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Low-Multiplicity Infection of Moloney Murine Leukemia Virus in Mouse Cells: Effect on Number of Viral DNA Copies and Virus Production in Producer Cells

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Mouse cells infected with Moloney murine leukemia virus (M-MuLV) were prepared by two methods, and the number of M-MuLV-specific DNA copies in the infected cells was measured. The number of M-MuLV-specific DNA copies detected varied from one to eight per infected cell in different cell lines. Cells in which multiple rounds of viral infection occurred during establishment had on the average more viral DNA copies than cells in which infection at low multiplicity was performed, followed by cloning of the cells. However, even in cells derived by the low multiplicity of infection method, most cell lines carried more than one copy of M-MuLV-specific DNA. Virus production per cell was also measured, and no strict correlation was observed between the number of M-MuLV DNA copies present and the amount of virus produced.

The genetic information of RNA tumor viruses is integrated into the chromosomal DNA of infected cells (21). Depending on the virus and host cell studied, the number of integrated virus-specific DNA copies varies widely. Rat cells infected with Rous sarcoma virus carry up to 20 copies per cell (20), whereas chicken fibroblasts infected with the same virus carry only two to four copies per cell genome equivalent (13, 15, 21). Mouse fibroblasts infected with murine leukemia virus carry between four and eight copies per diploid genome equivalent (3, 7), whereas animals infected at the preimplantation stage with Moloney murine leukemia virus (M-MuLV) carry one copy of virus per diploid cell equivalent (9). In M-MuLV- and AKR-MuLV-induced lymphoma cells, virus-specific DNA sequences are present in six to eight copies per diploid cell equivalent (2, 10).

We were interested in studying whether the number of virus-specific sequences found in chronically infected cells can be influenced by the conditions of infection. Two general methods for preparing clonal isolates of RNA tumor viruses have been utilized. In one method (6), used especially for RNA tumor viruses which do not morphologically transform cells, a single infectious particle is used to infect a culture of cells; with serial transfer (and multiple cycles of infection), all of the cells eventually become infected by progeny of the originally infecting particle. In this cloning procedure, it is likely that the multiplicity of infection of the secondarily infected (majority) cells was greater than one, because infection occurs most efficiently by high-multiplicity infection from neighboring virus-producing cells. In the second method, cells are infected with a single infectious virus particle, and then the cells are cloned before secondary spread of infection occurs. The resultant virus-positive cells from such a procedure represent progeny of a single infectious event. Furthermore, because virus-producing cells are resistant to superinfection with the same virus, these virus-producing cells are the result of low-multiplicity infection.

In the results reported here, the two methods of cloning were compared for mouse cells infected with M-MuLV, and the number of copies of virus-specific DNA integrated, as well as the level of virus production, was measured.

MATERIALS AND METHODS

Cells and viruses. All cell lines were grown in Dulbecco-modified Eagle medium supplemented with 5% calf serum. M-MuLV clone 1 cells were described previously (6), and M-MuLV clone 2A cells were kindly provided by Robert Weinberg.

Infectious M-MuLV was obtained from tissue culture supernatant of M-MuLV clone 1 cells. The supernatant was clarified by low-speed centrifugation (10 min at 1,350 × *g*), and portions were stored at −70°C. One portion was thawed, and the infectivity titer of M-MuLV was determined by infection onto NIH-3T3 cells followed by the UV-XC cell plaque assay (14).

The multiple-infection-cycle cloning method has been described previously (6). Briefly, an M-MuLV

stock which had been titered for infectivity was used to infect replicate 9-cm tissue culture dishes of cells at a concentration of approximately 0.5 infectious units per tissue culture dish. Each infected culture was transferred serially three times, and then a representative dish from each infected culture was assayed for virus production. Those cultures found to be producing virus were used for further analysis. In the isolation described in this report, 10 initially infected cultures were tested, of which 2 were found to be producing M-MuLV. Because the infected cultures produced by this method were the result of many infection events, a subclone of one of the cultures (M-MuLV clone 3B) was also prepared (M-MuLV clone 3B-1b), and subclones of the M-MuLV clone 1 cells were also prepared.

The low multiplicity of infection method of cloning was performed similar to that of D. Smotkin and R. Weinberg (personal communication). A 9-cm tissue culture dish was seeded with 6×10^5 cells, and after 18 h (during which time the cells doubled) the cells were infected with 6×10^5 XC units of M-MuLV in 1 ml of growth medium containing 20 μg of polybrene (Aldrich) per ml. After 1 h at 37°C, 9 ml of additional medium was added, and the cells were placed in a 37°C incubator for 6 h. The infected cells were then trypsinized and diluted to 5 cells per ml, and 0.1 ml of the diluted cells was seeded onto individual wells of a microtiter plate made of tissue culture plastic (Falcon). The microtiter plate was then placed in a 37°C incubator and monitored after 1 to 2 weeks for the growth of individual cell colonies in the microtiter wells. Cells from wells containing individual colonies were trypsinized and transferred to tissue culture dishes. The cells were then assayed for virus production by the appearance of viral reverse transcriptase in the medium (see below). In three different isolations, 5 out of 12, 2 out of 15, and 4 out of 21 clones were found to produce virus.

Measurement of cellular DNA content. The DNA content of the producer cells was determined by fluorescent staining with the dye mithramycin (generously provided by Pfizer) by the method of Crissman and Tobey (4). Briefly, trypsinized cell suspensions were fixed and stained for 1 h at room temperature with 100 μg of mithramycin per ml. The stained cell suspension was then analyzed by flow microfluorimetry with an argon laser, and the fluorescence intensity of individual cells was measured. The fluorescence intensity of the cells in the G_1 portion of the cell cycle was proportional to the DNA content of the cell. As a standard, diploid mouse embryo fibroblasts were measured in the same manner.

Measurement of virus production. Virus production in the cell clones was measured by reverse transcriptase activity in tissue culture supernatant with exogenously added poly(rA):oligo(dT) template-primer. To obtain reliable estimates, it was important to assay exponentially growing cells and to take the number of cells into account. Tissue culture supernatant (10 ml) from 9-cm dishes containing exponentially growing cells at less than one-quarter confluency was harvested after 5 h of incubation at 37°C and clarified by low-speed centrifugation. The virus was then pelleted from the clarified supernatant by centrifugation for 45 min at 45,000 rpm in a Beckman 50 Ti rotor. The pelleted virus was resuspended in 50 μl of 0.01 M Tris (pH 7.4)–5 mM NaCl. Samples (5 and 10 μl) of the resuspended virus were assayed for reverse transcriptase in an exogenous reverse transcriptase assay. Briefly, the reaction volume was 0.1 ml, and contained 50 mM Tris (pH 8.3), 60 mM NaCl, 20 mM dithiothreitol, 0.6 mM $MnCl_2$, 1.0 μg of poly(rA) per ml, 0.3 μg of oligo(dT) per ml, 25 μCi of [^{3}H]TTP per ml (50 Ci/mmol), and 0.1% Nonidet P-40. Incubation was for 15 min at 37°C, and the reaction was then assayed for acid-precipitable radioactivity.

After removal of the tissue culture supernatant for reverse transcriptase assay, the cells were removed from the tissue culture dish by trypsinization and counted in a Coulter Counter (Coulter Electronics). A virus production index for the cells was calculated by dividing the reverse transcriptase activity by the number of cells present. A virus production index of 1 represents 1 unit of reverse transcriptase activity (1 pmol of [^{3}H]TTP incorporated in 15 min) liberated from 10^6 cells in 5 h. Typically, 2×10^4 to 5×10^5 cpm of [^{3}H]TTP incorporation was measured.

One possible artifact that may occur in assays of total tissue culture supernatant is that cellular nucleic acid polymerase which can also recognize the poly(rA):oligo(dT) template-primer complex might be present in pelleted cell debris (22). However, the same relative production indexes were obtained if the pelleted virus was assayed for reverse transcriptase with a poly(rC):oligo(dG) template-primer instead, although the total radioactive incorporation was approximately 10-fold lower (22). The poly(rC):oligo(dG) assay is specific for viral reverse transcriptase, and cellular polymerases are not detected (22).

cDNA. Virus-specific DNA probes were prepared from M-MuLV stocks in the presence of calf thymus primer (17), as described previously (8, 9). To remove sequences from the M-MuLV complementary DNA (cDNA) which cross-hybridize with other endogenous viruses, the cDNA was selected by failure to hybridize to DNA from BALB/c mice as described (2, 9). The selected cDNA hybridized to 6% to normal mouse DNA and to 92% to DNA extracted from M-MuLV-infected cells.

Extraction of cell DNA. DNA from tissue culture cells or from mouse tissues was extracted by the Kirby method (12) and purified as described (8). The nucleic acids were precipitated with ethanol and banded in CsCl-ethidium bromide. The dye was removed with isopropanol, the nucleic acids were boiled in 0.2 M NaOH for 15 min and neutralized, and the CsCl was removed by ethanol precipitation. The purified DNA had an average size of 5 to 6S on alkaline sucrose gradients.

Molecular hybridization. Molecular hybridization in excess cDNA was performed essentially as described by Berns and Jaenisch (2) and Jaenisch (9). Hybridizations were performed in a volume of 15 μl at 68°C in 1 M NaCl. DNA concentrations were chosen to give between 10 and 30% hybridization. This was achieved by annealing 7×10^{-5} to 10×10^{-5} μg of M-MuLV cDNA (2×10^7 cpm/μg) to 10 to 30 μg of cell DNA. The sizes of both the cDNA probe and the cell DNA were 5 to 6S. After 120 h of hybridization, the S_1

nuclease resistance of the cDNA was determined. Kinetic analyses have shown that the final level of hybridization is reached under these conditions.

After correction for background hybridization to BALB/c DNA and for maximum hybridization to leukemia DNA in cell DNA excess, the relative amount of cellular sequences complementary to the cDNA was computed from the equation: $X/(1 + X)$ = fraction hybridized, in which X is the fraction of M-MuLV-specific sequences in the cell DNA as compared with the input cDNA (1 in this equation). The number of M-MuLV copies per haploid mouse genome equivalent was computed from the equation: number of copies = [(X × input cDNA)/(input mouse DNA)] × (molecular weight of haploid mouse DNA/molecular weight of specific cDNA). The molecular weight of the haploid mammalian genome was assumed to be 1.96×10^{12}, and the complexity of the selected M-MuLV cDNA was assumed to be 10^6 (assuming a 33% representation of the probe; see reference 9).

RESULTS

To perform these experiments, a very sensitive method for quantifying low numbers of viral DNA copies present in infected cells was necessary, and such a method has been developed by one of us (9). Total cellular DNA was extracted from cells and annealed with M-MuLV-specific cDNA in cDNA excess. Because M-MuLV cDNA has considerable homology to DNA from uninfected mouse cells (3, 10), the probe was first selected by preparative annealing to uninfected BALB/c mouse DNA followed by removal of the annealed sequences, as described previously (9). When annealed to excess mouse DNA, the selected probe hybridized 92% to DNA from infected cells and 6% to normal BALB/c DNA.

The selected M-MuLV-specific cDNA was annealed to end point with amounts of unlabeled DNA from cells containing integrated M-MuLV DNA in a 3- to 10-fold molar excess of cDNA, and the final extent of cDNA annealing could be used to calculate the relative M-MuLV-specific DNA concentration in the unlabeled cellular DNA (9). To calculate the absolute number of M-MuLV-specific sequences by this method, the complexity of the probe must be known. The complexity of the selected cDNA probe was not directly determined due to the limited amounts available, but indirect evidence suggests that it represented 30 to 35% of the M-MuLV genome (9).

To calibrate these measurements, annealings were compared with annealings with DNA from mice genetically transmitting the gene for M-MuLV. Table 1 gives the final hybridization values of a cDNA excess hybridization with DNA extracted from animals homozygous or heterozygous for the M-MuLV locus (BALB/Mo mice). The number of M-MuLV-specific copies in these animals has been determined by independent criteria to be one and two copies per diploid cell genome equivalent, respectively (8, 9). Using a 33% representation of the specific probe to calculate the number of M-MuLV copies in Table 1, we arrive at 0.98 and 2.0 copies per diploid cell equivalent for heterozygous and homozygous BALB/Mo embryo DNA, respectively. Therefore, in all hybridization experiments, each selected cDNA preparation was annealed with DNA extracted from animals heterozygous and homozygous at the M-MuLV locus to standardize the hybridization reactions. All virus copy numbers calculated in the following experiments were standardized to the number of M-MuLV-specific sequences calculated in DNA extracted from heterozygous and homozygous BALB/Mo embryos.

TABLE 1. *Hybridization in cDNA excess: number of M-MuLV DNA copies per diploid mouse genome equivalent*[a]

Source of DNA	Fraction S_1 resistance	X value	No. of copies
Heterozygous BALB/Mo embryo	0.095	0.105	0.98
Homozygous BALB/Mo embryo	0.178	0.215	2
M-MuLV clone 2A	0.125	0.142	1.3
M-MuLV clone 1	0.301	0.430	4

[a] DNA extracted from heterozygous and homozygous BALB/Mo embryos and from M-MuLV-infected cell lines was hybridized to excess cDNA as described in the text. Mouse DNA was at 30 μg and M-MuLV cDNA was at 8×10^{-5} μg. The fraction of cDNA hybridized to BALB/c DNA (6%) was subtracted from the hybridization levels obtained and the X value, and the number of copies per diploid mouse genome equivalent was calculated as described in the text.

In preliminary experiments on clonally infected fibroblasts, two cultures of M-MuLV-infected NIH-3T3 cells infected with M-MuLV were studied. M-MuLV clone 1 cells had been prepared by the multiple cycle of infection method (6), and M-MuLV clone 2A cells had been prepared by the low multiplicity of infection method. The results of these measurements are also shown in Table 1. Approximately three times as many M-MuLV-specific DNA copies were present in the M-MuLV clone 1 cells as in the clone 2A cells, and, by comparison to the DNA from animals heterozygous and homozygous for genetically transmitted M-MuLV, these cell lines contained 4.0 and 1.3 M-MuLV DNA copies per diploid cell equivalent, respectively.

TABLE 2. *Virus-specific DNA content of different infected cells*[a]

Producer cell line	Method of cloning	Parental cell line	No. of M-MuLV DNA copies per diploid cell equivalent
M-MuLV clone			
1-1c[b]	Multiple infection cycle	NIH-3T3	4.8
1-5a	Multiple infection cycle	NIH-3T3	3.8
1-1a	Multiple infection cycle	NIH-3T3	4.0, 3.2, 2.9[c]
M-MuLV clone			
3A	Multiple infection cycle	NIH-3T3	2.5, 2.8
3B	Multiple infection cycle	NIH-3T3	2.5, 2.3
3B-1B[d]	Multiple infection cycle	NIH-3T3	2.2
M-MuLV clone			
2A	Low multiplicity	NIH-3T3	1.4, 1.4, 1.7
4A	Low multiplicity	NIH-3T3	1.0, 1.3, 1.2
4B	Low multiplicity	NIH-3T3	4.2, 4.3
M-MuLV clone			
H3	Low multiplicity	NIH-3T3	2.8
B7	Low multiplicity	NIH-3T3	1.0, 1.0
A9	Low multiplicity	NIH-3T3	0.6, 0.5
E7	Low multiplicity	NIH-3T3	4.1
A4	Low multiplicity	NIH-3T3	3.2
E5	Low multiplicity	NIH-3T3	1.5
C11	Low multiplicity	NIH-3T3	2.2
B10	Low multiplicity	NIH-3T3	1.7
M-MuLV clone			
T2-C6	Low multiplicity	SV-T2	1.0, 0.9
T2-D2	Low multiplicity	SV-T2	1.0, 1.0

[a] The number of M-MuLV DNA copies per diploid cell DNA equivalent in M-MuLV infected cells prepared by the two cloning methods was determined as in Table 1. The parental cell lines of the infected cells are also indicated.

[b] Three subclones of the M-MuLV clone 1 cells.

[c] Multiple values represent separate hybridization experiments.

[d] A subclone of the M-MuLV clone 3B cells.

These results suggested a possible multiplicity of infection effect on the number of viral DNA copies present in the infected cells.

To further investigate the question, additional clonal isolates of M-MuLV infected into NIH-3T3 cells or into simian virus 40-transformed BALB/c cells (SV-T2) were prepared by the two cloning methods, and the number of M-MuLV DNA copies in the cells was determined. The results are shown in Table 2. The additional two cultures of M-MuLV-producing cells prepared by the multiple cycle of infection method also had more than two copies of M-MuLV-specific DNA per diploid cell equivalent, similar to the M-MuLV clone 1 cells. When the additional M-MuLV-producing cultures prepared by the low multiplicity of infection method were examined, a considerable number had less than two copies of M-MuLV DNA per diploid cell equivalent, similar to the M-MuLV clone 2A cells, but some of the culture also had more copies. In summary, all three cultures prepared by the multiple cycle of infection method had greater than two copies of M-MuLV per diploid cell equivalent, whereas a majority (8/13) prepared by the low multiplicity of infection method had less than two copies per diploid cell equivalent. These results therefore indicate that low multiplicity of infection may increase the possibility that the infected cells will contain a low number of M-MuLV DNA copies. However, this effect is not absolute, because some cultures contained as much or more viral DNA than cultures prepared by the multiple cycle of infection method.

Some of the M-MuLV-producing cultures of Table 2 were further analyzed to determine whether a gene dosage effect on virus production could be observed. First, a determination of the number of viral DNA copies per cell (rather than per diploid DNA equivalent) was made. To convert the value of Table 2 to number of viral DNA copies per cell, it was necessary to measure the DNA content of the infected cells. The DNA content was determined by staining cells with

the fluorescent dye mithramycin followed by quantitation of fluorescence in a flow microfluorimeter, as shown in Fig. 1. By comparison with diploid mouse embryo secondary fibroblasts, the cell lines derived from the NIH-3T3 cells were subtetraploid, whereas the cell lines derived from the SV-T2 cells were pseudodiploid, similar to the parental cell lines (5). From the relative DNA content of the M-MuLV-producing cells and the number of copies of M-MuLV DNA per diploid DNA equivalent in Table 2, the number of M-MuLV DNA copies per cell could be calculated, as shown in Table 3. Virus production was also measured from these cultures by harvesting tissue culture supernatant from exponentially growing cells and quantifying the amount of virus released by enzymatic assay for viral reverse transcriptase. A virus production index is shown in Table 3. The results indicate that, whereas the best virus-producing line (M-MuLV clone 1) had several (six to eight) copies of M-MuLV DNA, other cell lines showed a wide fluctuation in virus production independent of the number of M-MuLV DNA copies integrated. Therefore, a strict gene dosage effect on virus production was not observed, and other regulatory mechanisms for the amount of virus production must also exist. One interesting pair are the lines M-MuLV clone B7 and clone A9, which were both derived by low-multiplicity infection on NIH-3T3 cells. Clone A9, which contains only one copy of M-MuLV DNA per cell, produces large amounts of virus, whereas clone B7, which contains twice the number of M-MuLV DNA copies, produces very little virus.

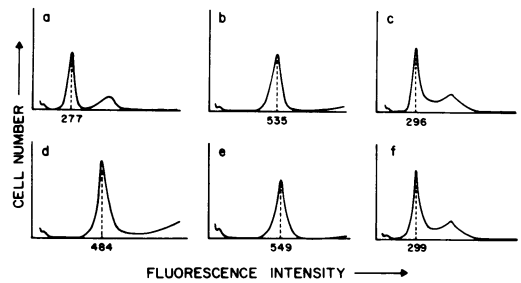

FIG. 1. *Cellular DNA content of infected and uninfected cells. Infected and uninfected cells were stained with mithramycin and analyzed individually for fluorescence in a flow microfluorimeter. The number of cells as a function of fluorescence intensity (in arbitrary units) is shown. The fluorescence intensity is proportional to the DNA content of each cell, and those cells in the peak of low fluorescence (best seen in a, c, and f) are cells in the G_0 and G_1 portions of the cell cycle (4). In cell lines with high DNA content, only the G_0 and G_1 cells were visible in the display. The fluorescence channel containing the peak of G_0 and G_1 cells is indicated. (a) Diploid secondary mouse embryo fibroblasts; (b) uninfected NIH-3T3 cells; (c) uninfected SV-T2 cells; (d) M-MuLV clone 1 cells; (e) M-MuLV clone A9 cells; (f) M-MuLV clone T2-D2 cells.*

TABLE 3. *Virus production and DNA copy number in infected cells*[a]

Producer cell line	DNA content per cell (times diploid)	Viral DNA copies per cell	Virus production index
M-MuLV clone			
1	1.74	7.0	26.4, 19.6
1-1a	1.79	6.0	5.2
3a	1.67	4.4	6.2
M-MuLV clone			
4A	1.83	2.1	4.0
4B	1.77	7.5	8.4
M-MuLV clone			
B7	2.00	2.0	0.6, 1.70
A9	1.98	1.1	15.2, 13.4
M-MuLV clone			
T2-C6	1.07	1.0	4.8
T2-D2	1.08	1.1	5.4
NIH 2° fibroblasts	1.00		
NIH-3T3	1.93		

[a] The total cellular DNA content per cell of some of the cell lines in Table 2 was measured in comparison to diploid mouse cells by fluorescent staining and flow microfluorimetry as shown in Fig. 1, and the values from Table 2 were then converted to number of M-MuLV DNA copies per cell. The amount of virus produced by the different cell lines was measured as described in the text, and virus production indexes are shown. Uninfected cell supernatants gave production indexes of 1.0 to 1.5 (average, 1.3), presumably due to cellular DNA polymerase which can recognize the poly(rA): oligo(dT) template-primer combination (22); the production indexes were corrected for the uninfected cell values.

DISCUSSION

These results indicate that low numbers of M-MuLV-specific DNA copies are stably integrated into mouse cells after infection and that the number of copies differs in different cell lines. Infection at low multiplicity followed by cloning of individual cells before virus spread occurred can result in infected cells which contain only one copy of M-MuLV-specific DNA. However, other infected cells derived in the same manner have more copies. Cloning in which multiple cycles of infection occur appears to increase the probability that the infected cells will have multiple M-MuLV-specific DNA copies, perhaps due to the higher multiplicity of

infection involved. Even in cells derived by the multiple infection cycle method, the number of viral DNA copies present is relatively low (less than 10/cell), which may indicate that there are a limited number of integrated sites for M-MuLV DNA in mouse cells. Studies on the integration of viral DNA copies in leukemic tissues of MuLV-infected mice and also of chicken fibroblasts infected with avain RNA tumor viruses also indicate that a limited number of integration sites for viral DNA may exist (2, 10, 11).

It should be noted that the virus-producing cell lines studied here were examined many cell generations after the original infection event(s). Therefore, the virus-specific DNA copies measured were those which became stably associated with the cell, and a different situation of virus-specific DNA may have existed in the cells shortly after infection (see below). Additionally, selection of a subpopulation of the infected cells may have occurred, especially in cells derived by the multiple infection cycle method.

The fact that infection at multiplicities of less than one resulted in cells with more than one copy of M-MuLV-specific DNA was somewhat surprising. We initially assumed that cells infected with a single infectious unit of M-MuLV would contain virus-specific DNA which was transcribed only from that infectious particle, as suggested by studies on duck cells infected with avian sarcoma virus (18). However, the results reported here indicate that infection with a single infectious unit of M-MuLV leads to the production of more than one virus-specific DNA molecule in the cell. Several explanations could account for this result: (i) multiple copies of viral DNA might be transcribed from the same viral RNA genome of the infecting virus particle; (ii) the multiple viral DNA copies might reflect the fact that the fraction of M-MuLV virus particles which are infectious is quite small, and that noninfectious (defective) particles might also be reverse transcribed and integrated at early stages of infection; (iii) the infectious particles might represent aggregates of virus particles; (iv) during culture of the infected cells, reinfection by virus produced from the same or a neighboring cell might occur, although the existence of stable cell lines with only one M-MuLV DNA copy argues against this.

The appearance of virus-specific DNA in chicken cells infected with avian RNA tumor viruses at different times after infection has been studied (1, 11). After infection, a relatively large number of viral DNA molecules are present in the cells, which are mostly unintegrated. With continued tissue culture transfer, a low number of the viral DNA molecules are established in the cells as integrated DNA molecules. Therefore, not all viral DNA molecules synthesized in cells after infection are stably integrated into the cellular DNA. In one study (11), the maximum number of viral DNA molecules (at 40 h post-infection) was somewhat dependent on the multiplicity of infection, but, even at a multiplicity of less than one infectious particle per cell, multiple unintegrated virus-specific DNA copies were present. These observations may explain our results, if a similarly large number of viral DNA copies is synthesized in mouse cells after infection with M-MuLV and if this number is multiplicity dependent. Infection at high multiplicity might thus increase the probability that more M-MuLV-specific DNA molecules are integrated. Even at low multiplicity more than one intracellular copy of unintegrated viral DNA might be present early after infection, and it would be possible for more than one M-MuLV-specific DNA copy to be integrated.

It is likely that the M-MuLV-specific DNA in the infected cells studied here was integrated in the host chromosomal DNA, even though evidence for continued presence of unintegrated viral DNA copies in duck cells infected with avian sarcoma virus has been reported (19). Two lines of evidence support this. First, the existence of stable virus-producing cell lines which contain only one copy of M-MuLV DNA is best explained by this hypothesis. Second, "blot-transfer hybridization" experiments (16) of restriction enzyme-digested cellular DNA indicate that the great majority of M-MuLV-specific DNA in the infected cells is integrated into host cell DNA sequences (L. Bacheler and H. Fan, Mod. Trends Leuk. Res., in press).

It should be noted that the selected M-MuLV-specific cDNA probe used in these experiments was by necessity not representative of the entire M-MuLV genome. Integration of portions of M-MuLV DNA complementary to those sequences removed from the M-MuLV cDNA probe during the selection procedure was therefore not measured. It is thus possible that additional integration of partial copies of the M-MuLV DNA representing those regions may have occurred, and these molecules would not have been detected. Similarly, it is possible that the M-MuLV-specific DNA measured in our experiments may have represented integration of only a fraction of the M-MuLV DNA genome.

These results indicate that several sites for integration of M-MuLV-specific DNA are available in tissue culture fibroblasts. Furthermore, a situation in which only one or a subset of the total available integration sites is occupied by

M-MuLV DNA is a stable situation for producer cells. Because these cells have been repeatedly transferred without any observed increase in the number of M-MuLV DNA copies, the intracellular synthesis and reintegration of viral DNA is apparently a rare phenomenon for the cells studied here.

The number of M-MuLV DNA copies in tissues of mice genetically transmitting M-MuLV or AKR strain MuLV has also been identified. In non-tumor tissues, the number of viral DNA copies is one per haploid genome for M-MuLV-transmitting mice (8, 9) and two for AKR strain mice (2). An amplification of the number of viral DNA copies is observed in tumor cells, with two to four additional DNA copies present per haploid genome. It is unclear whether these additional sites of viral DNA integration in leukemic lymphoblasts are the same sites or different from those available during in vitro infection of fibroblasts as studied here. This question is presently being investigated.

The infected cell lines with only one copy of M-MuLV DNA are of considerable utility for a variety of biochemical analyses. These cell lines should make it more easy to study the integration site of the M-MuLV DNA, because only one copy of the integrated DNA is present. These cell lines should also be very useful to study the expression of M-MuLV at the level of chromatin organization and viral RNA transcription. In cell lines which contain more than one copy of viral DNA, it is unclear which of the viral DNA sequences are transcribed into viral RNA. The cell lines which contain only one copy of viral DNA remove this uncertainty.

A gene dosage effect on the amount of virus produced was not observed in these experiments. Several explanations might account for this result: (i) whereas several integration sites might exist for M-MuLV DNA, only one might be effective in yielding progeny virus; (ii) the amount of virus-specific RNA synthesis might be regulated in infected cells, such that a constant amount of virus-specific RNA is synthesized regardless of the number of template DNA molecules; (iii) the level of viral protein synthesis might be regulated in the cell independently of the number of viral DNA and/or RNA molecules; (iv) some of the M-MuLV-specific DNA copies detected by molecular hybridization may have been defective and unable to express viral functions. Further study of the cell lines described here with respect to the viral DNA integration sites, virus-specific RNA synthesis and virus-specific protein synthesis might elucidate this phenomenon.

Recent results indicate a note of caution in handling cell lines such as those described here. Storage of frozen cells in liquid nitrogen followed by thawing and subsequent growth resulted in an increase of M-MuLV DNA copy number in some of the cell lines. For instance, after thaw, the A9 cell line was found to have increased in M-MuLV DNA copy number fourfold, whereas the B7 cell line showed no increase. One possible reason for this may have been the presence of dimethylsulfoxide in the cell-freezing medium: this agent greatly enhances the permeability of cells, and it may have been able to circumvent the normal surface resistance to superinfection of MuLV-producing cells. Another possible explanation is that a minority population of cells with different numbers of M-MuLV DNA copies may have been selected out during the process of freezing and thawing. In any event, it may be advisable to avoid freezing of cells if the number of M-MuLV DNA copies present in a cell lines is important.

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