JOURNAL OF VIROLOGY, July 1991, p. 3887–3890
0022-538X/91/073887-04$02.00/0

Vol. 65, No. 7

Production of High-Titer Helper Virus-Free Retroviral Vectors by Cocultivation of Packaging Cells with Different Host Ranges

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Received 22 January 1991/Accepted 21 March 1991

The titer of retroviral vectors can be increased by cocultivation of retrovirus packaging cells that produce a vector with packaging cells having a different host range. Multiple rounds of infection occur in such cultures, producing an amplification of vector copy number and titer. Production of a vector with a very high titer of over 10^{10} CFU per ml of conditioned medium has been reported, although replication-competent helper virus was also present. Since helper-free virus is a requirement for many applications of retroviral vectors, we repeated this procedure with a modified vector and achieved a 2- to 10-fold amplification of vector titer in the absence of helper virus, up to 2×10^7 CFU/ml. We have also repeated these experiments with the same vector and methods described previously or have assayed virus from the high-titer vector-producing cell line reported previously and observed maximum titers of 10^8 CFU/ml, invariably accompanied by helper virus. Thus, while amplification of vector titer in the absence of helper virus is possible, some unexplained difference in the assays for virus titer must account for our inability to obtain the exceptionally high vector titers that were reported previously.

Retroviral vectors allow efficient transfer of genes into a variety of cell types both in culture and in animals. Efficient gene transfer in some cell types, such as hematopoietic stem cells, is critically dependent on a high titer of the retroviral vector. Recently the production of a retrovirus vector with a titer of $>10^{10}$ CFU per ml of culture medium, or about 3 orders of magnitude greater than previous vectors, has been described by Bodine et al. (6). The method involved cocultivation of a retrovirus packaging cell line producing a retroviral vector with another packaging cell line having a different host range, so that the vector could "ping-pong" between the cell lines, resulting in an increase in vector copy number and thus an increase in the titer of vector produced. Unfortunately, this virus also contained replication-competent helper virus and thus is not appropriate for many applications. Here we have repeated this procedure and have solved the problem of helper virus production, although the titers that we measured are not as high as reported previously.

Retroviral vectors. The retroviral vectors used in the cocultivation experiments include N2 (2), which was the vector used in the previous report by Bodine et al. (6), and LN (14), both of which carry a neomycin phosphotransferase (*neo*) gene (Fig. 1). The large region of viral sequences between the 5′ long terminal repeat (LTR) and the *neo* gene in both vectors promotes efficient packaging of viral RNA into virions and the production of high-titer virus (1, 2, 4). However, the presence of this region also increases the potential for helper virus production in retrovirus packaging cells containing the vectors, presumably by homologous recombination between these sequences (which include part of the *gag* coding region) and viral coding sequences present in the packaging cells (10, 12, 15). To reduce the potential for helper virus production, the LN virus has been modified by alteration of the *gag* start codon to a TAG stop codon and by the removal of *env* coding sequences present in N2 between *neo* and the 3′ LTR (14). Depending on the packaging cell line, these modifications reduce or eliminate the potential for helper virus production by homologous recombination (10) and have been shown to be effective by using stringent tests for helper virus production (14).

Virus was produced from the DNA forms of the two vectors by using PA317 amphotropic packaging cells (12). Previously described clonal cell lines producing the vectors were used, PA317/N2 c11 (12) and PA317/LN c11 (14). Methods of cell culture and virus production were as described before (14). The selectable marker gene for histidinol dehydrogenase (*hisD*) was introduced into each cell line by calcium phosphate-mediated transfection of pSV2his (7) (gift from R. C. Mulligan) followed by selection of the cells in standard culture medium plus 4 mM histidinol. This dominant selectable marker provides a method for isolation of the cells after cocultivation with other packaging cells. The titer of virus produced by populations of transfected, histidinol-selected cells was comparable for the N2 and LN vectors, being about 10^6 CFU/ml when assayed on HeLa cells and about 10^7 CFU/ml when assayed on NIH 3T3 cells (Table 1).

Virus production after cocultivation of packaging cells. The amphotropic packaging cells producing the vectors described above were cocultivated with GP+E86 ecotropic packaging cells (9) to allow vector amplification. The choice of this cell line was based on its use in the previous report by Bodine et al. (6) and the reduced potential for helper virus generation predicted for this packaging cell line (9, 10). The cells were mixed at different initial ratios (1:3, 1:1, and 3:1) and were subcultured every 3 days. The cells were split at ratios designed to keep the cells as confluent as possible and to provide for efficient virus spread between cells, but not so confluent as to cause cell death. After 2 weeks of cocultivation, the titer of N2 or LN vector produced by the cell mixtures was assayed by using either HeLa or NIH 3T3 cells as recipients, and potential helper virus production was measured by using the S^+L^- assay (Table 1). While there was an increase in the titer of N2 virus of up to fivefold after cocultivation, this increase was accompanied by the production of helper virus at high titer. There was little if any

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TABLE 1. Virus titers before and after cocultivation of packaging cells[a]

Virus-producing cells	Vector	GP+E86/PA317 cell ratio	Virus titer (CFU/ml) assayed on:		Helper virus present[b]
			HeLa cells	NIH 3T3 cells	
PA317 (*hisD*)	N2		1×10^6	2×10^7	−
	LN		4×10^5	1×10^7	−
Packaging cells cocultivated for 2 wk	N2	1:3	4×10^6	6×10^7	++
		1:1	5×10^6	4×10^7	++
		3:1[c]	2×10^6	1×10^7	++
	LN	1:3	3×10^5	5×10^6	−
		1:1	1×10^6	9×10^6	−
		3:1	1×10^6	1×10^7	−
Cells selected with histidinol after cocultivation	N2	1:3	2×10^6	2×10^7	++
		1:1	7×10^6	5×10^7	++
		3:1	1×10^7	1×10^8	++
	LN	1:3	5×10^5	7×10^6	−
		1:1	3×10^6	1×10^7	−
		3:1	4×10^6	2×10^7	−

[a] HeLa and NIH 3T3 cells were plated at 10^5 per 6-cm dish the day before infection. For infection, recipient cells were fed with fresh medium containing Polybrene (4 μg/ml), and various amounts of the test virus were added. The day after infection, the medium was changed to medium containing G418 at 1.0 and 0.5 mg of active compound per ml for HeLa and NIH 3T3 cells, respectively.

[b] Helper virus was measured in 1-ml samples of virus-containing medium by the S^+L^- assay as described before (13). Symbols: −, <1 FFU/ml; ++, >200 FFU/ml.

[c] Virus-producing cells in this assay were not as confluent as the other cells (twofold-lower cell number) at the time of virus harvest, so the titers measured are underestimates.

increase in the titer of LN virus after cocultivation, and in this case, no helper virus was detected.

PA317 cells were isolated from the cocultivated packaging cell mixtures by selection in histidinol. These selected cells were also analyzed for virus production by using HeLa or NIH 3T3 cells as recipients (Table 1). N2 vector titers were increased up to 10-fold over the titer of the starting cell line PA317/N2 (*hisD*), and high levels of helper virus were again detected. LN vector titers were increased up to 10-fold on HeLa cells and up to 2-fold on NIH 3T3 cells compared with the starting cell line PA317/LN (*hisD*), and no helper virus was detected.

To determine whether clonal lines isolated from the cocultivated histidinol-selected cells might produce still higher titers of virus, as previously observed by Bodine et al. (6), six clones were isolated from the PA317/LN (*hisD*) cells. No further improvement in virus titer on either HeLa or NIH 3T3 cells (Table 2) was noted in comparison with virus titers produced by the uncloned population (Table 1). We did not examine clones from the PA317/N2 cocultivated cells because these cells produced helper virus. In summary, an increase in vector titer after cocultivation of up to 10-fold was found for the N2 vector and was associated with the production of helper virus. An increase in vector titer of up to 10-fold was also observed for the LN vector, although the maximum titer was about 5-fold lower than that of the N2 vector, and production of helper virus was not detected.

The highest-titer virus production that we were able to achieve by cocultivation of packaging cell lines producing the N2 virus was 10^7 CFU/ml by using HeLa cells as recipients, quite different from the titer of 2×10^{10} CFU/ml obtained by Bodine et al. (6) with the same vector, packaging cell lines, and recipient cells. We obtained a sample of the high-titer vector-producing cells (N263A2) and the packaging cell line used for cocultivation to make the high-titer line (N263) (gifts from David Bodine). We assayed virus production from these cell lines with our assay (Table 3) and found that the N2 virus titer produced by the high-titer line N263A2 was similar to the titers that we had found after cocultivation and histidinol selection of PA317/N2 (*hisD*) cells (Table 1). However, the titer of virus produced by the N263 cells was 10- to 20-fold lower than that of the PA317/N2 (*hisD*) cells

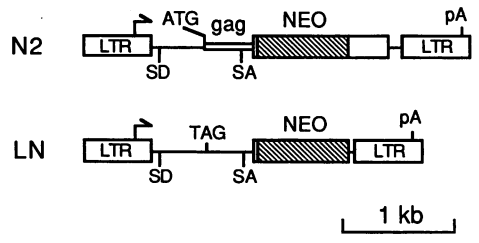

FIG. 1. Retroviral vectors. Lines represent viral sequences, the thin box represents the *gag* coding region, and the large boxes represent the viral LTRs or the *neo* cDNA, with coding region hatched. Other abbreviations: SD, splice donor; SA, splice acceptor; pA, polyadenylation signal; ATG, *gag* start codon; TAG, mutated *gag* start codon; kb, kilobase pairs.

TABLE 2. Virus titers produced by clonal PA317/LN cell lines isolated after cocultivation[a]

PA317/LN (*hisD*) amplified clone no.	Virus titer (CFU/ml) assayed on:	
	HeLa cells	NIH 3T3 cells
c1	1×10^6	5×10^6
c2	2×10^6	1×10^7
c3	$<10^5$	2×10^6
c4	7×10^5	3×10^6
c5	3×10^6	2×10^7
c6	4×10^5	4×10^6

[a] Clonal cell lines were isolated from PA317/LN (*hisD*) cells cocultivated at GP+E86/PA317 ratio of 3:1 and selected after cocultivation in histidinol. Virus production was measured as described in Table 1, footnotes *a* and *b*. No helper virus production was detected (<1 FFU/ml) in any of the clonal cell lines.

TABLE 3. Virus titers produced by cell lines N263 and N263A2[a]

Location	Virus-producing cells	Virus titer (CFU/ml) assayed on:		Helper virus present[b]
		HeLa cells	NIH 3T3 cells	
Seattle, Wash.	N263	1×10^5	1×10^6	−
	N263A2	1×10^7	4×10^7	++
	PA317/LN (*hisD*)	1×10^6	1×10^7	−
Bethesda, Md.	N263	6×10^6	ND[c]	−
	N263A2	2.6×10^9	ND	ND
	PA317/LN (*hisD*)	6.6×10^8	ND	ND

[a] Virus production was measured by our group in Seattle as described in Table 1, footnote *a*, and by Bodine et al. in Bethesda as described previously (6). The N263A2 cells were sent to Seattle, where they were assayed and then returned to Bethesda for reassay, and these values are reported in the table. Early-passage stocks of N263A2 cells have given titers of 2×10^{10} when assayed in Bethesda (6 and more recent data). The PA317/LN (*hisD*) cells assayed were not amplified by cocultivation, and the samples assayed by both groups were obtained from the same frozen stock of cells.

[b] See Table 1, footnote *b*.

[c] ND, not determined.

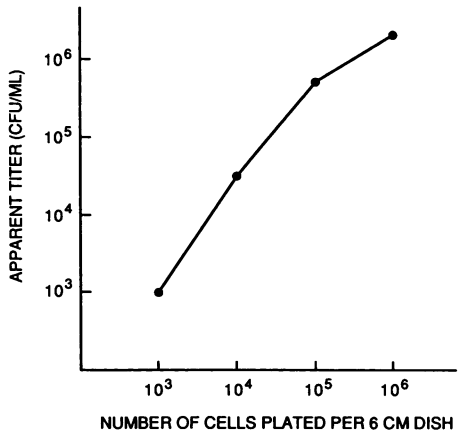

FIG. 2. Dependence of apparent virus titer on the density of recipient cells. NIH 3T3 cells were plated at the indicated densities the day before infection, infected in the presence of Polybrene (4 μg/ml) with 0.01 to 10 μl of virus harvested from PA12/N2 c2 vector-producing cells (12), and selected in G418 at 1 mg of active compound per ml the day after infection. The cells initially plated at 10^6 per dish were split 1:20 prior to selection because of the high cell density (and the observed colony number was multiplied by 20 to correct for the split), but the other dishes were selected without trypsinization and splitting.

used here to initiate cocultivation cultures for the production of high-titer cells (Table 1). Thus, while the fold increase in virus titer achieved by cocultivation in the previous experiments (40- to 100-fold) (6) was higher than that observed here (10-fold), the final titer after cocultivation was similar.

Differences in assays for vector production. Differences in the method used to assay vector titer can result in dramatic differences in the observed titer. For example, we plated NIH 3T3 cells at densities from 10^3 to 10^6 per 6-cm dish, infected the cells with various amounts of N2 virus produced by using PA12 cells (13), selected the cells 1 day after infection in G418 at 1 mg/ml (active), and counted colonies after 5 days of selection. As expected, the number of colonies was directly proportional to the amount of virus added at each cell density (not shown), but more important, the apparent titer of the vector was almost directly proportional to the initial plating density of the target cells (Fig. 2). We interpret this result to imply that virus either can stick to open areas of the culture dish or cannot efficiently diffuse to sparsely plated cells. These results demonstrate that the titer of retroviral vectors is markedly influenced by the assay used and may help to explain differences in reported titers for similar vectors.

Because there are slight differences in the method we have used here to measure virus titer and that used by Bodine et al. (6), we measured vector titer by both assays in parallel. Both assays involve plating recipient cells at 10^5 per 6-cm dish 1 day before infection, but in the previously described assay, selection was applied 2 days after infection (compared with 1 day used here), a lower dose of G418 was used (0.5 versus 1.0 mg of active compound per ml), the selective medium was replaced daily (compared with no medium replacement during 5 to 6 days of selection), and the selection period was longer (10 to 14 days) before colonies were counted. In spite of these differences between the two assays, similar estimates of virus titer were obtained with either assay (data not shown).

As an aside, the standard assay that we often use for assay of vector titer involves plating the recipient cells at 5×10^5 per 6-cm dish 1 day before infection, which we believe maximizes the infection rate because the cells are neither too sparse to be efficiently infected (Fig. 2) nor too dense to grow at maximal rates, which is also required for efficient infection (16). One day after infection, the cells are split 1:20 into selective medium to allow rapid drug selection. If the cells are not split, it takes a long time for uninfected cells in the resultant confluent layer to die, and much cell debris is formed during the selection. Averaging over many experiments, this assay gives about a fivefold-higher apparent titer than the assays that involve plating the cells at 10^5 per 6-cm dish with no split prior to selection. Using the assay in which target cells are plated at 5×10^5 per 6-cm dish, we found our best titers for cocultivated, histidinol-selected packaging cells carrying the N2 vector to be about 10^9 CFU/ml and for the LN vector to be about 10^8 CFU/ml on NIH 3T3 cells. In the experiments described elsewhere in this article, we used the more conservative method involving plating the cells at 10^5 per 6-cm dish so that our virus assay would be similar to that used by Bodine et al. (6).

In summary, we have found that cocultivation of vector-producing packaging cells with another packaging cell line having a different host range can result in up to a 10-fold increase in vector titer. The highest vector titer that we have observed when using HeLa cells as targets for infection was 10^7 CFU/ml. When NIH 3T3 cells were used as targets for infection, the apparent titer of the vectors was about 10-fold higher than with HeLa cells (Tables 1, 2, and 3), and the highest titer observed was 10^8 CFU/ml. The maximum titer observed here is higher than but in the same range as the maximum titer reported after amplification of a vector carrying a human growth hormone gene by cocultivation of packaging cell lines (1.7×10^7 CFU/ml [5, 8]), and after amplification of a vector carrying a mutant dihydrofolate reductase gene in the presence of helper virus by selection of the cells in increasing concentrations of methotrexate (6×10^7 CFU/ml [13]). Despite efforts to achieve reproducible conditions, titers obtained in our laboratory (Table 3) are about 100-fold lower than those obtained by Bodine et al. (Table 3) for the same cell lines. Our inability to obtain very high titer vector-producing cells after cocultivation is appar-

ently not due to the methods we used, the fact that previously cocultivated lines also produced interleukin-3 and interleukin-6, or to the possibility that if we had isolated many more clones after amplification, we would have found a very high titer producer, because the cell line previously reported to produce titers of over 10^{10} CFU/ml (N263A2 [6]) produced a virus titer of only 4×10^7 CFU/ml in our hands, which is no higher than the titers that we have been able to obtain.

Amplification of the N2 vector by the cocultivation technique always resulted in the production of helper virus, similar to results observed previously after cocultivation of a variety of packaging cells producing the N2 vector (17). Helper virus was not detected after amplification of the LN vector, which was designed to reduce homologous overlap between vector sequences and viral coding sequences in the packaging cells (14). However, the final titer of the LN vector after amplification was also about fivefold lower, indicating that the presence of helper virus facilitates high-titer vector production. This cocultivation technique may also have utility for improving the titer of vectors that have a low titer to begin with, although vector rearrangement to yield more highly transmissible vectors will likely be a problem (3, 11).

We thank David Bodine and Arthur Nienhuis for the N263 and N263A2 cell lines and for data on virus titers as assayed in their laboratories and Victor Garcia for comments on the manuscript.

This work was supported by a grant from Genetic Therapy Incorporated (C.M.L.) and grants from the National Heart, Lung, and Blood Institute of the National Institutes of Health (A.D.M.).

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