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Overcoming interference to retroviral superinfection results in amplified expression and transmission of cloned genes

(gene expression/gene amplification/retroviral packaging)

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ABSTRACT **A procedure is described for stably expressing cloned genes at high levels in vertebrate cells and for obtaining these genes in high-titer virus preparations. The process uses retroviral vectors and mixtures of two "packaging cell lines" that incorporate retroviral genomes into virions with different host-range envelopes. In these cocultures, interference barriers to superinfection are overcome, retroviral vectors can replicate in the absence of a transmissible helper virus, and the cells become infected with multiple copies of the provirus that contains the cloned gene. This procedure was used to amplify expression of the membrane glycoprotein that is encoded by Friend spleen focus-forming virus, a retrovirus that is replication defective in other cell cultures. Amplifications were measured at the DNA provirus, RNA, and protein levels. In addition, the human growth hormone gene was inserted into retroviral vectors and we observed amplifications of growth hormone synthesis and secretion. The amplified growth hormone was properly processed as indicated by immunoblot analyses. A vector is described (pSFF) that is exceptionally active in coculture amplification.**

Retroviruses that are replication competent contain the *gag*, *pol*, and *env* genes (i.e., *trans* genes) that encode the proteins necessary for packaging the genomic RNA into transmissible virions. In addition, such replication-competent viruses contain *cis* sequences that enable the genomic RNA to become incorporated into virions and to be reverse transcribed to form functional proviral DNA (1–3). Because replication-defective retroviral genomes contain these *cis* sequences but not all of the *trans* sequences, they can be transmitted in nature only in the presence of a replication-competent "helper virus" (1). In addition, retroviral packaging cell lines have been recently described (4–7). These can be formed by stably transfecting cells with DNAs that encode functional gag, pol, and env proteins but that lack some or all of the requisite *cis* sequences (4–7). When retroviral vectors are introduced into retroviral packaging cell lines, the cells can release virions that contain the retroviral vector genomic RNA (4–7). These helper-free virions have been used to stably transfer genes into cell cultures or animals (2, 4–11). However, retroviral expression systems have not yet been useful for producing vertebrate proteins in large quantities.

Retroviruses have been classified into different host-range/interference groups (1, 2, 12–15). These group differences are determined by the viral *env* genes that encode glycoprotein "knobs" on the virion membrane envelopes (1, 15). These envelope glycoproteins mediate viral binding to specific receptors that are present only on the surfaces of susceptible cells (1, 15–17). For example, murine leukemia viruses are classified as ecotropic (able to infect only mice and rats), amphotropic (able to infect most vertebrate cells), or xenotropic (able to infect all mammalian cells except mice and rats) (1, 12–15). Moreover, cells infected with a retrovirus are resistant to superinfection by a second virus of the same host-range group (1, 2, 12–15). This interference occurs because the synthesis of an env glycoprotein by a cell somehow blocks the corresponding receptor sites on the cell (1, 15).

Packaging cell lines have been described that produce retroviruses with different host ranges (4–7). The ψ-2 cell line (4) releases a virus with an ecotropic host range, whereas the PA12 and PA317 cell lines (5, 6) release virions with an amphotropic host range. Each of these cell lines is resistant (because of interference) to superinfection by the virus it releases. However, these packaging cells are susceptible to infection by virions of the other host-range group. Here we describe a simple procedure that uses retroviral vectors and packaging cells to amplify expression of genes. The vectors can be rapidly and efficiently amplified in cocultures that package retroviruses into different host-range coats.

MATERIALS AND METHODS

Cell Cultures and Viruses. Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cell lines included NIH/3T3 fibroblasts, ψ-2 ecotropic packaging cells (4), PA12 amphotropic packaging cells (obtained from D. Miller, Fred Hutchinson Cancer Research Center) (5), PA317 amphotropic packaging cells (American Type Culture Collection no. CRL 9078) (6), and Friend 745 erythroleukemia cells (clone PC-4) (18). DNA was transfected by the $CaPO_4$ technique (19, 20). Selection of cells expressing the neomycin-resistance gene was with G418 (21) at 500 μg/ml for 10–14 days. Virus was harvested by placing fresh culture medium on nearly confluent monolayers of virus-producing cell lines for 16–24 hr, removing the medium, and filtering it through a 0.2-μm filter. Cells were infected as follows: cells were seeded for 16–24 hr with virus-containing medium and then incubated for 2 hr at 37°C in the presence of Polybrene (8 μg/ml).

Plasmid Constructions. The plasmid pL2-6K encodes a colinear molecular clone of Friend spleen focus-forming virus (SFFV) (20). As shown in Fig. 1, pL2-6K modified to include a 215-base-pair *Hin*dIII fragment of simian virus 40 (SV40) (nucleotides 1493–1708) (22), was named pSVSF (C. Spiro, B. Gliniak and D.K., unpublished data). Plasmid pSFF was also constructed from pL2-6K. The *Eco*RI/*Bam*HI fragment was removed from a subclone of the *pol* gene region, and the cut ends were blunt ended and religated. The *Bam*HI/*Eco*RI fragment in the *env* gene region was then deleted. The ends were blunted and then ligated to a *Xho* I linker. This resulted in reconstruction of these *Bam*HI and *Eco*RI sites. Plasmid

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Abbreviations: SFFV, Friend spleen focus-forming virus; gp55, the glycoprotein encoded by SFFV; SV40, simian virus 40.

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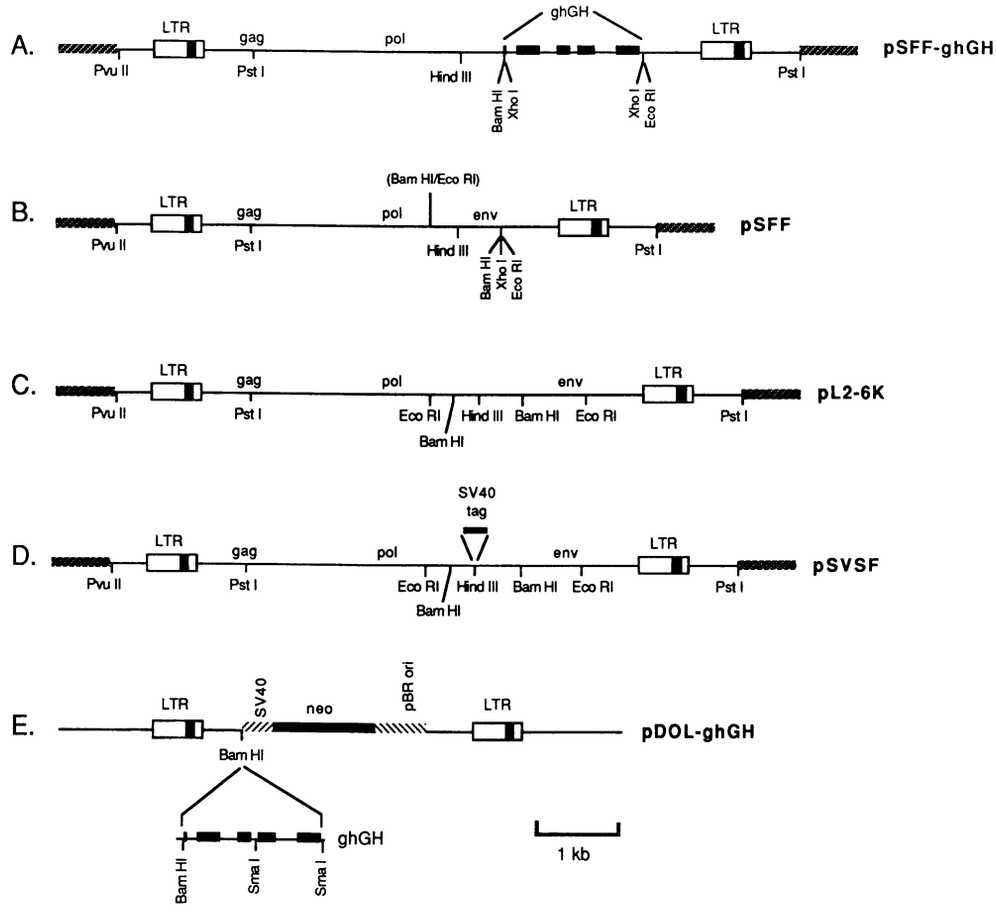

FIG. 1. Structures of the retroviral clones used in this study. pL2-6K (*C*) is the previously described (20) full-length colinear SFFV molecular clone. It was used to derive pSFF-ghGH (*A*), pSFF (*B*), and the SV40 "tagged" construct pSVSF (*D*). The SV40 fragment is a 215-base-pair *Hin*dIII fragment. ghGH is the coding region of the human growth hormone gene.

pSV2neo-ghGH (23) was the source of the genomic human growth hormone (ghGH) gene and was generously provided by T. Burgess (University of California, San Francisco). A 1.5-kilobase (kb) *Bam*HI/*Sma* I fragment containing the genomic human growth hormone coding sequences but lacking the poly(A) addition signal was inserted into the *Bam*HI site of pDOL (24) to generate the plasmid pDOL-ghGH. pSFF-ghGH was made by subcloning the same 1.5-kb fragment into pUC 19. The resulting plasmid was cut with *Bam*HI and *Eco*RI and then blunt ended and ligated to *Xho* I linkers. After digesting with *Xho* I, the insert was ligated into the *Xho* I site of pSFF.

Blotting Methods. Immunoblot detection of the SFFV-encoded glycoprotein (gp55) with ^{125}I-labeled protein A was described (20, 25). Methods used for RNA slot blots (26, 27), for Southern blotting (20, 21, 28), and for densitometric scanning of autoradiograms (29) have been described. The antiserum to human growth hormone was from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases.

Analysis of Human Growth Hormone Expression. *Protocol 1:* The pDOL-ghGH plasmid was stably transfected into ψ-2 and PA12 cultures and the released viruses were used to infect the other packaging cell line. Since pDOL contains the bacterial neomycin-resistance gene, it was possible to select infected clones with G418 (21). Clones were then analyzed for secretion of human growth hormone. The genomic human growth hormone gene contains introns (23) that can be properly excised during transmission in retroviral vectors (30). Several clones of each packaging cell line meeting these criteria were isolated and used in coculture studies. For assay of secreted growth hormone, cell cultures at 50% confluency were incubated in 1 ml of fresh medium per 10-cm^2 surface area. After 24 hr, this medium was harvested and used for growth hormone immunoassay and for immunoblotting. Radioimmunoassay (sensitivity, 1.5 ng/ml) was performed as recommended by the manufacturer (Kallestad Laboratories, Austin, TX).

Protocol 2: Plasmids (10 μg) encoding growth hormone were transfected as $CaPO_4$ precipitates (19, 20) into 25-cm^2 culture dishes that contained a 1:1 mixture of ψ-2 and PA12 or PA317 cells at a total cell concentration of 2×10^5 cells per dish. The cultures were then grown and assayed for growth hormone secretion as described above.

RESULTS

Amplification of SFFV Gene Expression. Plasmid pSVSF is a full-length colinear clone of SFFV that contains a 215-base-pair SV40 "tag" inserted into its nonfunctional *pol* gene region (see Fig. 1). This plasmid was stably transfected into the ψ-2 ecotropic packaging cell line (4) by cotransfecting it with pSV2neo (21) and screening the individual G418-resistant colonies for expression of the SFFV-encoded membrane glycoprotein gp55 (data not shown). One clone, ψ-2/SVSF, was selected and was shown to release helper-free tagged SFFV (C. Spiro, B. Gliniak, and D.K., unpublished data). When ψ-2/SVSF cells were mixed with either PA12 or PA317 amphotropic packaging cells (5, 6), gp55 expression was rapidly amplified. As detected by immunoblotting (Fig. 2) and by densitometric scanning of the autoradiograms (29), the amplified quantities of gp55 (lanes 2, 3, 6, and 7) were at least 50 times larger than the unamplified quantity (lane 4). Indeed, the amplified amounts of gp55 were larger than we

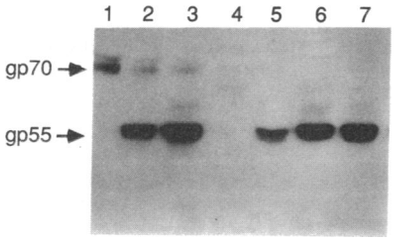

FIG. 2. Immunoblotting of SFFV-encoded envelope glycoproteins after amplification in the mixed culture system. The ψ-2/pSVSF cells were cocultured with either PA12 or PA317 cells. The immunoblot reacted with the 7C10 monoclonal antibody (20). Each lane contains lysate derived from the same number of cells (2.5×10^5). Lanes: 1, cocultivated ψ-2 and PA12 cells; 2 and 3, ψ-2/SVSF and PA12 cells cocultured for 12 and 17 days, respectively; 4, ψ-2/SVSF cells; 5, NRK clone 1 SFFV nonproducer fibroblasts; 6 and 7, ψ-2/SVSF and PA317 cells cocultured for 12 and 17 days, respectively. Although the gp55 band in lane 4 is weak, longer exposures of this blot showed this component.

had observed in any other cells. NRK clone 1 cells (lane 5) were previously our highest-expressing line of fibroblasts. The quantities of gp55 per mg of protein were approximately twice as high in the amplified cultures as in Friend 745 erythroleukemia cells. In the latter cells, gp55 constitutes ≈0.3% of total cellular protein synthesis (D.K., unpublished results). Similar results were obtained by using the untagged colinear SFFV clone pL2-6K (see Fig. 1) and SFFV clones with site-directed mutations in their gp55 genes (20). In those experiments, the SFFV plasmid DNAs were transfected without other DNAs directly into ψ-2/PA12 cocultures, and the amplifications occurred without selections. Therefore, isolation of stable transfectants is not a prerequisite for amplification (see below).

These results implied that the virus released from ψ-2/SVSF cells spreads into PA12 or PA317 cells and that virus subsequently released from the latter cells can then reinfect ψ-2/SVSF cells. This process can be repeated a number of times, resulting in continued amplification. This interpretation predicted that SFFV transcription would increase during amplification. As shown by the RNA slot blot analysis in Fig. 3, the amount of SFFV-specific RNA in the ψ-2/SVSF cells was relatively very low (slots 1). This RNA was increased 50- to 100-fold in the cocultures (slots 2, 3, and 4).

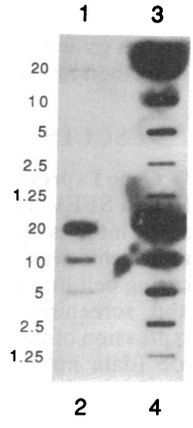

FIG. 3. RNA slot blot from mixed cultures of ψ-2/SVSF and PA12 cells. Total cellular RNA was used. Serial dilutions (2-fold) were applied to nitrocellulose. The hybridization probe was the 215-base-pair SV40 fragment. The quantity of RNA (μg) applied to each slot is shown on the left. Slots: 1, ψ-2/SVSF cells only; 2, 3, and 4, ψ-2/SVSF cells cocultured with PA12 for 6, 15, and 19 days, respectively.

The numbers of integrated SVSF proviruses were determined by isolating high molecular weight DNA from the mixed ψ-2/SVSF and PA12 or PA317 cocultures and comparing them with DNA isolated from either the ψ-2/SVSF line or from splenic erythroblasts that had been generated from diseased mice as a result of infection with the helper-free tagged SVSF virus. These splenic erythroblasts form by clonal expansion of infected cells and they have a single proviral copy number (C. Spiro, B. Gliniak, and D.K., unpublished data). Southern blots prepared with these DNAs are shown in Fig. 4. The DNAs were digested with *Eco*RI, which has sites on both sides of the SV40 tag and should produce a 2.1-kb fragment that hybridizes to the SV40 probe (see Fig. 1). Based on densitometric comparison with the single-copy erythroblast DNA (lane 1), the ψ-2/SVSF transfectant cells (lane 3) contained ≈15 copies of the tagged SFFV DNA. Previous studies of stable transfectants suggest that these copies are probably linked together with pSV2neo at one chromosomal site in the host cells (31). The 1:1 cocultures of ψ-2/SVSF with either PA12 or PA317 (lanes 4 and 5, respectively) contained, on average, 37–45 tagged SFFV copies per cell. Therefore, coculturing resulted in increases of 30–38 copies of tagged SFFV DNA per cell (see Fig. 4, legend). The fact that gp55 synthesis was increased 50- to 100-fold, whereas tagged DNA copy number only increased 2- to 3-fold, is consistent with evidence that retroviral vector DNA is expressed relatively poorly when it is transfected into cells compared to the levels obtained after proviral integration (32). The same DNAs were also digested with either *Pst* I, which has a single site in the provirus and should produce proviral–cellular junction fragment DNAs, or with *Xho* I, which does not cut in the provirus. These blots were hybridized with the SV40 probe and smears of the expected sizes (e.g., >5 kb for the *Pst* I digests) were detected in the DNAs

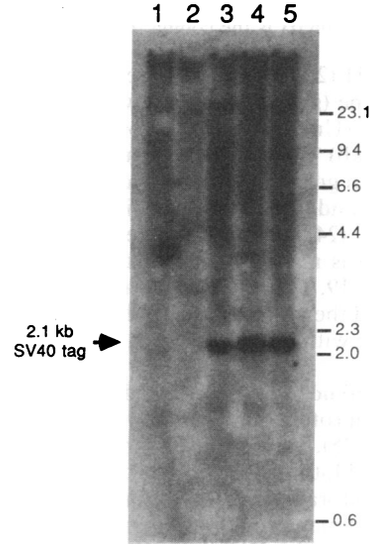

FIG. 4. Southern blot of DNA from pre- and postamplification cell cultures. High molecular weight DNA was digested with *Eco*RI. Each lane contained 5 μg of DNA. The hybridization probe was the 215-base-pair SV40 DNA fragment. Lane 1, splenic erythroblast DNA from mice infected 21 days earlier with ψ-2/SVSF virus. The latter cells contain only one copy of tagged virus per cell; lane 2, DNA from NIH/3T3 cells; lane 3, ψ-2/SVSF; lane 4, cocultured ψ-2/SVSF and PA12 cells; lane 5, cocultured ψ-2/SVSF and PA317 cells. The cell mixtures used in lanes 4 and 5 were cocultured for 21 days. Appropriately exposed autoradiograms were densitometrically scanned. Proviral copy numbers were measured relative to the single copy standard (lane 1). Amplified copy numbers in the 1:1 cocultures were calculated as the average total copy numbers in the cocultures minus one-half the copy number in the pure ψ-2/SVSF cells.

from the cocultures (results not shown). These results support the hypothesis that amplification results from new proviral integrations at different sites in the host-cell chromosomes.

Amplification of the Human Growth Hormone Gene. To determine whether this process could be used for other genes, we constructed pDOL-ghGH with the human growth hormone gene, and we assayed expression by measuring growth hormone in the culture medium (23, 33). When coculturing was initiated by mixing packaging cells that had been preselected for expression of pDOL-ghGH (e.g., *Protocol 1* in *Materials and Methods*), growth hormone synthesis became amplified throughout the next 2–4 weeks (Fig. 5*A*). However, the kinetics and extents of amplification varied in different experiments (data not shown). Moreover, amplification to stable expression did not occur when the pDOL-ghGH plasmid was simply transfected into the cocultures without preselection (i.e., see *Protocol 2*). This deficiency was not overcome by using other test genes (i.e., cDNA clones of human adenosine deaminase and β-hexosaminidase) or another retroviral expression vector [pLNSL (34)].

Because the latter amplifications were inferior to those obtained with SFFV, we constructed the SFFV-based expression vector pSFF and inserted the genomic human growth hormone gene (see Fig. 1). When this plasmid was transfected into ψ-2/PA12 or ψ-2/PA317 cocultures (*Protocol 2*), spontaneous amplification of growth hormone synthesis rapidly and reproducibly ensued. A representative amplification with a ψ-2/PA12 coculture is shown in Fig. 5*B*. Using this protocol, we have obtained 0.54 μg of human growth hormone from the culture medium overlying 10^6 cells (0.3% of the cellular protein).

Amplification Involves an Infectious Process. Cocultures with amplified retroviral vector gene expression contained high titers of the corresponding viruses in their media. For example, in one study the SFFV clone pL2-6K (see Fig. 1) and other SFFV clones with site-directed in-frame insertions

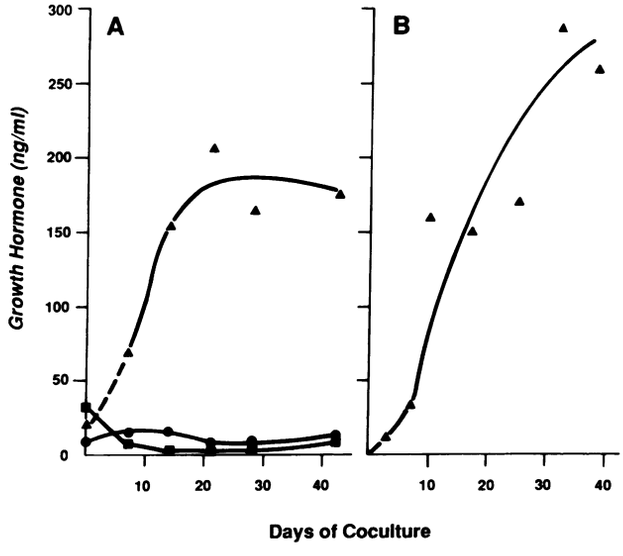

FIG. 5. Amplification of human growth hormone expression. (*A*) Protocol 1 was used for the coculture amplification (see *Materials and Methods*). ψ-2 cells and PA317 cells were first stably infected with pDOL-ghGH virus. These infected cells were then either cultured separately or in a 1:1 coculture. Rates of hormone secretion were measured at the times indicated. The ψ-2 (■) and PA317 (●) cell cultures released relatively little hormone compared to the 1:1 coculture (▲). The day 0 data for the coculture is the calculated average of the two pure cultures. (*B*) The pSFF-ghGH plasmid was simply transfected into a 1:1 coculture of ψ-2 and PA12 cells. The culture was then grown without selection. Controls with pure ψ-2 and PA12 cultures did not release hormone.

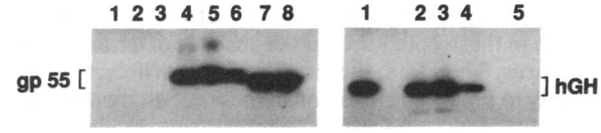

FIG. 6. Immunoblot evidence that the coculture media contain high titers of the corresponding viruses. The results are from cultures infected with amplified SFFV virions (*Left*) or with growth hormone virions (*Right*). For the SFFV study, virions were harvested from ψ-2/PA12 cocultures that had been transfected with wild-type pL2-6K plasmid or with site-directed mutant plasmids (20) that contained in-frame insertions (Pst, R1) or deletions (Sm1 and Sm2) in their gp55 genes. The cells that were lysed for analysis were as follows: lane 1, PA12 cells; lane 2, ψ-2 cells; lane 3, uninfected NIH/3T3 cells; lanes 4–8, NIH/3T3 cells infected with media from cocultures that had been transfected 14 days earlier with wild-type, Pst, R1, Sm1, and Sm2, respectively. The light bands in lanes 4 and 5 above the gp55-related major components are more highly processed forms that occur on cell surfaces (20). Only the pathogenic SFFVs encode these components (20). For the growth hormone study, the lanes are as follows. Lane 1, ^{125}I-labeled human growth hormone from the radioimmunoassay kit; lane 2, medium from a ψ-2/PA12 coculture that had been transfected with pSFF-ghGH; lanes 3 and 4, media from mouse NIH/3T3 and mink CCL64 fibroblasts, respectively, that had been infected several days previously with virus from the coculture medium; lane 5, medium from an untransfected ψ-2/PA12 coculture. Typically, as measured by immunofluorescence, the coculture media contained approximately 4 × 10^5 and 2 × 10^4 virions per ml of growth hormone virions as measured by infection of NIH/3T3 and CCL64 cells, respectively. This is not a quantitative measure of ecotropic and amphotropic virions because both host-range classes infect mouse cells and because our amphotropic murine leukemia virus also gives higher titers on NIH/3T3 than on CCL64 cells. The cell cultures appear to synthesize the major M_r 22,000 growth hormone and also a small proportion of the normal M_r 20,000 variant (35).

or deletions in their gp55 genes (20) were transfected without other DNAs directly into ψ-2/PA12 cocultures, and the amplifications occurred spontaneously. The coculture supernatants contained SFFV virions that could efficiently infect NIH/3T3 fibroblasts (Fig. 6 *Left*). Based on the SFFV assay method described (8), the coculture medium routinely contained 10^6–10^7 SFFV per ml, whereas the titers from ψ-2/SVSF cells were several orders of magnitude lower (data not shown).

The medium from ψ-2/PA12 cocultures that produced human growth hormone (e.g., Fig. 5*B*) was also analyzed for the corresponding virus. As shown by immunoblot analysis in Fig. 6 (*Right*), the medium from the cocultures contained immunoreactive growth hormone (lane 2) that coelectrophoresed with an authentic growth hormone standard (lane 1). Murine NIH/3T3 and mink CCL64 fibroblasts infected with virus from the coculture medium also synthesized growth hormone (lanes 3 and 4, respectively).

DISCUSSION

Genes cloned into retroviral vectors can be amplified in cocultures that contain cells for packaging retroviruses into different host-range envelopes. Amplification results in enhanced synthesis of the encoded protein (Figs. 2 and 5) and RNA (Fig. 3), in increased copy numbers of integrated proviral DNAs (Fig. 4), and in high-titer virion preparations that can be used to transfer the gene expression into other cells (e.g., Fig. 6). Glycoproteins and secretory proteins synthesized in these cocultures are properly glycosylated and processed to cell surfaces (20).

A model of the process that results in retroviral vector amplification is mentioned above. It involves an unusual back-and-forth (Ping-Pong) process of infection in which the virions released from ψ-2 cells can only infect PA12 or PA317

cells and vice versa. Interestingly, retroviral vectors can replicate in these cocultures in the absence of a transmissible helper virus. Therefore, the term "replication defective" does not apply to Ping-Pong infection in these coculture conditions.

Initially, it was uncertain whether this process would function. Cells that synthesize retroviral envelope glycoproteins (e.g., ψ-2, PA12, and PA317 cells) shed substantial quantities of these glycoproteins into the culture medium (1). Moreover, genomic RNA is not required for retroviral budding from cell surfaces so that cultures of packaging cells also shed noninfectious virion particles (1, 29, 36, 37). These genome-free particles and soluble envelope glycoproteins would be expected to cause "early interference" (1, 38) by binding to receptors (16). Our results demonstrate that this form of interference is not always sufficient to prevent retroviral vector amplification.

Fortunately, in our initial studies we used SFFV. SFFV was amplified relatively rapidly, extensively, and reproducibly. Accordingly, we constructed the SFFV-based vector pSFF and found it to be superior to pDOL for mixed culture amplifications. The pDOL-ghGH plasmid only functioned in coculture amplification when the process was initiated by mixing cells that had been preselected for stable expression of growth hormone (see *Results*). With the pSFF vector, abundant growth hormone synthesis reproducibly occurred when the plasmid was simply transfected as a $CaPO_4$ precipitate into the cocultures. Recent evidence indicates that the packaging signal of murine leukemia viruses extends into the *gag* gene (39). pSFF contains the same *gag*, long-terminal repeat, and splice donor and acceptor sites as SFFV.

According to our model for this back-and-forth (Ping-Pong) process of vector replication (see above), amplification could theoretically proceed indefinitely, until the two cell types are separated by cloning or until killing occurs from excessive proviral integrations. However, amplifications of RNA and protein synthesis appear to stop after several weeks (Figs. 2, 3, and 5; unpublished results), and the cocultures remain healthy. The reason(s) for the cessation are uncertain but could include a saturation of transcription factors. Conceivably, replication-competent helper viruses could form by recombination in the cultures and establish interference blocks to further amplification (6).

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