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Evidence that retroviruses integrate into post-replication host DNA

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We have studied the question of whether a retrovirus integrates into the chromosomal DNA of the host cell before or after the DNA is replicated during the S phase of the cell cycle. We have infected single NIH-3T3 cells with BAG, a replication-incompetent retroviral vector which encodes the *lacZ* gene, then observed the clones derived from these cells to discover whether all the cells carry a copy of the proviral DNA. We have discovered that only half of the progeny of an infected cell carries a copy of the provirus. This indicates that the virus only integrates into post-replication DNA. We discuss the implications of this result for applications of retroviruses, such as gene therapy and cell lineage, which use them as vehicles for gene transfer into stem cells.

Key words: cell lineage/gene transfer/integration/retrovirus

Introduction

Retroviruses have become a very popular means of gene transfer. They have been used for cell lineage studies (Lemischka *et al.*, 1986; Sanes *et al.*, 1986; Price *et al.*, 1987), to transfer specific cDNAs (Williams *et al.*, 1986; vanBeusechem *et al.*, 1992) and for gene knockout (Galileo *et al.*, 1992). It has become axiomatic in all such studies that the retroviral genome is inherited by all the progeny of an infected cell. However, there is an aspect to this inheritance that is unclear because of an ambiguity associated with the process of retroviral integration.

Integration is a crucial step in the retroviral life cycle. It is the process by which a DNA copy of the viral genome becomes inserted into the chromosomal DNA of the infected cell (Panganiban, 1985; Goff, 1990). After reverse transcription from the viral RNA genome, the linear viral DNA gains access to the host cell chromosomal DNA as part of a nucleoprotein complex (Bowerman *et al.*, 1989). As a consequence primarily of the viral integrase activity (Brown *et al.*, 1989), this viral DNA becomes integrated into the chromosomal DNA. This event has two primary consequences. First, the viral genome can be transcribed and translated using the cell's normal machinery. Second, the provirus is inherited by the progeny of the infected cell.

Although the integration process is driven primarily by virus-encoded enzymes, various aspects of the host cell physiology are thought to be important. The most obvious of these is the cell cycle. In general, cells stationary in G_1 cannot integrate viral DNA (Harel *et al.*, 1981; Chen and Temin, 1982; Hsu and Taylor, 1982; Richter *et al.*, 1984; Springett *et al.*, 1989; Miller *et al.*, 1990), although this is not true of all cells and all retroviruses (Lewis *et al.*, 1992). Several studies have indicated that S phase is critical for integration (Harel *et al.*, 1981; Humphries *et al.*, 1981; Hsu and Taylor, 1982; Springett *et al.*, 1989; Miller *et al.*, 1990), although precisely what takes place during this phase is not clear. Certainly the virus can gain entry into the cell prior to S phase, but it seems unable to integrate. Humphries *et al.* (1981), studying Rous sarcoma virus, were able to follow the timing of the appearance of integrated provirus following the release from block in G_1. They found that the integrated DNA appeared synchronously with S phase, suggesting that that was when the process of integration occurred. The timing of integration, however, has since been reinvestigated by Roe *et al.* (1993) using murine leukaemia virus. They suggest that it occurs later in the cell cycle.

This ambiguity raises a question of fundamental importance to those applications of retroviruses mentioned above: does the retroviral DNA integrate before or after the chromosomal DNA replicates? The cell's chromosomal DNA is replicated during S phase. If integration were to take place then, the viral DNA could theoretically integrate into the host cell chromosome either before or after it had replicated. If it integrated into pre-replication DNA, then both daughter cells would inherit a copy of the provirus at the next cell division. If, however, the viral DNA were to integrate exclusively into replicated DNA, then only one daughter cell would inherit the provirus. Integration during G_2 or M must naturally be into post-replication DNA with only one daughter cell inheriting the provirus.

This point is clearly significant for studies using retroviruses, especially those in which embryonic or adult precursor cells are being labelled. If the infected cell were an asymmetrically dividing stem cell, the two daughters could give rise to quite different cell types. So it is important to know whether one or both halves of a lineage are inheriting the provirus.

We have addressed this question by infecting single NIH-3T3 cells with replication-incompetent murine leukaemia virus (MLV) vectors that carry a histochemical marker gene. Using both PCR and the expression of the marker, we have asked whether one or both daughters of the infected cell inherits a copy of the provirus when the infected cell subsequently divides. We have discovered that approximately half the progeny of an infected cell inherits the provirus. Thus we conclude that proviral integration is into post-replication DNA.

Results

NIH-3T3 cells were plated at single cell density in Terasaki multiwell plates. The cells were fluorescently labelled so that after 12–15 h they could be viewed under an epifluorescence microscope to determine unequivocally which wells

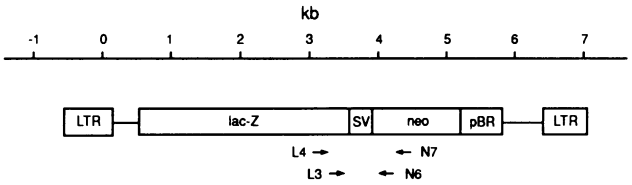


Fig. 1. The BAG vector and PCR primers. The BAG vector is as previously described (Price *et al.*, 1987). It contains the *lac-Z* and *neo* genes, the former driven from the endogenous retroviral promoter in the long terminal repeat (LTR) and the latter from the SV40 early promoter (SV). The construct also includes the pBR origin of replication (pBR). The arrows indicate the position and orientation of the PCR primers.

contained a single cell. Immediately after this was determined, single cells were infected with BAG (Figure 1), a replication-incompetent retrovirus encoding β-galactosidase (Price *et al.*, 1987). The division of cells was monitored 24 and 48 h post-infection. After 4–5 days, the plates were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). With the low titre of virus used, an average of 3% of single cells exposed to virus gave clones which included blue (stained) cells. There were 49 such multiple-cell clones from 15 experiments (Table I). Thirty-nine (80%) of these had blue cells together with unstained cells (Figure 2). On average, the ratio of blue to unstained cells was 177:217, a ratio of 0.82:1. The remaining 10 (20%) were composed entirely of blue cells, an anomalous result which is discussed below. In many cases the plated single cell failed to divide at all within the first 72 h. None of the 244 such cells we observed subsequently stained blue, supporting the hypothesis that non-dividing cells cannot integrate viral DNA. We did observe cases, however, in which a cell did not divide within 24 h of exposure to virus, yet divided during the subsequent 24 h and gave rise to blue progeny. Thus, >24 h can elapse between exposure to virus and the infected cell's next mitosis, yet the viral DNA can still integrate.

The finding that the progeny of single infected cells are mixed blue and unstained could be interpreted in two different ways: either the provirus had been inherited by only half the progeny of the infected cells, or all of the progeny carried a provirus but half of the cells had turned off expression. To distinguish between these possibilities, we infected clones as above, allowed them to grow for 7–8 days, then subcloned the progeny as single cells. These subclones were allowed to grow for up to 3–7 days, then stained with X-gal. In total, 36 such subclones were analysed; 35 were composed entirely of either blue or unstained cells. The single exception was probably the result of two cells being plated in one well at the subcloning stage. The fluorescent labelling to verify single cell plating was not used during subcloning because the number of cells was so small, and the risk of losing or damaging them so great.

After staining the subclones, their DNA was extracted and amplified by PCR to detect integrated provirus. Blue subclones invariably gave a positive PCR signal even when the subclone was composed of a single cell; unstained subclones invariably failed to give a signal (Figure 3). Thus, there is a perfect correlation between X-gal stain and the presence of provirus as detected by PCR.

Cell death

The finding that 20% of clones were all-blue raised the possibility that in some cases both of the progeny of an infected cell inherit the provirus. There are, however, two other possible explanations of this finding. Either the infected cell could have acquired two copies of the provirus which went on to segregate between the two progeny, or one daughter cell (the one without a copy of the provirus) could have died after the first division. Since each viral particle is thought to generate a single provirus, the first explanation would have to mean that the cell was infected by more than one particle. This is unlikely for two reasons. (i) These experiments were conducted with viral titres which produce such a low rate of infection (see above) that according to the Poisson distribution, double infections would be expected in less than one in 1000 clones. (ii) In another series of experiments, we infected single cells with equal titres of BAG and of a second virus, DAP, which carries the alkaline phosphatase gene (Fields-Berry *et al.*, 1992). We observed mixed stained and unstained clones with both viruses, but no clones stained for both viruses (data not shown), although this would have been expected if double infections were occurring.

Cell death, however, was a factor in these experiments. In many cases, we observed a loss of cells from clones during the period of culture. Often single cells disappeared, or two-cell clones were reduced to a single cell. Sometimes we noticed this specifically in clones that subsequently proved to be composed of one blue cell (e.g. clones 9.1, 13.3, 14.5 and 14.11; Table I). We measured the rate of cell loss in these experiments and found that 31% ± 12 of cells disappeared during the first 24 h in culture, and 37% ± 15 during the subsequent 24 h. Thus about a third of infected clones will lose a cell between the first and second division, i.e. a third of two-cell clones will lose a cell. In half of these cases, the blue cell will die leaving a completely unstained (and hence undetected) clone. In the remaining half (i.e. roughly 16% of the total), the unstained cell will die leaving an all-blue clone. This accords well with the observed frequency of all-blue clones (20%). Thus, the all-blue clones are of the frequency we would expect as a result of cell death, and are not evidence for the inheritance of provirus by both daughters of an infected cell.

Discussion

These results show that the integration of viral DNA is predominantly into post-replication DNA. In ~80% of clones, approximately half the progeny of an infected cell carry the provirus and half do not. The observed proportion of blue cells in these clones is actually slightly less than half, a fact which could reflect a slight growth disadvantage for cells carrying the transgene. This is probably not the case, however, since the discrepancy can be entirely accounted for by the single largest clone (10.4) being disproportionately unstained. In the remaining 20% of clones all the cells were blue. We do not take this as evidence for integration into pre-replication DNA, however, because all-blue clones will occur at approximately this frequency simply as a result of cell death. Indeed, in most cases where a single blue cell resulted from an infection, we had previously observed that

Table I. The result of X-gal staining clones of NIH-3T3 cells derived from single BAG-infected cells

Exp. No.	Single cells plated	Clone No.	Clone size	Blue:Unstained	1D/2D
Exp. 1	62	1.1	9	4:5	1D
		1.2	4	2:2	1D
		1.3	4	1:3	1D
		1.4	2	2:0	2D
		1.5	6	2:4	2D
		1.6	9	4:5	1D
Exp. 2	120	2.1	14	4:10	1D
		2.2	12	2:10	2D
		2.3	10	10:0	1D
Exp. 3	64	3.1	35	17:18	2D
		3.2	32	16:16	1D
		3.3	9	7:2	1D
Exp. 4	82	4.1	10	6:4	n.d.
		4.2	3	2:1	1D
		4.3	12	6:6	n.d.
		4.4	subcloned		1D
		4.5	subcloned		2D
Exp. 5	86	5.1	3	3:0	1D
		5.2	6	2:4	2D
		5.3	16	11:5	1D
Exp. 6	150	6.1	4	4:0	1D
		6.2	5	3:2	2D
Exp. 7	110	7.1	5	3:2	1D
		7.2	2	1:1	1D
Exp. 8	124	8.1	subcloned		1D
		8.2	6	6:0	n.d.
Exp. 9	159	9:1	1	1:0	2D
Exp. 10	109	10:1	3	2:1	1D
		10.2	3	3:0	1D
		10.3	26	14:12	1D
		10.4	59	13:46	n.d.
Exp. 11	141	11.1	7	7:0	2D
		11.2	500	~1:1	2D
Exp. 12	155	12.1	5	3:2	2D
Exp. 13	175	13.1	2	2:0	1D
		13.2	6	4:2	1D
		13.3	1	1:0	1D
		13.4	18	9:9	2D
		13.5	23	11:12	1D
		13.6	5	1:4	1D
Exp. 14	178	14.1	2	1:1	n.d.
		14.2	4	2:2	n.d.
		14.3	7	3:4	1D
		14.4	7	7:0	2D
		14.5	1	1:0	1D
		14.6	9	3:6	1D
		14.7	3	2:1	1D
		14.8	2	1:1	n.d.
		14.9	8	7:1	1D
		14.10	12	4:8	1D
		14.11	1	1:0	n.d.
		14.12	3	3:0	2D
Exp. 15	57	15.1	7	3:4	1D
Total B:U				177:217	

Fifty-three clones (49 multiple-cell clones plus four one-cell blue clones) were generated from 15 experiments and either stained with X-gal or subcloned. The second column records the number of wells which contained single cells after 15 h. The third column records those cells that subsequently divided to give a clone of cells that stained partly or wholly with X-gal. The clone size is recorded (column 4), as is the number of stained (blue) and unstained cells in each clone (column 5). Also recorded (final column) is whether the infected cell divided within 1 day (1D) or 2 days (2D) of infection. For some clones this was not determined (n.d.). The total figure of blue and unstained cells does not include the data from those clones that were entirely blue. Note that clone 11.2 was handled atypically in that it was subcloned onto a 90 mm dish and grown to ~500 cells before staining. The proportion of blue to unstained cells was not accurately determined, but was ~1:1.

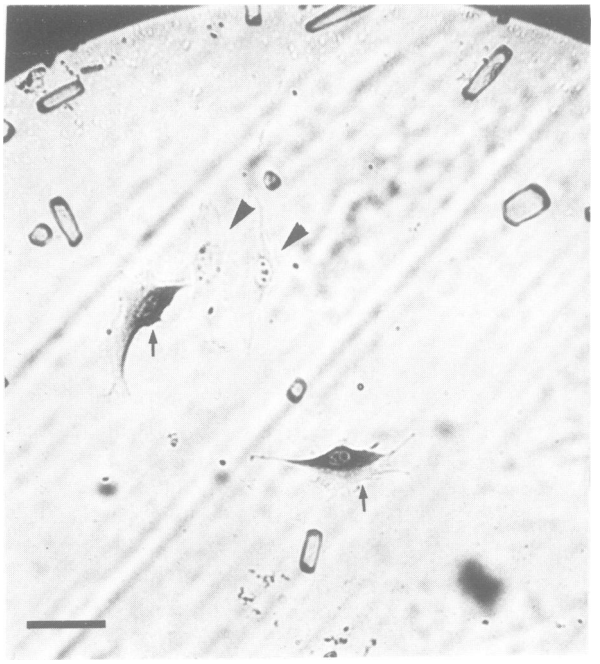


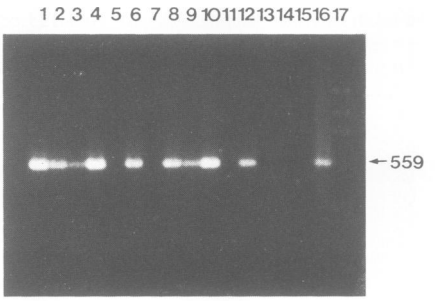
Fig. 2. A clone of BAG-infected NIH-3T3 cells. The photomicrograph shows a clone of four cells stained with X-gal, which were derived from a single, BAG-infected cell. Two cells (arrows) show positive staining for X-gal. The other two (arrowheads) are negative. Scale bar = 85 μm.

the clone had been composed of two cells, one of which died. Naturally, these results still leave open the possibility that a very small proportion of proviruses integrate into pre-replication DNA, but that hypothesis is not required to explain these data.

The subcloning experiments and the PCR analysis are important here because they indicate a perfect correlation between retroviral gene expression and viral integration. They allow us, therefore, to exclude two hypotheses: that a proportion of the progeny of the infected cell is unstained because they failed to express the provirus; and also that expression could result from transient expression of unintegrated virus.

Our results mean either that integration takes place after S phase of the cell cycle, or that it occurs during S phase and is specifically directed towards newly synthesized DNA. The latter explanation seems unlikely because it would require specific factors to direct integration that would only be available in S phase. There is no evidence for such factors, and studies of integration *in vitro* suggest that viral DNA is not specifically directed since it can integrate into such unlikely substrates as λ DNA (Brown *et al.*, 1987). The former explanation is more likely in light of the evidence from Roe *et al.* (1993) that the viral DNA only gains access to the chromosomal DNA with the breakdown of the nuclear membrane at the start of M phase. Our data would concur with this suggestion.

We found that cells never subsequently stained blue had they not divided. Thus there is a perfect correlation between division and integration in our assay. Nonetheless, in a third of our infected cells >24 h elapsed between exposure to virus and their next mitosis, yet successful integration still resulted. This argues for a stable intermediate form of the virus that can survive the 24 h between infection and integration. This contrasts with the results of Miller *et al.*



Clone Number	Subclone Number	Blue:Unstained	PCR lane
4.4	4.4.1	0:1	
	4.4.2	0:1	
	4.4.3	0:1	
	4.4.4	0:2	5
	4.4.5	0:9	7
	4.4.6	2:0	6
	4.4.7	18:0	4
	4.4.8	2:0	3
	4.4.9	3:0	2
	4.4.10	2:0	1
4.5	4.5.1	1:0	8
	4.5.2	6:0	
	4.5.3	21:0	9
	4.5.4	1:0	
	4.5.5	14:0	10
	4.5.6	3:0	
	4.5.7	11:0	12
	4.5.8	0:1	11

Other PCR lanes

13	Uninfected cells plus 1st. primer set 3/6
14	Uninfected cells plus 2nd. primer set 4/7
15	No DNA
16	Infected cell DNA
17	ΦX DNA + Hae III

Fig. 3. The analysis of subclones. Subclones, taken from the clones shown in Table I, were stained with X-gal. Each subclone was invariably composed of either blue or unstained cells (column 3, Blue:Unstained). In three experiments, the DNA from the subclones was amplified by PCR in order to detect the presence of the BAG provirus. The result of one such PCR experiment is shown. In each case the blue subclones gave the expected band of 559 bp, even when the subclone was a single cell (see lane 8); in no case did an unstained subclone give a band. The subclones analysed by PCR are indicated by a lane number referring to the figure.

(1990) who failed to find evidence for such a stable intermediate. They infected non-dividing cells and found that the cells failed to integrate viral DNA when subsequently released from block. Thus they argued that the cells did not form a stable intermediate while blocked in G_1. The obvious difference between their experiment and ours is that our cells are not prevented from passing through S phase, so perhaps that is when a stable intermediate is generated. We have to acknowledge, however, that we do not know precisely when infection occurs in relation to the point of

exposure to virus, although we do know that exposure to virus was for a maximum of 4 h.

These considerations notwithstanding, the principal importance of our conclusion is its significance for applications of retroviruses. Consider, for example, a plate of cells infected with a viral vector, which after 2–3 days is assayed to detect virus. The fraction of labelled cells will not be equal to the fraction of cells that was infected. Rather, it will equal half the fraction infected; i.e. if 10% of the cells carry virus then 20% of the original population were infected; 100% infection of the cells (with single multiplicity) would theoretically give only 50% labelled cells, assuming no selection is made for cells carrying the virus. This factor is not currently taken into consideration in assays of efficiency of infection with retroviruses.

For some studies, the implications are more profound. In many studies, such as those of cell lineage (Lemischka *et al.*, 1986; Sanes *et al.*, 1986; Price *et al.*, 1987), gene transfer with a view to gene therapy (Williams *et al.*, 1986; vanBeusechem *et al.*, 1992), or gene knockout (Galileo *et al.*, 1992), retroviruses are used to infect precursor cells. Only half of the progeny of such cells will carry the provirus. If the precursor cell divides symmetrically to give two cells similar to itself, then effectively the lineage has been labelled just one cell cycle later than if both daughter cells had inherited the provirus. Frequently, however, retroviruses are used to transfer genes into stem cells that are known to divide asymmetrically to give another stem cell and a committed cell (Williams *et al.*, 1984). Again only one of these two daughter cells will inherit the provirus, but in only 50% of cases will this be the stem cell; in the other 50%, it will be the committed daughter. Thus although the infected cell was a stem cell, possibly with a broad developmental potential, half of the infected clones will be made up entirely of committed progeny. Moreover, this will be true however early or late in the proliferative history of this lineage the infection takes place. This effect will be particularly dramatic in the nervous system where the committed daughter cell is often a post-mitotic neurone. This seems a likely explanation of why so many one-cell neuronal clones have been discovered following the infection of neural precursor cells with a retrovirus (Price and Thurlow, 1988; Williams *et al.*, 1991; Moore and Price, 1992; Walsh and Cepko, 1992).

Materials and methods

Single cell cloning

NIH-3T3 cells were trypsinized from stock plates and labelled in suspension with the fluorescent dye, CFSE (5-carboxyfluorescein diacetate, succinimidyl ester, Molecular Probes, 30 μM) for 45 min in normal tissue culture medium [Dulbecco's modified Eagle's medium (DMEM) + 10% newborn calf serum (NCS)]. The cells were washed and plated at single cell density into 72-well Terasaki plates. After 12–15 h, the cells had attached and were inspected using a fluorescence microscope. The CFSE label allowed us to determine unequivocally whether or not a well contained a single cell. Single cells were then infected with BAG, prepared as previously described (Price *et al.*, 1987), or in some cases a combination of BAG and DAP, a virus encoding alkaline phosphatase (Fields-Berry *et al.*, 1992) in equal titres. These viruses had been screened and found free of helper virus. We used 400 c.f.u. of virus plus polybrene (Sigma, 0.01 mg/ml), as titred on NIH-3T3 cells, a titre which preliminary experiments indicated would infect a low proportion of single cells (see Results). After 3.5–4 h, the virus was removed and the cells were grown in 45% DMEM, 5% NCS and 50% NIH-3T3-conditioned medium. The next day and every subsequent 24 h, the growth of each clone was monitored. The medium was replaced every 2 days. After 4–5 days in culture, most cultures were fixed with 0.5% glutaraldehyde and stained with the β-galactosidase substrate X-gal as previously described (Price *et al.*, 1987). The wells which contained blue cells were identified and the number of blue and unstained cells was counted (see Table I).

Subcloning

In some experiments, single cells were grown as above until the well contained ~25 cells. The cells were then trypsinized from the multiwell dish and replated at single cell density. Subclones were then allowed to grow in each well, then stained with X-gal as described above for the primary clones. Since clones were of necessity subcloned without prior staining, they included the great majority of clones that were uninfected. Consequently, most clones gave subclones that were entirely unstained. A subset, however, gave subclones that were either blue or unstained. The DNA was then prepared from these cells for amplification by PCR.

PCR

Subclones were fixed and stained with X-gal (see Table I). Blue and unstained subclones were identified. The cells were then frozen and thawed twice in 15 μl of 1 mM EDTA pH 8.0, overlaid with 70 μl of light mineral oil (Sigma), heated to 93°C for 30 min in order to inactivate cellular enzymes and denature the DNA and associated proteins (Handyside *et al.*, 1992). This DNA was then amplified using the 'hot-start' procedure according to the standard Perkin-Elmer protocol. Nested sets of primers were used. The first set was L4 (GAA GAA GGC ACA TGG CTG AAT ATC GAC GGT) from *lacZ* region and N7 (GTC CAG ATA GCC CAG TAG CTG ACA TTC ATC) from the *neo* region of the proviral DNA (Figure 1; Bosch *et al.*, 1993). The mixture was subjected to 70 PCR cycles with each set of primers: 95°C for 45 s, 52°C for 45 s and 72°C for 90 s. The inner primers were L3 (TTC CAT ATG GGG ATT GGT GGC GAC GAC TCC) and N6 (CGG ACT GGC TTT CTA CGT GTT CCG CTT CCT). All primers were purified on 10% acrylamide gel electrophoresis. Finally, 50 μl from each PCR reaction were separated by electrophoresis on 1% agarose gel, then stained with ethidium bromide and visualized with UV light.

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