2}$ and since the method of internal standardization gave results compatible with published findings, we then investigated the MSV DNA content of the MSV-transformed mammalian cells.

Each M-MSV-transformed cell system contained MSV-specific DNA (Table 3; Fig. 1). The uninfected or helper virus-infected cat, dog, or human cells did contain some (16 to 17%) M-MSV-related DNA sequences; however, as demonstrated previously, the hybridizing frac-

tion had a melting profile reduced by 20°C (18). Normal mouse cells were again shown to contain about one quite exact ($T_m = 80^\circ\text{C}$) copy of MSV-specific DNA. Infection of normal mouse cells with MuLV did not cause an increase of endogenous MSV-specific nucleotide sequences. In addition, the infection of human cells with SSAV or of cat cells with FeLV had no effect on the detection of MSV-specific nucleotide sequences in DNA over the related nucleotide sequences found in normal cells.

Examination of copy numbers of MSV-specific nucleotide sequences in the DNA of S+L-cells showed considerable variation. Dog S+L-cells had the lowest value, less than one copy per haploid genome. In mouse or human S+L-cells, about one copy was regularly detected. Cat S+L-cells had a somewhat higher number, about 3 to 4 copies of MSV DNA. To get cells with both MSV and helper virus infection, two techniques were used. In the first case, S+L- MSV-transformed mammalian cells were infected with helper virus. These were superinfected cell lines and produced MSV and helper virus. We did this with the cat 81 and FeLV-81 cells, mouse 3B11 and 3B11-IC cells, and human HUT and S-HUT cells. In the second case, normal mammalian cell lines were infected with a mixture of MSV and helper virus, and a producer clone was isolated. This was done with the cat CCC and P521 and dog DK and 6D-DK cells. These coinfecting cells also produced MSV and helper virus. When M-MSV-producing cells were examined by hybridizing viral cDNA to total cellular DNA, significantly lower $C_0t_{1/2}$ values were regularly observed with M-MSV-producing cell DNA-superinfected or -coinfecting cells when compared with S+L- cell DNA. The increased rate of association indicated that the number of M-MSV copies in cell DNA increased between 3- and 10-fold in each virus-producing cell system. Cat S+L- 81 cells had initially higher copy numbers; it is of interest that, in this system, the endogenous CCC virus and MSV(CCC) were readily detected (17; Table 1). Two types of M-MSV(FeLV)-producing cells were examined; one was the chronic producer P521 of M-MSV(FeLV) and FeLV in culture for several years and was obtained by an infection of CCC cells with M-MSV(FeLV)-FeLV complex. The other was the 81 S+L- clone superinfected with FeLV, which then produced MSV(FeLV) and FeLV. No significant differences were observed in the final extent of hybridization or copy numbers between the two M-MSV producer lines. No significant changes in DNA copy number in M-MSV-producing cat cells were found in over 1 year of observation. In dog

TABLE 3. Quantity of MSV nucleotide sequences in M-MSV-transformed mammalian cells^a

Cell	Description	cDNA	Maximal hybridization (%)	C ₀ t _{1/2} (mol·s/liter)	Copy no./haploid genome
CCC	Normal cat cell	MSV-MuLV	12	— ^b	—
CCC-FeLV	Cat cell infected with FeLV	MSV-MuLV	13	—	—
81	M-MSV-transformed cat cell line	MSV-MuLV	38-54	300	3-4
P521	M-MSV(FeLV) + FeLV-coinfected cat cell line	MSV-MuLV	67-72	125	9-16
FeLV-81	81 cells superinfected with FeLV	MSV-MuLV	64	150	8
F49-1	Normal human cell	MSV specific	19	—	—
S-HEMS	Human embryonic muscle-skin infected with SSAV	MSV specific	19	—	—
HUT	M-MSV-transformed human cell line	MSV specific	31	1,200	1
S-HUT	SSAV-superinfected HUT cell line	MSV specific	63	200	5
DK	Normal dog cell	MSV-MuLV	10	—	—
H-6	M-MSV-transformed dog cell line	MSV-MuLV	42	3,000	0.3
6D-DK	M-MSV(CCC) + infected dog cell line	MSV-MuLV	45	390	4
3T3FL	Normal mouse cell	MSV specific	50	2,000	1
IC-3T3	3T3FL mouse cell infected with M-MuLV	MSV specific	50	2,500	1
FG10	M-MSV-transformed mouse cell line	MSV specific	45	2,500	1
3B11	M-MSV-transformed mouse cell line	MSV specific	45	2,600	1
3B11-IC	3B11 cells superinfected with MuLV	MSV specific	54	500	5

^a The hybridizations were performed as described in the text. The internal standard gave unique-sequence DNA C₀t_{1/2} values of 1,000 to 3,000 mol·s/liter, as shown in the legend to Fig. 1. Additionally, CCC, CCC-FeLV, F49-1, S-HEMS, DK, 3T3FL, and IC-3T3 DNAs had unique-sequence C₀t_{1/2} values of 2,300, 714, 2,700, 1,800, 2,200, 1,500, and 1,000 mol·s/liter, respectively.

^b —, Not applicable.

cells producing M-MSV, the quantity of M-MSV DNA increased by about 10-fold. Human S+L- cells superinfected with SSAV displayed a fivefold increase of MSV-specific DNA. Infection of mouse cells with M-MSV and M-MuLV similarly resulted in a fivefold amplification of MSV-specific DNA.

A problem in the hybridization experiments was the imprecision in copy number estimates. For all cell lines tested the hybridization reaction was performed three to four times. The error in reproducibility was 100% for 1 copy per haploid genome, 50% for copy numbers less than 5 per haploid genome, and 30% for copy numbers of 10 to 15 per haploid genome. This estimate of error is based only on the reproducibility of the experiment. Systematic errors would not be expected to be as great, since the internal standard of cellular unique sequences was tested on each experiment. Hence, differences of one copy per cell, as in the case of S+L- cells and normal mouse cells (Table 3),

are too small to detect, as noted before (18). However, differences of 3- to 10-fold (5 to 10 proviral copies per cell) are significant. An additional problem was the variability of the final extent of hybridization, which demonstrated in all cases that maximal hybridization increased with a greater copy number. This appears to be an artifact based on specific reconstruction experiments mixing high-MSV-copy number cat cell DNA (P521) with normal cat CCC cell DNA and comparing it to DNA with a low MSV copy number (81). As demonstrated previously, the maximal extent of hybridization was decreased in mixed DNA experiments (20; see Discussion).

Linked amplification of MSV-specific and MSV-common nucleotide sequences in the DNA of M-MSV producer cells. The DNA of cat cells producing M-MSV(FeLV) and FeLV had an increased number of copies of M-MSV DNA, as determined by the unfractionated cDNA representing the entire M-MSV genome. It was of interest to determine whether frac-

FIG. 1. Hybridization curves of MSV-MuLV and MSV-specific cDNA with various cellular DNAs. Hybridizations were performed as described in the text. MSV-MuLV cDNA was used for the cat and dog cell lines, and MSV-specific cDNA was used for the mouse and human cell lines. Equivalent results were obtained when MSV-MuLV-common cDNA was used for the cat cell lines (Table 4). Also, similar copy numbers were obtained with MSV-MuLV-common cDNA with cat cell lines. The cell lines used are described in Table 1; nonproducing cell lines are represented by ○, and producer cell lines are represented by ●. (A) FG-10 cell DNA and 3B11-1C cell DNA; (B) HUT cell DNA and S-HUT cell DNA; (C) 81 cell DNA and P521 cell DNA; (D) H6 cell DNA and 6D-DK cell DNA. The curves were calculated by a computer-determined second-order least-squares fit. Actual percentages of hybridization are plotted. The maximal hybridization of the MSV-MuLV cDNA with homologous viral RNA is 90%, with a 5% background. The maximal hybridization of the MSV-specific cDNA with MSV(FeLV) viral RNA is 80%, with a 6% background. The internal standard unique-sequence cellular DNA had $C_{\theta_{1/2}}$ values of 1,100 and 2,500 mol·s/liter for FG10 and 3B11-1C cell DNAs, respectively; $C_{\theta_{1/2}}$ values of 840 and 1,700 mol·s/liter for HUT and S-HUT cell DNAs, respectively; and $C_{\theta_{1/2}}$ values of 1,000 and 2,000 mol·s/liter for H6 and 6D-DK cell DNAs, respectively. The experiments presented represent one set of three complete sets of experiments. The error in copy number was 50% for 2 to 5 copies per haploid genome and 30% for 8 to 20 copies per haploid genome.

tionated cDNA representing the MSV-specific region and the cDNA representing the nucleotide sequences in common with MuLV were equally increased. As described previously, the subsets of cDNA's of M-MSV were highly specific for each region of M-MSV RNA and were representative to the degree that low molar ratios (two- to threefold excess) of cDNA were adequate to reach a plateau value of protection in experiments subjecting iodinated M-MSV RNA to RNase digestion. Table 4 compares the quantitative increase of M-MSV DNA in M-MSV-producing cat cells. Both MSV-specific and MSV-common DNA nucleotide sequences were increased by a similar degree over those found in S+L- cat cells, except in the use of one clone of FeLV-81 cells.

Detection of leukemia virus proviral sequences in various infected mammalian cells. It was of interest to gain some understanding of the role of leukemia virus in the amplification of DNA sequences of M-MSV after helper virus infection. The extent of hybridization and the quantity of proviral DNA sequences were measured for a number of leukemia helper-type RNA tumor viruses in the producer cells studied above and in leukemia virus-infected mammalian cells in the absence of M-MSV. The cell systems examined initially comprised well-characterized murine, feline, and primate helper viruses infecting cells in which the viruses were not endogenous. cDNA's were prepared that hybridized well with homologous viral RNAs (Table 5). In each case viral cDNA

was reacted with DNA of homologous virus-infected cells, and the extent of hybridization and $C_0t_{1/2}$ values were determined. In each measurement case the cellular unique-sequence $C_0t_{1/2}$ was determined by optical density, as described. In the MSV producer cell lines containing a heterologous helper virus, there were one (6D-DK) or three (S-HUT) proviral DNA copies of the corresponding helper virus. In the MSV producer cell lines containing a helper virus with related endogenous sequences in the host DNA, there were five (3B11-IC) or ten (P521 and FeLV-81) proviral

DNA copies of the helper virus (Table 5). However, the uninfected mouse and cat cells contained a similar number of copies of helper virus-related sequences (Table 5). Hence, it is difficult to estimate the number of added proviral DNA copies per cell.

The controls for these experiments, mammalian cells infected with murine, cat, or primate leukemia viruses, gave the expected proviral DNA copy numbers (Table 5).

Further examination of cat cells infected with FeLV yielded the following results. The T_m of the FeLV cDNA hybrid with uninfected

TABLE 4. Presence of different subsets of *M*-MSV nucleotide sequences in the DNA of *M*-MSV-transformed cat cells^a

cDNA	Cell	Maximal hybridization (%)	$C_0t_{1/2}$ (mol·s/liter)	Copy no./haploid genome ^b
M-MSV-MuLV, unfractionated	81	54	300	4
	P521	64	150	8
	FeLV-81	64	150	8
MSV-MuLV common	81	49	740	2
	P521	64	270	5
	FeLV-81	35 ^c	150	5
MSV specific	81	40	300	4
	P521	64	150	7
	FeLV-81	55	300	5 ^d

^a The hybridization reactions were performed as described in the text. The internal standard unique-sequence cellular DNA reannealed at least 75% with a $C_0t_{1/2}$ of 1,200 to 2,000 mol·s/liter.

^b Calculated as in Tables 2 and 3.

^c 1,000 cpm of MuLV cDNA used.

^d One clone of FeLV-81 cell DNA had an MSV-specific $C_0t_{1/2}$ of 1,300 mol·s/liter with a cellular $C_0t_{1/2}$ of 1,300 mol·s/liter and a copy number of 1/haploid genome.

TABLE 5. Leukemia virus nucleotide sequences in mammalian cell DNA^a

Cell ^b	Leukemia virus produced	Hybridization of leukemia virus cDNA with homologous viral RNA (%)	Maximal hybridization with cell DNA (%)	$C_0t_{1/2}$ (mol·s/liter)	Cellular unique-sequence $C_0t_{1/2}$ (mol·s/liter)	Copy no./haploid genome ^c	Published copy no./haploid genome
Experimental							
S-HUT	SSAV	84	62 (human)	400	1,200	3	
6D-DK	RD-114	68	52 (dog)	1,930	1,970	1	
3B11-IC	M-MuLV	90	62 (mouse)	200	1,000	5	
FeLV-81	FeLV	79	57 (cat)	120	1,200	10	
P521	FeLV	79	70 (cat)	100	1,000	10	
3T3FL-IC	M-MuLV	90	50 (mouse)	300	1,500	5	
CCC-FeLV	FeLV	79	60 (cat)	100	1,000	10	
Control							
AKR-NRK	AKR-MuLV	87	42 (rat)	2,000	940	0.5	1 (10)
BMuX-NRK	MuX	81	49 (rat)	650	1,090	2	1-2 (6)
BALB-2-A673	MuX	81	70 (human)	500	1,800	3	1-2 (6)
SSAV-NRK	SSAV	84	82 (rat)	140	1,060	8	
S-HEMS	SSAV	84	83 (human)	140	1,200	9	
RD-114	RD-114	68	61 (human)	600	1,150	2	1-2 (6)
FeLV-HEL	FeLV	79	67 (human)	500	1,500	3	

^a RNA hybridizations were performed as described in the text. Internal standards are shown in the table. The known copy numbers per haploid genome, with references, are also shown in the table.

^b Cells were infected with the leukemia virus and cloned and retested for virus production.

^c Copy number per haploid genome is calculated as in Tables 2 through 4.

CCC cat cell DNA was 78°C. The T_m of the FeLV cDNA hybrid with the heterologously infected human cell DNA, FeLV-HEL DNA, was 86°C. The T_m of cat cell DNA infected with FeLV, P521, or CCC-FeLV cell DNA was 82°C. These observations suggest that the endogenous FeLV sequences are different from the sequences in the infecting FeLV and that the infected cat cells may have acquired some of these sequences, hence leading to a higher T_m .

DISCUSSION

Four mammalian cell systems were examined for proviral M-MSV copy numbers, firstly as S+L- cells and secondly as M-MSV-producing cells. Each cell system was transformed with the S+L- isolate of the M-MSV genome, and S+L- was transformed with the S+L- isolate of the M-MSV genome, and S+L- or producer cell lines were examined several months to several years after the initial infection, which should preclude the reported initial variability of integrated DNA copy numbers (23). The findings were uniform. In each of mouse, cat, dog, and human cells, a significant and stable increase in M-MSV copy number occurred when the S+L- cells were infected with helper virus to become producers or when a productive dual infection with M-MSV and helper was started initially. In dog S+L- cells there appeared to be less than one copy of M-MSV DNA per cell; it might be possible that some cells were revertants, which could have lost the M-MSV genome as demonstrated previously (13). However, five out of five dog S+L- cells cloned and tested had rescuable MSV. In cat or dog cells, cDNA representing the entire M-MSV genome could be used. In mouse cells or human S+L- cells infected with SSAV, fractionated, M-MSV-specific cDNA had to be used because of endogenous murine RNA tumor virus sequences in mouse cells related to both M-MSV and the MuLV genomes (21) or because, as demonstrated, SSAV shared homology with the "common" and not the leukemia- or MSV-specific parts of the M-MSV-MuLV virus complex. In cat cells both the unfractionated and the fractionated cDNA's gave similar values for M-MSV DNA proviral copy numbers. The MSV-specific and MSV-common cDNA's were found to increase in equal proportions in M-MSV producer cells over the S+L- cells, demonstrating that there was no selective amplification of a part of the M-MSV genome.

An interesting continuing observation in all systems was that a lower M-MSV copy number apparently gave a lower maximal hybridization value, suggesting that there might be qualitatively less M-MSV DNA. This question has

been dealt with in reconstruction experiments, where it was initially determined at the beginning of the experiment and at the end that essentially all cat S+L- clones yielded a normal M-MSV after rescue. Secondly, infection of S+L- cat cells with FeLV gave rise to a higher maximal hybridization. Thirdly, appropriate mixing of cat M-MSV DNA copy per cell resulted in a reduced maximal hybridization value (20; unpublished data). With a 10⁶-fold cell DNA excess over probe and one copy of a DNA that is 10⁻⁶ of the cell DNA, a hybridization maximum of 50% might be expected. As copy number increases, so should maximum hybridization levels in such a situation. Accordingly, where $C_{t,1/2}$ values show low copy number, caution is in order relative to assigning the proportion of the genome present or absent.

Data regarding the quantity of integrated leukemia virus DNA in heterologous mammalian cells was in general agreement with previous findings, with several notable exceptions. Specifically, SSAV DNA proviral copy numbers in two unrelated cell systems were much higher than those of some other mammalian eco- or xenotropic viruses. Secondly, most, if not all, FeLV DNA can be found as related nucleotide sequences in normal cat cells, and the infection of normal or S+L- cat cells resulted in an increase of some additional DNA copies into cat cell DNA (cf. reference 33 and Results). Whatever the role of leukemia helper virus might be in the amplification of M-MSV DNA proviral nucleotide sequences, there does not appear to be a simple relationship in the number of leukemia virus DNA copies to the number of M-MSV DNA copies integrated in the same cell.

Mechanistically, an increase of the M-MSV DNA copy number could have occurred in four ways: (i) reinfection with newly generated pseudotype M-MSV, (ii) a new round of reverse transcription with the enzyme supplied by helper virus, (iii) a "saltatory" replication of M-MSV DNA sequences, (iv) a select chromosomal endoreduplication. The latter can be most easily ruled out because normal, S+L- and P521 producer cat cells are all hypodiploid (modal number, 30) and no significant reproducible differences in chromosomes were seen (manuscript in preparation). Reinfection is possible but difficult to quantitate because of coexistence of helper virus in all M-MSV stocks. Varmus and Shank have found some evidence against reinfection as a mechanism controlling DNA copy number in ASV-infected duck cells (41). At this time none of the first three possibilities can be excluded.

The mode by which multiple RNA tumor virus copies may promote loss of growth control is

not known; however, since normal cells may have transforming RNA tumor virus-specific sequences as part of their genetic makeup, these genes may be under the influence of normal contiguous *cis* elements. Indeed, such an explanation was offered by Cooper and Temin in explaining the failure of transfection experiments with normal avian DNA that had an intact avian leukosis provirus (11). It can be inferred that proviral transcription could occur in a less restricted fashion at multiple sites that do not have these controlling nucleotide sequences.

Increased DNA copying of an RNA tumor virus could be important relative to neoplasia. An important observation made by Jaenisch was that mice into which M-MuLV was genetically introduced by the sire contained about one copy per cell of M-MuLV DNA in all organs. When the animals developed leukemia, up to four copies per cell were detected in leukemic organs (22). A similar increase in copy number was observed in leukemic organs of AKR mice (8). In the present system the morphology of all the S+L- cells with a low M-MSV DNA copy number was only slightly abnormal, whereas after leukemia virus infection the cells became hyperrefractile, loosely adherent, and could be grown in suspension cultures. Concomitant with the morphological changes, the P521 producer cat cells contained about 10-fold more M-MSV-specified viral RNA than found in the S+L- cells (20). It may well be that amplification of proviral DNA nucleotide sequences involved with neoplastic transformation may be widespread. The present availability of cDNA's representative of specific parts of transforming RNA tumor viruses allow further testing of cells transformed by both viruses and chemicals.

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LITERATURE CITED

- Aaronson, S. A., and F. R. Stephenson. 1974. Differential cellular regulation of three distinct classes of type C RNA viruses endogenous to mouse cells. Cold Spring Harbor Symp. Quant. Biol. 39:1129-1137.
- Aaronson, S. A., G. J. Todaro, and E. M. Scolnick. 1971. Induction of murine C-type virus from clonal lines of virus-free BALB/3T3 cells. Science 174:157-159.
- Bassin, R. H., N. Tuttle, and P. J. Fischinger. 1970. Isolation of murine sarcoma virus transformed mouse cells which are negative for leukemia virus from agar suspension culture. Int. J. Cancer 6:95-107.
- Bassin, R. H., N. Tuttle, and P. J. Fischinger. 1971. Rapid cell culture assay technique for murine leukemia viruses. Nature (London) 229:546-566.
- Benveniste, R., and G. J. Todaro. 1973. Homology between type-C viruses of various species as determined by molecular hybridization. Proc. Natl. Acad. Sci. U.S.A. 70:3316-3320.
- Benveniste, R. E., R. Heinemann, G. L. Wilson, R. Callahan, and G. J. Todaro. 1974. Detection of baboon type C viral sequences in various primate tissues by molecular hybridization. J. Virol. 14:56-67.
- Benveniste, R. E., and G. J. Todaro. 1974. Multiple divergent copies of endogenous type-C virogenes in mammalian cells. Nature (London) 252:170-173.
- Berns, A., and R. Jaenisch. 1976. Increase of AKR-specific sequences in tumor tissues of leukemic AKR mice. Proc. Natl. Acad. Sci. U.S.A. 73:2448-2452.
- Chattopadhyay, S. K., D. R. Lowy, N. M. Teich, A. S. Levine, and W. P. Rowe. 1974. Qualitative and quantitative studies of AKR-type murine leukemia virus sequences in mouse DNA. Cold Spring Harbor Symp. Quant. Biol. 39:1085-1101.
- Chattopadhyay, S. K., W. P. Rowe, and A. S. Levine. 1976. Quantitative studies of integration of murine leukemia virus after exogenous infection. Proc. Natl. Acad. Sci. U.S.A. 73:4095-4099.
- Cooper, G. M., and H. M. Temin. 1976. Lack of infectivity of the endogenous avian leukosis virus-related genes in the DNA of uninfected chicken cells. J. Virol. 17:422-430.
- Duc-Nguyen, H., E. N. Rosenblum, and R. F. Zeigel. 1966. Persistent infection of a rat kidney cell line with Rauscher murine leukemia virus. J. Bacteriol. 92:1133-1140.
- Fischinger, P. J., C. S. Blevins, and S. Nomura. 1974. Simple, quantitative assay for both xenotropic murine leukemia and ectropic feline leukemia viruses. J. Virol. 14:177-179.
- Fischinger, P. J., C. O. Moore, and T. E. O'Connor. 1969. Isolation and identification of a helper virus found in the Moloney sarcoma-leukemia virus complex. J. Natl. Cancer Inst. 42:605-622.
- Fischinger, P. J., and T. E. O'Connor. 1969. Viral infection across species barriers: reversible alteration of murine sarcoma virus for growth in cat calls. Science 165:714-716.
- Fischinger, P. J., and T. E. O'Connor. 1970. Productive infection and morphologic alteration of human cells by a modified sarcoma virus. J. Natl. Cancer Inst. 44:429-438.
- Fischinger, P. J., P. T. Peebles, S. Nomura, and D. K. Haapala. 1973. Isolation of an RD-114-like oncornavirus from a cat cell line. J. Virol. 11:978-985.
- Frankel, A. E., and P. J. Fischinger. 1976. Moloney sarcoma virus-specific nucleotide sequences in mouse DNA and RNA. Proc. Natl. Acad. Sci. U.S.A. 73:3705-3709.
- Frankel, A. E., and P. J. Fischinger. 1977. Rate of divergence of cellular sequences homologous to segments of Moloney sarcoma virus. J. Virol. 21:153-160.
- Frankel, A. E., D. K. Haapala, R. L. Neubauer, and P. J. Fischinger. 1976. Elimination of the sarcoma genome from murine sarcoma virus transformed cat cells. Science 191:1264-1266.
- Frankel, A. E., R. L. Neubauer, and P. J. Fischinger. 1976. Fractionation of DNA nucleotide transcripts from Moloney sarcoma virus and isolation of sarcoma virus-specific complementary DNA. J. Virol. 18:481-490.
- Jaenisch, R. 1976. Germline integration and Mendelian transmission of the exogenous Moloney leukemia virus. Proc. Natl. Acad. Sci. U.S.A. 71:1260-1264.

23. Khoury, A. T., and H. Hanafusa. 1976. Synthesis and integration of viral DNA in chicken cells at different times after infection with various multiplicities of avian oncornavirus. *J. Virol.* 18:383-400.
24. Leder, P., J. Ross, J. Gielen, S. Packman, Y. Ikawa, H. Aviv, and D. Swan. 1973. Regulated expression of mammalian genes: globin and immunoglobulin as model systems. Cold Spring Harbor Symp. Quant. Biol. 38:753-761.
25. Lowy, D. R., W. P. Rowe, N. Teich, and F. W. Hartley. 1971. Murine leukemia virus: high frequency activation *in vitro* by 5-iododeoxyuridine and 5-bromodeoxyuridine. *Science* 174:155-157.
26. McAllister, R. M., J. Nicolson, M. B. Gardner, R. W. Ronney, S. Rasheed, P. S. Sarma, R. J. Huebner, M. Hatanaka, S. Oroszlan, R. V. Gilden, A. Kabigting, and L. Vernon. 1972. C-type virus released from cultured human rhabdomyosarcoma cells. *Nature (London) New Biol.* 235:3-6.
27. Nomura, S., P. J. Fischinger, J. W. Hartley, and C. F. T. Mattern. 1976. Induction of a novel transforming virus (MSV-Z) from sarcoma positive leukemia negative 3T3FL mouse cells. *Virology* 69:635-646.
28. Nomura, S., P. J. Fischinger, C. F. T. Mattern, P. T. Peebles, R. H. Bassin, and G. P. Friedman. 1972. Revertants of mouse cells transformed by murine sarcoma virus. I. Characterization of flat and transformed sublines without a rescuable murine sarcoma virus. *Virology* 50:51-64.
29. Oskarsson, M. K., W. G. Robey, C. L. Harris, P. J. Fischinger, D. K. Haapala, and G. F. VandeWoude. 1975. A p60 polypeptide in the feline leukemia virus pseudotype of Moloney sarcoma virus with murine leukemia virus p30 antigenic determinants. *Proc. Natl. Acad. Sci. U.S.A.* 72:2380-2384.
30. Peebles, P. T., P. J. Fischinger, R. H. Bassin, and A. G. Papageorge. 1973. Isolation of human amnion cells transformed by rescuable murine sarcoma virus. *Nature (London) New Biol.* 242:98-101.
31. Quintrell, N., H. E. Varmus, J. M. Bishop, M. O. Nicholson, and R. M. McAllister. 1974. Homologies among the nucleotide sequences of the genomes of C-type viruses. *Virology* 58:568-575.
32. Robey, W. G., M. K. Oskarsson, G. F. VandeWoude, R. B. Naso, R. B. Arlinghaus, D. K. Haapala, and P. J. Fischinger. 1977. Cells transformed by certain strains of Moloney sarcoma virus contain murine p60. *Cell* 10:79-89.
33. Ruprecht, R. N., C. Goodman, and S. Spiegelman. 1973. Determination of natural host taxonomy of RNA tumor viruses by molecular hybridization: application to RD-114, a candidate human virus. *Proc. Natl. Acad. Sci. U.S.A.* 70:1437-1441.
34. Schincariol, A. L., and W. K. Joklik. 1973. Early synthesis of virus-specific RNA and DNA in cells rapidly transformed with Rous sarcoma virus. *Virology* 56:532-548.
35. Shoyab, M., M. A. Baluda, and R. Evans. 1974. Acquisition of new DNA sequences after infection of chicken cells with avian myeloblastosis virus. *J. Virol.* 13:331-339.
36. Theilen, G. H., D. Gould, M. Fowler, and D. L. Dungworth. 1971. C-type virus in tumor tissue of a woolly monkey (*Lagothrix* spp.) with fibrosarcoma. *J. Natl. Cancer Inst.* 47:881-889.
37. Todaro, G. J., R. Benveniste, R. Callahan, M. Lieber, C. Scherr. 1974. Endogenous primate and feline type C viruses. Cold Spring Harbor Symp. Quant. Biol. 39:1159-1168.
38. Varmus, H. E., J. M. Bishop, and P. K. Vogt. 1973. Synthesis and integration of Rous sarcoma virus-specific DNA in permissive and non-permissive hosts, p. 373-379. In C. F. Fox and L. Silvestri (ed.), *Virus research*. Academic Press Inc., New York.
39. Varmus, H. E., R. V. Guntaka, W. J. Fan, S. Heasley, and J. M. Bishop. 1974. Synthesis of viral DNA in the cytoplasm of duck embryo fibroblasts and in enucleated cells after infection by avian sarcoma virus. *Proc. Natl. Acad. Sci. U.S.A.* 71:3874-3878.
40. Varmus, H. E., S. Heasley, and J. M. Bishop. 1974. Use of DNA-DNA annealing to detect new virus-specific DNA sequences in chicken embryo fibroblasts after infection by avian sarcoma virus. *J. Virol.* 14:895-903.
41. Varmus, H. E., and P. R. Shank. 1976. Unintegrated viral DNA is synthesized in the cytoplasm of avian sarcoma virus-transformed duck cells by viral DNA polymerase. *J. Virol.* 18:567-573.