Ping-Pong Amplification of a Retroviral Vector Achieves High-Level Gene Expression: Human Growth Hormone Production

SUSAN L. KOZAK AND DAVID KABAT*

Department of Biochemistry and Molecular Biology, School of Medicine, Oregon Health Sciences University, Portland, Oregon 97201-3098

Retroviral vectors offer major advantages for gene transfer studies but have not been useful for producing proteins in large quantities. This deficiency has resulted in part from interference to superinfection, which limits the numbers of active proviruses in cells. Recently, we found that these vectors amplify when they are added as calcium phosphate precipitates to cocultures of cells that package retroviruses into ecotropic and amphotropic host range envelopes. Helper-free virions from either cell type can infect the other without interference, resulting in theoretically limitless back-and-forth (ping-pong) vector replication. In initial studies, however, amplifications of a vector that contained the human growth hormone gene ceased when the hormone produced was 0.3% or less of cellular protein synthesis. This limit was caused by two factors. First, recombinant shutoff viruses that are replication defective and encode envelope glycoproteins form at a low probability during any round of the vector replication cycle and these spread in cocultures, thereby establishing interference. Single cells in shutoff cocultures therefore synthesize both ecotropic and amphotropic envelope glycoproteins, and they release promiscuous (presumably hybrid) virions. The probability of forming shutoff viruses before the vector had amplified to a high multiplicity was reduced by using small cocultures. Second, cells with large numbers of proviruses are unhealthy and their proviral expression can be unstable. Stable expresser cell clones were obtained by selection. Thereby, cell lines were readily obtained that stably produce human growth hormone as 4 to 6% of the total protein synthesis. A ping-pong retroviral vector can be used for high-level protein production in vertebrate cells.

Retroviruses are advantageous for gene transfer studies because they have broad host and tissue ranges and relatively innocuous effects on cells in culture and in animals (12, 34, 35, 37). Consequently, they are exceptionally useful for marking normal cell lineages in vivo and for studying genes that influence cellular differentiation.

Nevertheless, retroviral vectors have not always worked reliably and they have not been applicable to protein production (35, 37). This limitation occurs because proviral gene expression is a small percentage (ca. 0.1 to 0.3%) of cellular protein synthesis and because the number of proviruses that can be expressed in a cell is usually severely limited by viral interference (3, 20, 22, 39, 40). Retrovirions of any host range class cannot infect cells that synthesize envelope glycoproteins of that same class. Consequently, cells that release retrovirions cannot be superinfected by those virions. For the same reason, replication-defective viruses cannot spread efficiently when they are introduced into cell cultures in the presence of a replication-competent helper virus. Defective viruses are excluded from cells that first become infected with the helper. Replication-defective viruses therefore can only replicate efficiently or be detected if they cause cells to proliferate abnormally or if they contain a dominant selectable marker gene.

These problems have been addressed in several ways. One advance involves the use of retrovirus-packaging cell lines that do not release any replication-competent helper viruses (9, 26, 27, 29–31). After transfection with retroviral vector DNA, these cells release helper-free virions that contain the genomic RNA of the vector. For example, ψ-2 cells release

virions with an ecotropic host range (26), and PA12 or PA317 cells release virions with an amphotropic host range (29, 30). Second, a major effort has involved the construction and design of retroviral vectors (2, 6, 12, 31, 37). Typically, these vectors contain a dominant selectable marker gene in addition to a site for inserting the gene of interest. However, vectors with two genes generally express the gene of interest poorly, and they often become altered during the retroviral replication cycle (6, 13, 21, 31, 32).

Recently, we described a strategy that simplifies the use of retroviral vectors and that can potentially overcome several of these problems (4). We found that retroviral vectors become amplified when they are added as calcium phosphate precipitates to cocultures that contain ecotropic-packaging ψ-2 cells and amphotropic-packaging PA12 or PA317 cells. Because the vectors spread efficiently in the cocultures, dominant selectable genes are unnecessary, and the vectors can be constructed simply for very high levels of expression of one gene. Vector DNA that is transiently expressed in the transfected cells suffices to initiate the process. Helper-free virions released from either cell type are cross-infectious for the other cell type, and the result is a theoretically limitless back-and-forth (ping-pong) process of vector replication that spreads throughout the culture. Viruses that are replication defective in other conditions replicate in these cocultures in the absence of any replication-competent helper virus. We used this method to express a leukemogenic membrane glycoprotein gene and the gene for human growth hormone (ghGH) (4, 23). However, amplifications ceased when the gene products were 0.3% or less of total cellular protein synthesis. We have now identified and overcome several problems in the previously used procedures. The result is a simple method for protein production in vertebrate cells.
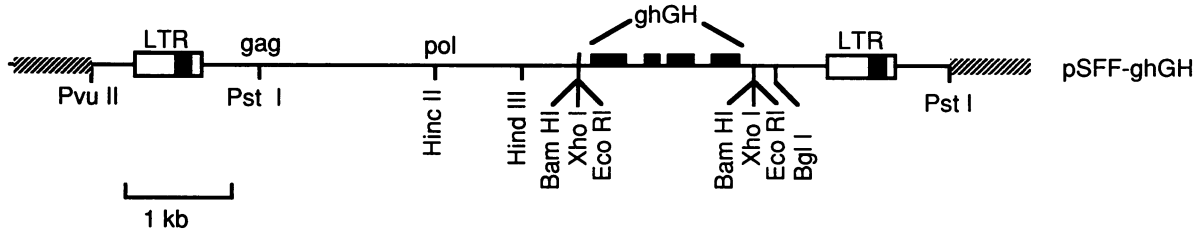
---

* Corresponding author.

FIG. 1. Structure of the pSFF-ghGH vector used for growth hormone production. Construction of this plasmid involved inserting the 1.55-kb ghGH into the expression site in the pSFF retroviral vector (4). The exons in ghGH are shown (■). ▨, pSP64 vector sequences. Restriction endonucleases HincII and BglI cut once in the viral sequence but not in the ghGH sequences. LTR, Long terminal repeat.

MATERIALS AND METHODS

Cell cultures and virus infections. Cell lines were maintained in 0.2 ml of medium per cm^2 surface area in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. Cell lines included NIH 3T3 fibroblasts, ψ-2 ecotropic-packaging cells (26), PA12 amphotropic-packaging cells (28), PA317 amphotropic-packaging cells (29), and CCL64 mink lung fibroblasts (American Type Culture Collection, Rockville, Md.). Virus was harvested by placing fresh culture medium on nearly confluent monolayers of virus-producing cell lines for 16 to 24 h, removing the medium, and filtering it through a 0.2-μm filter. NIH 3T3 and CCL64 fibroblasts were infected with helper-free human growth hormone (hGH)-encoding virus as follows. A total of 10^5 cells growing in 25-cm^2 dishes were incubated for 2 h at 37°C with 1 ml of 1:2 serial dilutions of virus in the presence of 8 μg of Polybrene per ml. The cells were then grown in fresh culture medium. After 48 h, the cultures were passaged, grown on cover slips, and assayed for the fraction of hGH-positive cells by immunofluorescence (see below). Titers were calculated as the cell number at the time of infection multiplied by the multiplicity of infection multiplied by the viral dilution. The multiplicity of infection was determined as $-\log P_0/0.44$, where P_0 is equal to the fraction of cells lacking hGH immunofluorescence.

Immunological and blotting assays. hGH radioimmunoassays (sensitivity, 1.5 ng/ml) were performed as recommended by the manufacturer (Kallestad Laboratories, Austin, Tex.). Immunoblot detection of murine retroviral envelope glycoproteins employed a goat antiserum made to ecotropic Friend murine leukemia virus gp70 (23). This antiserum has group reactivity and therefore combines with both ecotropic and amphotropic env glycoproteins. For detection of hGH, we used a rabbit antiserum to hGH donated by the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Md. Methods for protein electrophoresis, blotting onto nitrocellulose membranes, and detection with ^{125}I-protein A were described previously (23). DNA blots were performed as described elsewhere, using as the hybridization probe a nick-translated plasmid that contained ghGH cloned into pUC19 (4, 23).

For hGH immunofluorescence, cells grown on cover slips were fixed in 3.7% formaldehyde in phosphate-buffered saline at room temperature for 20 min and then permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 10 min. The cover slips were then incubated with 1:2,000 dilutions of rabbit antiserum to hGH in culture medium for 1 h at 37°C. The cover slips were washed twice by incubation for 5 min at 37°C with culture medium, and they were then incubated in culture medium with a 1:50 dilution of fluorescein-conjugated goat anti-rabbit immunoglobulin G (Tago Immunologicals, Burlingame, Calif.) for 1 h at 37°C. The

cover slips were then washed three times for 5 min by incubation in medium at 37°C. The cover slips were then rinsed with phosphate-buffered saline and mounted in phosphate-buffered saline that contained 10% glycerol. Negative controls were uninfected cells.

Retroviral vector amplifications. ghGH (10) was cloned into the XhoI site in the pSFF ping-pong retroviral vector as described previously (4). The resulting pSFF-ghGH construct is shown, with relevant restriction sites, in Fig. 1. Two protocols were used for amplications. In both protocols, pSFF-ghGH (10 μg) was transfected as a calcium phosphate precipitate (4, 23) into 25-cm^2 culture dishes that contained a 1:1 mixture of ψ-2 and PA12 cells at a total cell concentration of 10^5 cells per dish. Protocol A was previously described (4). The cultures were passaged every 3 to 4 days at confluency by seeding approximately 5×10^4 cells into new 25-cm^2 dishes. For protocol B, 24 h after transfection, the cultures were passaged by seeding 100 cells into 0.32-cm^2 wells of a 96-well culture plate. These minicocultures were maintained for 7 days, and they were then passaged to 9-cm^2 culture dishes for another 7 days. The culture media were then analyzed for hGH by radioimmunoassay, and positive cultures were subsequently grown and passaged into 25-cm^2 dishes as described above. This protocol enabled the critical early stage of amplifications to occur in small cocultures in the absence of recombinant shutoff viruses. After that stage, the exponential growth of hGH-encoding virus is assured because it has been given a head start. Recombinant shutoff virus that forms subsequently does not spread rapidly enough to impair hGH production. One modification of this protocol was to grow the minicocultures in 2-cm^2 dishes rather than to expand them into 25-cm^2 dishes. Another modification that also worked successfully was to wait 2 to 4 days after transfection before seeding the 100 cells into 96-well dishes. This gave a larger proportion of hGH-producing minicocultures.

RESULTS

Cells in cocultures become resistant to ping-pong amplification. When the pSFF-ghGH plasmid (Fig. 1) was transfected as a calcium phosphate precipitate into a coculture containing 10^5 total ψ-2 and PA12 cells (i.e., protocol A), hGH synthesis subsequently occurred (Fig. 2). However, maximum yields of hGH were low and variable in different experiments, and there were reproducible indications of a decline in the late stages of the process (see below).

Several lines of evidence suggested that the cells in shutoff cocultures were resistant to infection. First, these cultures continued to release hGH virions in titers sufficient to rapidly infect 100% of murine NIH 3T3 and mink CCL64 fibroblasts or pure ψ-2 and PA12 cells, as assayed by immunofluorescence for hGH. Nevertheless, as illustrated
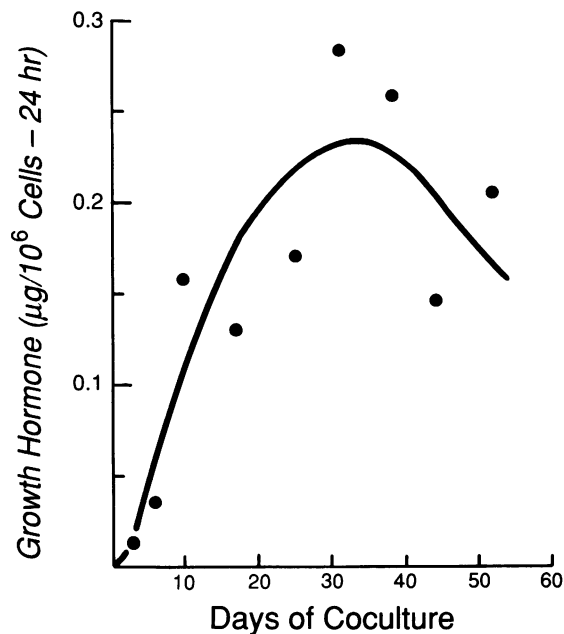
FIG. 2. Amplification of hGH expression in an initial coculture amplification experiment using protocol A (see Materials and Methods). The pSFF-ghGH plasmid was transfected into a 1:1 ψ-2-PA12 coculture in a 25-cm^2 dish. The cells were grown to confluency and passaged every 3 to 4 days. Fresh medium was added 24 h before confluency. This medium was collected after 24 h for hGH radioimmunoassay.

by the immunofluorescence results (Fig. 3, arrow), some cells in shutoff cocultures were uninfected with hGH-encoding virus. Different shutoff cocultures also varied in the percentage of the cells (between 10 and 86%) that contained hGH. These results suggested that shutoff was caused by cellular resistance to infection by hGH-encoding virions rather than by cessation of hGH-encoding virion production. Moreover, cellular resistance evidently occurred at different stages of amplification in different cocultures, resulting in a variable percentage of uninfected cells.

The hypothesis that a change in the cells made them resistant to ping-pong amplification was supported by studies in which we added fresh cells to the cocultures. Addition of ψ-2 cells had no effect, whereas PA12 cells reproducibly caused a substantial increase in hGH synthesis (Fig. 4). These results imply that shutoff cocultures contain a severe depletion or functional deficiency of PA12 cells. Surprisingly, the increases in hGH synthesis that occurred after additions of PA12 cells were followed by decreases. These results were explained by subsequent studies (see Discussion).

Shutoff viruses form in the cocultures. The resistance to infection could be caused by viral interference. According to this idea, transmissible viruses that encode envelope glycoproteins might form in the ψ-2 and PA12 cells. These viruses could spread in the cocultures, causing interference to further amplification of hGH-encoding virus. This possibility was tested by studying envelope glycoproteins in the cocultures and by searching for *env* gene-encoding viruses in the culture media.

As shown by protein immunoblot analysis, the ecotropic virus *env* glycoproteins in ψ-2 cells have faster electrophoretic mobilities than the amphotropic virus *env* glycoproteins
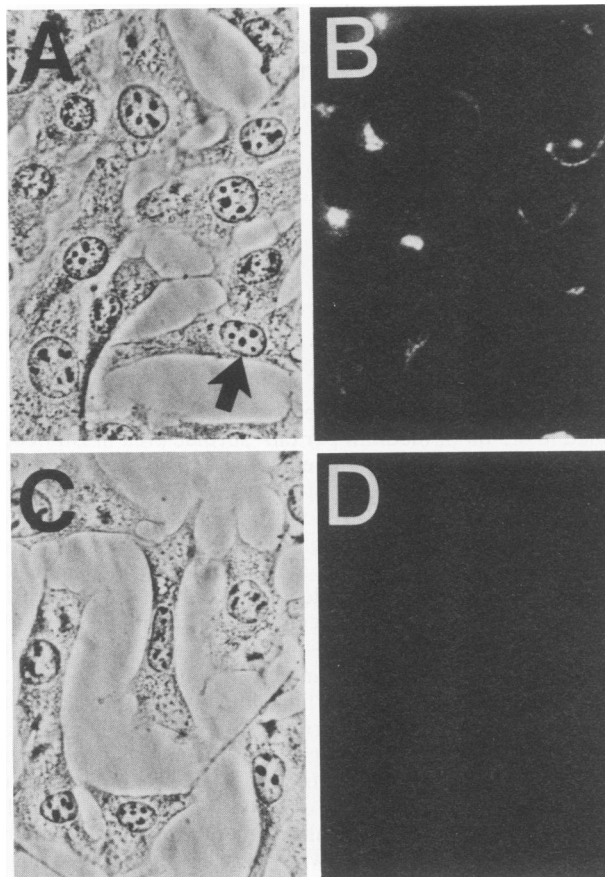

FIG. 3. Immunofluorescent detection of hGH in cells from a shutoff coculture. A cell coculture that had been used for pSFF-ghGH amplification according to protocol A shut off its hGH amplification, as illustrated in Fig. 2. After several additional weeks of culturing, the cells were grown on cover slips and were stained for hGH by immunofluorescence. Each field was photographed both in phase-contrast (A and C) and under fluorescent conditions (B and D). The arrow in panel A shows a cell that lacked hGH. In this culture, 86% of the cells contained hGH. However, the percentage of hGH-containing cells varied widely in independently made shutoff cocultures. The cell culture in panels C and D was a negative control that had not been transfected with the pSFF-ghGH vector.

in PA12 cells (Fig. 5A, compare lanes 1 and 2, respectively). These glycoproteins consist of mature gp70 products and smaller amounts of gPr90 precursors. As expected, NIH 3T3 and CCL64 fibroblasts lack these glycoproteins (Fig. 5A, lanes 3 and 4). The shutoff ψ-2-PA12 coculture contained both gp70s (Fig. 5A, lane 5). Cell-free medium from the latter shutoff coculture was then used to infect fresh ψ-2, PA12, NIH 3T3, and CCL64 cell cultures (Fig. 5A, lanes 6 to 9, respectively). Clearly, this medium contained transmissible virions that encoded both ecotropic and amphotropic *env* glycoproteins. Thus, after incubation with medium from the shutoff coculture, both ψ-2 cells (Fig. 5A, lane 6) and PA12 cells (Fig. 5A, lane 7) synthesized both types of glycoproteins. The amphotropic *env* virus evidently also transferred into the mouse NIH 3T3 and mink CCL64 fibroblasts (Fig. 5A, lanes 8 and 9), implying that the titer of this virus in the coculture medium was sufficient to infect a detectable fraction of the nonpackaging cells. Presumably, the ecotropic *env* virus was present at a lower titer and it could not spread
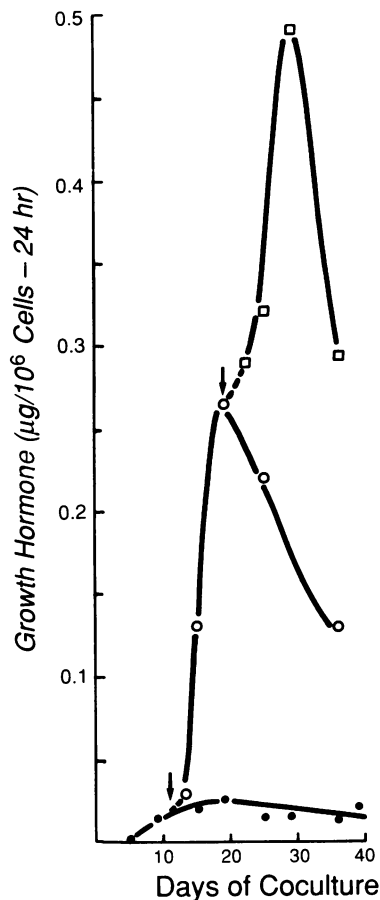
FIG. 4. Production of hGH is stimulated when fresh PA12 cells are added to a shutoff coculture. A 1:1 ψ-2-PA12 coculture was used for pSFF-ghGH amplification according to protocol A, and it shut off amplification at a low level of hGH synthesis. The shutoff coculture was passaged at 2- to 4-day intervals and was mixed 4:1 with fresh PA12 cells at the two times indicated by arrows. Culture medium was replaced 24 h before harvest of samples for hGH assays. Hormone synthesis was measured at the times indicated in the original coculture (●), in the spiked coculture (○), and in the twice-spiked coculture (□).

in the NIH 3T3 fibroblasts because it was not replication competent.

Additional experiments supported and extended these conclusions. For example, the *env* viruses recovered from the shutoff cocultures were tested for the ability to be serially transmitted in fresh ψ-2, PA12, NIH 3T3, and CCL64 cultures and between these different cell types. In one experiment, the virus from a shutoff coculture was first infected into ψ-2 cells (these infected ψ-2 cells were analyzed in Fig. 5A, lane 6). The secondary virus from the latter ψ-2 cells was then infected into fresh ψ-2, PA12, NIH 3T3, and CCL64 cells (Fig. 5B, lanes 3 to 6, respectively). Clearly, this secondary virus was promiscuous because it could infect both ψ-2 and PA12 cells. This was evident both from the additional envelope glycoproteins produced by these cells after infection (compare the infected cells in Fig. 5B, lanes 3 and 4, with the uninfected ψ-2 and PA12 cells in lanes 1 and 2, respectively) and from their synthesis of hGH (results not shown). As determined from these data, the secondary virus clearly contained both ecotropic and amphotropic *env* viruses. Consequently, the ψ-2 cells that released these sec-

ondary virions must have also contained both *env* viruses. These and other experiments indicated that both *env* viruses in the medium from shutoff cocultures were able to promiscuously infect both ψ-2 and PA12 cells. Presumably, such virus transmission must involve pseudotyping. For example, a ψ-2 cell that contained an amphotropic *env* virus would form dually infectious (presumably hybrid) virions that contained both types of envelope glycoproteins. These virions would infect either ψ-2 or PA12 cells. Similarly, an ecotropic *env* virus would also spread into both types of packaging cells. Our results clearly established that single cells in the shutoff cocultures contain both types of envelope glycoproteins and that they release promiscuously infectious virions. In addition, our results indicated that the *env*-recombinant viruses were not replication competent because they could not be serially passaged in the nonpackaging NIH 3T3 and CCL64 cells (results not shown). Specifically, NIH 3T3 and CCL64 cells that had been infected with shutoff viruses (e.g., Fig. 5A, lanes 8 and 9, and 5B, lanes 5 and 6) did not release secondary virions that could infect fresh NIH 3T3 or CCL64 cells and cause them to synthesize immunologically detectable *env* glycoproteins (results not shown).

A prediction of the results described above is that shutoff of amplification would occur when all cells in the coculture synthesize both *env* glycoproteins, whereas at earlier stages of amplification such cells should be absent or relatively rare. Figure 6 shows results from an experiment in which cells were cloned from a coculture before shutoff had occurred but after hGH-encoding virus amplification was substantially advanced. Clearly, both ψ-2 and PA12 cell clones were isolated from the coculture. Only one cell clone (Fig. 6, lane 17) contained both types of *env* glycoproteins. A skewing was evident, however, in that ψ-2 cells (Fig. 6, lanes 4, 6 to 9, and 11 to 16) were more prevalent than PA12 cells (Fig. 6, lanes 5, 10, and 18) (see Discussion).

Cell clone 98, analyzed in Fig. 6 (lane 13), produced a substantial titer (ca. 2×10^6/ml) of exclusively ecotropic host range hGH-encoding virus. This virus was used to repeatedly infect the PA12 cells (Fig. 6, lane 18) and the cells that contained both types of *env* glycoproteins (Fig. 6, lane 17). The cells that contained only amphotropic gp70 were efficiently infected by the ecotropic hGH-encoding virions, whereas cells with both gp70s were completely resistant (Fig. 7). Susceptibility to infection in this and other experiments agreed with expectations based on the envelope glycoprotein phenotype of the cell. We conclude that resistance to infection by hGH-encoding virions can be caused by *env*-encoding shutoff viruses that spread throughout the cocultures.

Ping-pong amplification in minicocultures overcomes the problem caused by shutoff viruses. Since *env*-encoding shutoff viruses are presumably recombinants that form by rare events and then spread throughout the cocultures, we reasoned that they would form less frequently in small cocultures than in larger ones. In minicocultures, fewer rounds of vector replication would be required to achieve a high multiplicity of proviruses per cell. Consequently, the probability of shutoff occurring before substantial hGH-encoding vector amplification should be reduced (see Discussion).

Indeed, ping-pong amplifications were much superior in minicocultures. Figure 8 shows a typical hGH-encoding virus amplification in a minicoculture that was seeded with only 50 ψ-2 and 50 PA12 cells (protocol B). The maximum 24-h yield, 1.65 μg of hGH per 10^6 cells, was much higher than we ever obtained by using larger cocultures (i.e., protocol A). In another minicoculture, the maximum 24-h
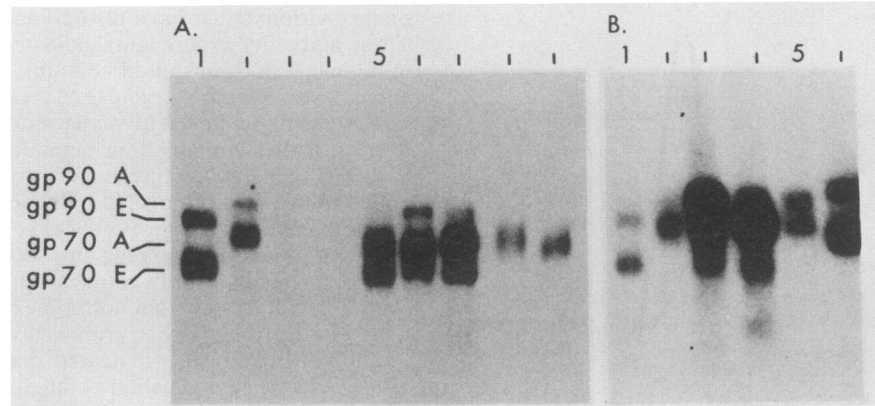
FIG. 5. Protein immunoblot analysis of shutoff viruses that encode *env* glycoproteins. (A) The *env* glycoproteins (or absence of such) in pure ψ-2, PA12, NIH 3T3, and CCL64 fibroblasts are shown in lanes 1 to 4, respectively. The ecotropic virus *env* glycoproteins in ψ-2 cells (the gPr90 precursor and the gp70 product) differ in size from the amphotropic virus *env* glycoproteins in PA12 cells. A shutoff coculture produced as described for Fig. 1 (protocol A) was analyzed in lane 5. Virus harvested from the latter shutoff coculture was also used to infect fresh ψ-2, PA12, NIH 3T3, and CCL64 cells, and the infected cells were then analyzed in lanes 6 to 9, respectively. (B) The *env* glycoproteins in pure ψ-2 and PA12 fibroblasts are shown for controls in lanes 1 and 2, respectively. The ψ-2 cells used in panel A, lane 6 (they had been infected with virus from the shutoff coculture medium) also released virus into the culture medium, and this secondary virus was used to infect ψ-2, PA12, NIH 3T3, and CCL64 cells that were then analyzed for *env* glycoproteins (panel B, lanes 3 to 6, respectively). These results show that *env*-encoding viruses occur in the medium of a shutoff coculture and that these *env* viruses can be transmitted from packaging cells into naive cell cultures. The amphotropic virus *env* glycoproteins synthesized in mink CCL64 cells (panel A, lane 9 and panel B, lane 6) had slightly different electrophoretic mobilities than those synthesized in mouse cells. This difference is caused by differences in oligosaccharides. A, Amphotropic; E, ecotropic.

yield was 2.1 μg/10⁶ cells. As determined by radioimmunoassays, 100% of the cells cloned from these high-expresser cocultures synthesized hGH.

Two other methods also reduced the problem of shutoff viruses. They both involved cloning cells from cocultures before shutoff had occurred and then screening the clones for hGH production and for envelope glycoprotein (e.g., as for Fig. 6). Thereby, we selected ψ-2 and PA12 hGH expresser cell clones that lacked shutoff viruses. In one method, these latter clones were remixed to allow ping-pong amplification to reinitiate. This resulted in rapid amplification of hGH production (i.e., 24-h yields as high as 2.2 μg of hGH per 10⁶ cells). In the other method, a selected expresser PA12 cell clone was superinfected with virus harvested from a selected expresser ψ-2 clone (e.g., as for Fig. 7). This also resulted in abundant hGH production.

High-level ping-pong amplifications were unhealthy for cells. Diverse morphological changes occurred, including

formation of multinucleated cells. The cell growth rate decreased, and cells became less able to recover from trypsinization and from freezing. With practice, we were able to use the morphological changes to monitor amplifications. Cells were then cloned from cocultures before they had become badly damaged, thereby separating ψ-2 and PA12 cells and terminating amplifications. Most of the resulting cell clones stabilized after several weeks and then produced a constant amount of hGH. However, the quantities of hGH produced by these stable cell lines were typically severalfold less than the maximum amounts of hGH that were initially synthesized. Table 1 shows yields of hGH obtained from representative stable hGH producer cell lines. The hGH produced by these high-expresser cells was homogeneous and coelectrophoresed with standard hGH (Fig. 9).

Properties of high-expresser cell lines. ghGH in the pSFF-ghGH plasmid is the complete gene with introns (Fig. 1). When cut with the restriction endonuclease *Eco*RI, an
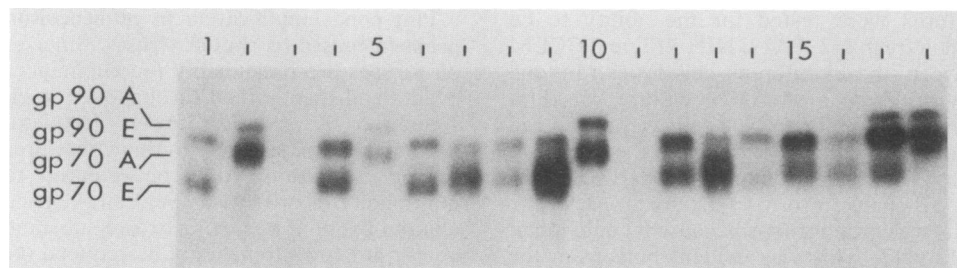

FIG. 6. Protein immunoblot analysis of *env* glycoproteins encoded by hGH-expressing cell clones. The pSFF-ghGH vector was amplified by ping-pong amplification in a 1:1 ψ-2-PA12 coculture, using protocol B. This procedure reduces shutoff and results in elevated hGH production (see Results). After the culture was expanded into a 25-cm² dish for several weeks, cells were cloned from the coculture and each clone was assayed for *env* glycoprotein phenotype. Lanes 1 and 2 show the *env* glycoproteins in pure ψ-2 and PA12 cells, respectively. Lanes 4 to 18 show the *env* glycoproteins in different clones of hGH producer cells. Only one clone (lane 17) contained both ecotropic and amphotropic *env* glycoproteins. One other clone (lane 11) lacked *env* glycoproteins, illustrating the hereditable shutoff of *env* synthesis that occurs occasionally in clonally prepared ψ-2 and PA12 cells (5). A, Amphotropic; E, ecotropic.
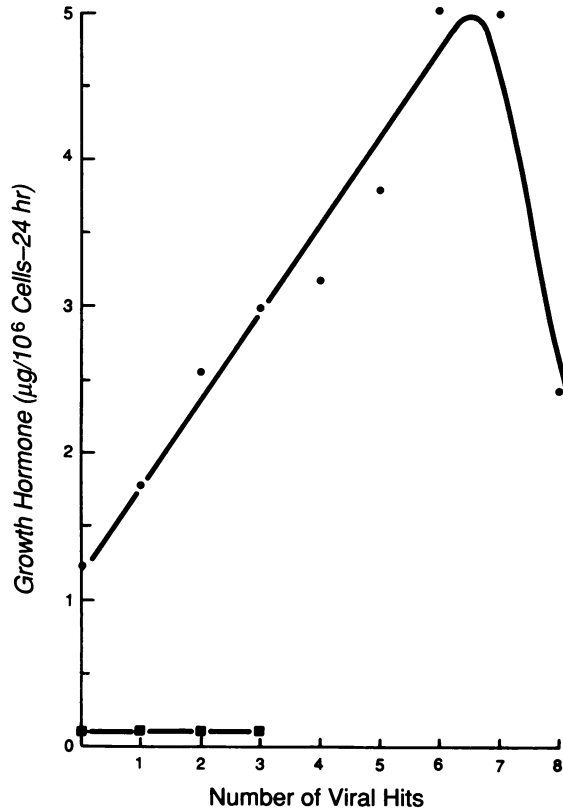
FIG. 7. Susceptibility to infection by hGH-encoding virions correlates with the *env* glycoprotein phenotype of cells. Clone 98 cells analyzed in Fig. 6 (lane 13) produced a substantial titer (ca. 2 × 10^6/ml) of pure ecotropic hGH-encoding virus. The virus was used to repeatedly infect hGH producer PA12 cells (●) (they were analyzed for *env* glycoproteins in Fig. 6, lane 18) and also cells that contained both ecotropic and amphotropic virus *env* glycoproteins (■) (see Fig. 6, lane 17). In this and other experiments, susceptibility to infection correlates with the *env* glycoprotein phenotype, and only cells that lack specific *env* glycoproteins are susceptible to infection by that host range of hGH-encoding virus. hGH production is unstable in very high-expresser cells, which accounts for the reduced expression in the cells that were infected eight times by the virus (see Results).

intron-containing fragment of 1.55 kilobase pairs (kb) would be expected. However, the gene without introns should yield a fragment of 0.77 kb. As shown by Southern blot analysis, the original pSFF-ghGH plasmid yielded the expected 1.55-kb fragment (Fig. 10A, lane 1). However, the DNA from a high-expresser cell clone yielded a 0.8-kb fragment that hybridized in stringent conditions with the human ghGH probe (Fig. 10A, lane 2). This suggests that ghGH amplified by ping-pong amplification was intronless. We also digested the cellular DNA with *Hinc*II and *Bgl*I restriction endonucleases that uniquely cut within the viral sequences of pSFF-ghGH but not within the ghGH sequences (Fig. 1). The number of fragments that hybridize to the ghGH probe should indicate the number of hGH-encoding proviruses integrated into the cellular DNA. The results shown in Fig. 10B suggest that these cells contain approximately 13 hGH-encoding proviruses. High-expresser cells also released hGH-encoding virions into the culture media (Table 1). The titers of amphotropic viruses from PA12 cells were much lower than the titers of ecotropic viruses released from ψ-2
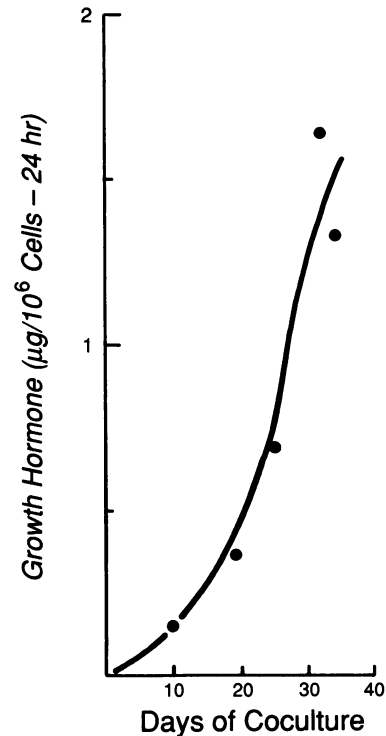

FIG. 8. Kinetics of hGH production during amplification of the pSFF-ghGH vector in a 1:1 ψ-2-PA12 coculture produced by using minicoculture protocol B (see Materials and Methods). The cells became unstable at the end of the amplification, and the cells were then isolated by cloning (see Results).

cells, even when the cells synthesized similar amounts of virus-encoded hGH.

DISCUSSION

Problem caused by shutoff viruses. The results described above strongly support earlier evidence (4) that ping-pong amplification is a back-and-forth process in which ecotropic virions from ψ-2 cells infect PA12 cells and amphotropic virions from PA12 cells infect ψ-2 cells. Although this process could theoretically proceed indefinitely, in our initial experiments, amplifications shut off when only a variable fraction (10 to 86%) of the cells had been infected by an hGH-encoding virus (e.g., Fig. 3). This premature shutoff was at least partially caused by formation of *env* gene-containing transmissible viruses. These shutoff viruses spread in cocultures, and they produced cells that contained both ecotropic and amphotropic envelope glycoproteins. Because of interference, such cells resisted infection by hGH-encoding virions.

Efficient spread of shutoff viruses in ψ-2-PA12 cocultures is evidently facilitated by formation of promiscuous virions. Thus, a packaging cell that synthesizes both ecotropic and amphotropic envelope glycoproteins produces virions (presumably hybrids) that can infect either ψ-2 or PA12 cells (Fig. 5). The spread of newly formed shutoff viruses in cocultures therefore short-circuits a strictly back-and-forth route of infectious transmission. Previous evidence has indicated that glycoproteins encoded by different enveloped viruses can copackage into hybrid virion particles (1, 8, 28, 40). However, dualtropic host range murine leukemia vi-

TABLE 1. Properties of stable growth hormone producer cell clones

Cell clone	Type of gp70	Titer of released virus/ml[a]	hGH yield		
			μg/10^6 cells[b]		% of total protein synthesis[c]
			24 h	96 h	
63	—[d]	—[d]	2.2		1.8
74	Ecotropic	1.7×10^7	2.0		1.3
75	Amphotropic	6.0×10^3	1.1	3.6	1.2, 1.3
98	Ecotropic	2.0×10^6	1.2	2.9	2.8, 1.0
104.16	Amphotropic	4.4×10^4	4.2	15.2	3.8, 5.5

[a] Virus titers were measured after 2-h virus adsorption onto NIH 3T3 cells. Retroviral titers depend on the cells and adsorption conditions, and they are therefore relative rather than absolute measures of virus concentrations. In addition, virus concentrations in medium were only slightly reduced after the 2-h adsorption time. Titers measured here are therefore less than they would be if we had used more cells and longer adsorption times (31).

[b] Cells were grown for 96 h in culture media. The medium in some cultures was changed 24 h before harvest. For other cultures, the medium was simply harvested at 96 h. hGH was measured by radioimmunoassay. In both cases, cells were counted at the time of harvest.

[c] Where two numbers are indicated, they were determined from the 24- and 96-h hGH harvests, respectively. Amounts of cellular protein were measured by the Coomassie blue method (Bio-Rad Laboratories, Richmond, Calif.). For the 24-h results, the percent yields of hGH were determined by measuring the total amount of cell protein that accrued in the cultures during the 24-h harvest period. For the 96-h results, percent yields were calculated by dividing the amount of hGH in the medium by the total cell protein at the time of harvest. The latter values will be underestimates for slowly growing cells. Clone 98 grows only slowly.

[d] —, Clone did not express either gp70 and accordingly did not produce virus.

ruses (also called mink cell focus-inducing viruses) are genomically masked when they are released from cells that also contain an ecotropic murine leukemia virus (14, 18, 36). Ecotropic env glycoproteins can apparently competitively exclude dualtropic but not amphotropic env glycoproteins from budding virions.

Presumably, shutoff viruses form by recombination between replicating hGH-encoding virions and endogenous env genes in packaging cells. Accordingly, shutoff viruses form efficiently only in cocultures that contain a replicating retroviral vector (results not shown). Since recombination would be a low-probability event during any single cycle of vector replication (11, 17, 31), we reasoned that it would cause fewer problems in small cocultures than in larger ones. For example, if a shutoff virus formed at a frequency of 10^{-4} per cycle of vector replication, a coculture of 10^5 cells would usually be shut off before all cells had acquired one proviral copy of the vector, whereas a coculture of 10^2 cells would
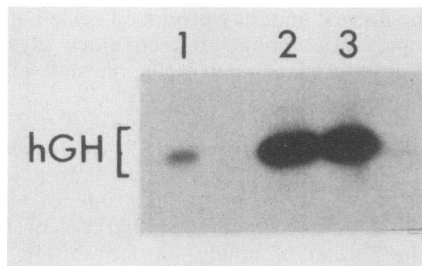

FIG. 9. Protein immunoblot analysis of hGH secreted from stable high-expresser hGH producer cell lines. Lane 1 contains an authentic ^{125}I-hGH standard supplied with the radioimmunoassay kit. The culture media from cell clones 75 and 98 were analyzed in lanes 2 and 3, respectively.
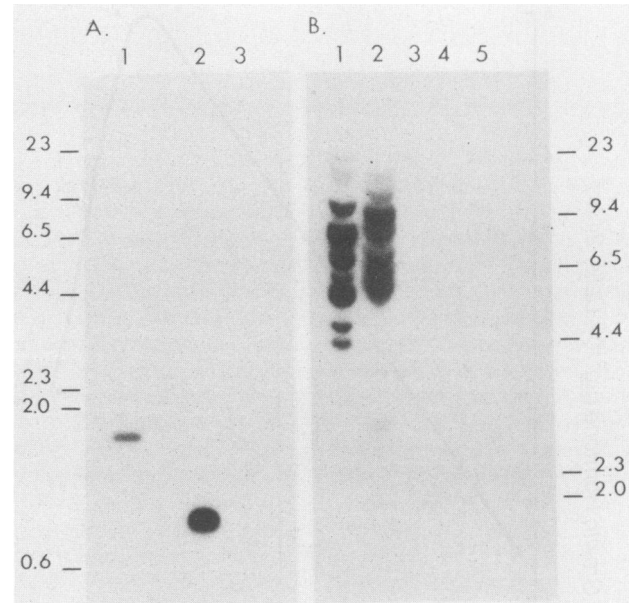

FIG. 10. DNA blot analysis of ghGH amplified in ψ-2-PA12 cocultures and detected by using a ^{32}P-hGH probe. (A) Analysis of EcoRI-digested DNAs from the original pSFF-ghGH plasmid (lane 1), from the genomic DNA of the high-expresser cell clone 98 (lane 2), and from the genomic DNA of normal mouse spleen (lane 3). (B) Analysis of HincII- and BglI-digested DNAs from clone 98 high-expresser cells (lanes 1 and 2, respectively) and normal mouse spleen DNA digested with EcoRI, BglI, and HincII (lanes 3, 4, and 5, respectively). Sizes of fragments (in kilobase pairs) are indicated.

become infected with vector to a high proviral multiplicity. In support of this reasoning, we reproducibly obtained higher levels of hGH synthesis when we initiated amplifications in small cocultures (protocol B) (e.g., Fig. 8). Two screening methods based on these principles also resulted in substantial yields of hGH (see Results).

For these reasons, we believe that the critical variable in protocol B is the small initial size of the coculture (i.e., 100 cells). Under that condition, the vector has a better chance to quickly amplify to a substantial proviral multiplicity in the absence of any shutoff viruses and without mutations (11). By the time the cocultures are grown to larger sizes, the titers of hGH-encoding virions are sufficiently high to ensure their continued exponential amplification. Any shutoff viruses that form after that time cannot replicate rapidly enough to limit hGH-encoding virus amplifications.

An alternative strategy to eliminate shutoff viruses would be to use packaging cells in which the resident env genes are altered to reduce their abilities to recombine with the vector. Several packaging cell lines (including PA317) are crippled in their abilities to produce replication-competent recombinants with gag, pol, and env genes (9, 27, 29, 31), but they were not constructed to reduce formation of the replication-defective env recombinants detected in these experiments. Although we continue to study this issue, our preliminary studies suggest that crippled packaging cells are not superior to ψ-2 and PA12 cells for ping-pong amplifications. We are currently modifying the pSFF vector to reduce its potential for homologous recombination with env genes.

Problem caused by instability of cells that contain numerous hGH-encoding proviruses. A major problem that continues to influence this process involves an instability of cells that contain many proviruses and that synthesize large amounts

of hGH. Such cells have reduced viabilities and growth rates, and their syntheses of hGH diminish with time. After several weeks, however, yields of hGH often stabilize and the resulting cell lines produce hGH in amounts as high as 4 to 6% of the total cellular protein synthesis (Table 1).

Many factors could conceivably contribute to the instability of hGH production. For example, the hGH protein, the many proviruses, or the large amounts of viral RNA could be toxic to the cells. Accordingly, low-expresser cell variants would have a growth advantage. Alternatively, a cellular mechanism (e.g., a stress-induced pathway) might inhibit cell growth and reduce retrovirus-specific gene expression (15, 42). Such a mechanism would be compatible with the rapid reduction in hGH production that we have observed in single cell clones. Instabilities of retroviral expression have been described previously (5, 7, 11, 17, 25).

An important phenomenon that appears related to this cellular instability is the selective depletion of PA12 cells that occurs in cocultures during hGH-encoding virus amplifications (e.g., Fig. 4 and 6). As a consequence, spiking these cocultures with PA12 cells can cause bursts of hGH production (Fig. 4). This depletion was unexpected because we seeded ψ-2 and PA12 cells in a 1:1 ratio and because our PA12 cell line grows slightly faster than our ψ-2 cell line. Reproducibly, however, we have found that ψ-2 cells release at least 10 times higher titers of virus than PA12 cells when both cell types express similar amounts of a retroviral vector (Table 1). Therefore, during the ping-pong amplification process we would expect PA12 cells to become infected more rapidly than ψ-2 cells. The resulting growth disadvantage would be expected to cause selective reductions in proportions of PA12 cells. The addition of naive PA12 cells to such depleted cocultures would be expected to result in their rapid infection to high multiplicities. As observed, this would cause a burst of hGH production, followed by subsequent depletion of these high-expresser PA12 cells (Fig. 4).

Applications to protein production. We conclude that retroviral vectors can be used for high-level protein production in vertebrate cell cultures. The maximum yields of hGH obtained in these initial experiments (and in other studies using the human erythropoietin gene [results not shown]) are in the useful range of several milligrams per liter of medium from confluent cultures. Moreover, increased yields should be possible by modifying the vector and by using different producer cells (see below). With the improved protocols described here, cell clones that produce the desired protein as several percent total protein synthesis can be routinely obtained within 2 to 3 months. We have used this method to produce hGH, the erythroblast mitogenic glycoprotein encoded by Friend erythroleukemia virus (4, 23), human erythropoietin, and the mouse ecotropic retrovirus receptor (unpublished results). The erythropoietin·receptor gene has also been expressed in substantial yields by using the pSFF vector by this method (24), as has a brain protein (B. Chesebro, personal communication). This method, therefore, appears to be widely applicable for producing different proteins.

An important feature of the method is its potential use with any human or animal cell line that can be infected with a retrovirus. Cells specialized for secretion or able to grow in serum-free medium (19) would probably be advantageous for protein production, compared with ψ-2 and PA12 mouse fibroblasts. Also, ψ-2 and PA12 cells lift from culture dishes shortly after they reach confluency, whereas many other cells can be maintained for prolonged periods at high densities. This is an important factor that contributes to the

apparent high yields of secretory proteins reported for other production methods (16, 38). Posttranslational modifications, including glycosylation, are also often host and tissue specific (33, 41). For these reasons, adapting the method to other cells would potentially improve the yields and properties of the protein products.

The ping-pong amplification method is exceptionally useful for functional studies and for site-directed mutagenesis because genes are rapidly expressed in substantial amounts and because the high-titer by-product virions can efficiently transfer the mutated and wild-type genes into diverse cell types from different species, or even into animals, for functional assays (4, 23, 24; unpublished results). Previous methods for producing high-titer retrovirions are relatively complex and uncertain (6, 12, 31, 37). They require use of a dominant selectable marker gene that can reduce virus yields and expression (6, 13, 21, 31, 32).

LITERATURE CITED

1. **Adams, S. E., K. M. Dawson, K. Gull, S. M. Kingsman, and A. J. Kingsman.** 1987. The expression of hybrid HIV:Ty virus-like particles in yeast. Nature (London) **329:**68–70.
2. **Armentano, D., S.-F. Yu, P. W. Kantoff, T. von Ruden, W. F. Anderson, and E. Gilboa.** 1987. Effect of internal viral sequences on the utility of retroviral vectors. J. Virol. **61:**1647–1650.
3. **Bassin, R. H., S. Ruscetti, I. Ali, D. K. Haapala, and A. Rein.** 1982. Normal DBA/2 mouse cells synthesize a glycoprotein which interferes with MCF virus infection. Virology **123:**139–151.
4. **Bestwick, R. K., S. L. Kozak, and D. Kabat.** 1988. Overcoming interference to retroviral superinfection results in amplified expression and transmission of cloned genes. Proc. Natl. Acad. Sci. USA **85:**5404–5408.
5. **Bestwick, R. K., C. A. Machida, E. E. Polonoff, and D. Kabat.** 1984. Frequent hereditable shutdown of murine retrovirus gene expression in murine cells. Mol. Cell. Biol. **4:**908–914.
6. **Bowtell, D. D. L., S. Cory, G. R. Johnson, and T. J. Gonda.** 1988. Comparison of expression in hemopoietic cells by retroviral vectors carrying two genes. J. Virol. **62:**2464–2473.
7. **Chiswell, D. J., P. J. Enrietto, S. Evans, K. Quade, and J. A. Wyke.** 1982. Molecular mechanisms involved in morphological variation of avian sarcoma virus-infected rat cells. Virology **116:**428–440.
8. **Clapman, P., K. Nagy, and R. A. Weiss.** 1984. Pseudotypes of human T-cell leukemia virus types 1 and 2: neutralization by patients' sera. Proc. Natl. Acad. Sci. USA **81:**2886–2889.
9. **Danos, O., and R. C. Mulligan.** 1988. Safe and efficient generations of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc. Natl. Acad. Sci. USA **85:**6460–6464.
10. **Denoto, F. M., D. D. Moore, and H. M. Goodman.** 1981. Human growth hormone DNA sequence and mRNA structure: possible alternative splicing. Nucleic Acids Res. **9:**3719–3730.
11. **Dougherty, J. P., and H. M. Temin.** 1989. Determination of the rate of base-pair substitution and insertion mutations in retrovirus replication. J. Virol. **62:**2817–2822.
12. **Eglitis, M. A., and W. F. Anderson.** 1989. Retroviral vectors for introduction of genes into mammalian cells. BioTechniques **6:**608–614.
13. **Emermann, M., and H. M. Temin.** 1986. Quantitative analysis of gene suppression in integrated retrovirus vectors. Mol. Cell. Biol. **6:**792–800.
14. **Fishinger, P. J., C. S. Blevins, and N. M. Dunlap.** 1978. Genomic masking of nondefective recombinant MuLV in Moloney virus

stocks. Science 201:457–459.

15. Fornace, A. J., Jr., D. W. Nebert, M. C. Hollander, J. D. Luethy, M. Papathanasou, J. Fargnoli, and N. J. Holbrook. 1989. Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. Mol. Cell. Biol. 9:4196–4203.

16. Friedman, J. S., C. L. Cofer, C. L. Anderson, J. A. Kushner, P. P. Gray, G. E. Chapman, M. C. Stuart, L. Lazarus, J. Shine, and P. J. Kushner. 1989. High-expression in mammalian cells without amplification. Bio/Technology 7:359–362.

17. Gillespie, D. A. F., K. A. Hart, and J. Wyke. 1985. Rearrangements of viral and cellular DNA are often associated with expression of Rous sarcoma virus in rat cells. Cell 41:279–287.

18. Haas, M., and V. Patch. 1980. Genomic masking of dual-tropic murine leukemia viruses: role of pseudotype virions in viral lymphomagenesis. J. Virol. 35:583–591.

19. Hamilton, W. G., and R. G. Ham. 1977. Clonal growth of Chinese hamster cell lines in protein-free media. In Vitro 13:537–547.

20. Ikeda, H., and H. Sugimura. 1989. Fv-4 resistance gene: a truncated endogenous murine leukemia virus with ecotropic interference properties. J. Virol. 63:5405–5412.

21. Joyner, A. L., and A. Bernstein. 1983. Retrovirus transduction: segregation of the viral transforming function and the herpes simplex virus *tk* gene in infectious Friend spleen focus-forming virus thymidine kinase vectors. Mol. Cell. Biol. 3:2191–2202.

22. Kabat, D. 1989. Molecular biology of Friend viral erythroleukemia. Curr. Top. Microbiol. Immunol. 148:1–42.

23. Li, J.-P., R. K. Bestwick, C. S. Spiro, and D. Kabat. 1987. The membrane glycoprotein of Friend spleen focus-forming virus: evidence that the cell surface component is required for pathogenesis and that it binds to a receptor. J. Virol. 61:2782–2792.

24. Li, J.-P., A. D. D'Andrea, M. F. Lodish, and D. Baltimore. 1990. Activation of cell growth by binding of Friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. Nature (London) 343:762–764.

25. Li, X., J.-K. Yee, J. A. Wolff, and T. Friedmann. 1989. Factors affecting long-term stability of Moloney murine leukemia virus-based vectors. Virology 171:331–341.

26. Mann, R., R. C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell 33:153–159.

27. Markowitz, D., S. Goff, and A. Bank. 1989. Construction and use of a safe and efficient amphotropic packaging cell line. Virology 167:400–406.

28. Metsikkö, K., and H. Garoff. 1989. Role of heterologous and homologous glycoproteins in phenotypic mixing between Sendai virus and vesicular stomatitis virus. J. Virol. 63:5111–5118.

29. Miller, A. D., and C. Buttimore. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol. 6:2895–2902.

30. Miller, A. D., M.-F. Law, and I. M. Verma. 1985. Generation of helper-free amphotropic retroviruses that transduce a dominant-acting, methotrexate-resistant dihydrofolate reductase gene. Mol. Cell. Biol. 5:431–437.

31. Miller, A. D., and G. J. Rosman. 1989. Improved retroviral vectors for gene transfer and expression. BioTechniques 7:980–990.

32. Miller, C. K., and H. M. Temin. 1986. Insertion of several different DNAs in reticuloendotheliosis virus strain T suppresses transformation by reducing the amount of subgenomic mRNA. J. Virol. 58:75–80.

33. Paulson, J. C., and K. J. Colley. 1989. Glycosyltransferases: structure, localization and control of cell type-specific glycosylation. J. Biol. Chem. 264:17615–17618.

34. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. In J. Sambrook, E. F. Fritsch, and T. Maniatis (ed.), Molecular cloning: a laboratory manual, 2nd ed., book 3, p. 16.1–16.81. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

35. Sambrook, J., and M. J. Gething. 1988. Vectors for high level expression of proteins in mammalian cells, p. 41–48. In N. Sasavage (ed.), Focus, vol. 10. Bethesda Research Laboratories/Life Technologies, Inc., Gaithersburg, Md.

36. Sitbon, M., J. Nishio, K. Wehrly, and B. Chesebro. 1985. Pseudotyping of dual-tropic recombinant viruses generated by infection of mice with different ecotropic murine leukemia viruses. Virology 140:144–151.

37. Temin, H. M. 1989. Retrovirus vectors: promise and reality. Science 246:983.

38. Van Brunt, J. 1986. Immobilized mammalian cells: the gentle way to productivity. Bio/Technology 4:508.

39. Weiss, R. 1981. Retrovirus receptors and their genetics, p. 187–199. In K. Lonberg-Holm and L. Philipson (ed.), Virus receptors, part 2: animal viruses. Chapman & Hall, Ltd., London.

40. Weiss, R. 1982. Genetics of retroviruses, p. 209–260. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

41. Yan, S. C. B., B. W. Grinnell, and F. Wold. 1989. Post-translational modifications of proteins: some problems left to solve. Trends Biochem. Sci. 14:264–268.

42. Young, R. A., and T. J. Elliot. 1989. Stress proteins, infection and immune surveillance. Cell 59:5–8.