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Generation of a Helper Cell Line for Packaging Avian Leukosis Virus-Based Vectors

PIERRE SAVATIER,[1]* CLAUDE BAGNIS,[2] PIERRICK THORAVAL,[1] DIDIER PONCET,[3] MUSTAPHA BELAKEBI,[1] FRANÇOIS MALLET,[1] CATHERINE LEGRAS,[1] FRANÇOIS-LOÏC COSSET,[1] JEAN-LUC THOMAS,[1] YAHIA CHEBLOUNE,[1] CLAUDINE FAURE,[1] GÉRARD VERDIER,[1] JACQUES SAMARUT,[2] AND VICTOR NIGON[1]

Laboratoire de Biologie Cellulaire, INRA, CNRS UA92, Université Claude Bernard Lyon I, 43 Boulevard du 11 novembre 1918, 69622 Villeurbanne Cedex,[1] Laboratoire de Biologie Moléculaire et Cellulaire, Ecole Normale Supérieure de Lyon, 69364 Lyon Cedex 07,[2] and INRA, Centre de Recherches de Jouy en Josas, 78350 Jouy en Josas,[3] France

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We constructed an avian leukosis virus-based packaging cell line, pHF-g, containing Rous-associated virus DNA with several alterations to abolish RNA packaging. One of them is a 52-base-pair deletion encompassing the putative encapsidation signal in the leader region. The 3′ long terminal repeat was also removed and replaced by the polyadenylation sequence from the herpes simplex virus thymidine kinase gene. When pHF-g cells were transfected by an avian leukosis virus-based vector, they produced replication-defective virus at high titer but they did not release any replication-competent particles. Proviral DNA was shown to be correctly integrated as well as correctly expressed. Viral RNAs were shown to be correctly translated into *gag*-related polypeptides.

Retrovirus vectors have become helpful tools for the efficient transfer of exogenous genes into eucaryotic cells. However, they are replication-defective viruses and need helper virus to replicate. In mammals, several retrovirus-packaging cell lines are available (1, 4, 21–24, 41) which release replication-defective retrovirus vectors in the absence of helper virus. Such vectors infect cells and integrate into the host genome but they cannot replicate and spread.

We and others are currently attempting to insert exogenous genes into the chicken germ line using avian leukosis virus (ALV)-based retrovirus vectors (13, 26–28, 36, 37, 39, 40). Some chicken strains have natural helper cells owing to constitutive expression of *ev-3*, *ev-6*, and *ev-9* endogenous viral sequences (35). However, only subgroup E *env* proteins are produced by these cells, and most chicken strains are not susceptible to infection by subgroup E retroviruses. In addition, these endoviruses are poorly expressed in culture (34); a 200-fold discrepancy in virus titer has been reported between low and high producing cell lines following transfection by a replication-defective vector (12). The highest titer has been observed with chicken cell lines whose *gag* gene is weakly expressed. Therefore, only *env* defective viruses could be packaged with these cells. Also, cells from these chicken strains display high recombination frequencies, giving rise to infectious viruses (33). Therefore, none of these naturally occurring helper cell lines was found suitable for production of helper-free retrovirus vectors. An avian packaging cell line has previously been constructed with the genome of the reticuloendotheliosis helper virus (49) but it cannot package ALV-based vectors. A packaging cell line suitable for packaging ALV-derived vectors has recently been reported (43). However, it still releases some replication-competent particles, and therefore, spreading of helper virus following germ line transformation of domestic animals might not be completely ruled out.

* Corresponding author.

In every genetically engineered packaging mutant generated from the Moloney murine leukemia virus (21, 23, 24), a sequence involved in RNA packaging has been deleted. This sequence is located in the 5′ untranslated segment of viral RNAs between the 5′ long terminal repeat (LTR) and the initiator codon for *gag* message. This sequence can be removed without detectable effect on *gag*, *pol*, and *env* gene expression. However, these mutants still produce a very low amount of helper virus and they are subject to frequent recombinations yielding replication-competent virus (23, 24). Other murine packaging cell lines have been constructed harboring more heavily deleted helper proviruses either with deletion of primer-binding site and polypurine tract sequences involved in initiation of reverse transcription and/or deletion of the 3′ LTR (23, 41) or from generation of two packaging mutants, one carrying *gag* and *pol* genes, the other one carrying the *env* gene, both driven either by the metallothionein promoter (1) or by the ALV LTR (22). None of these packaging mutants produces helper virus.

In avian retroviruses, a segment involved in RNA packaging has also been described from both naturally occurring packaging mutants of avian sarcoma virus (17, 19, 38), spleen necrosis virus (48), and genetically engineered mutants of ALV (16). This segment has been called the packaging signal. We constructed a packaging mutant from Rous-associated virus type 1 (RAV-1, an ALV) by deleting a 52-nucleotide (nt) segment homologous to the packaging signal described in ALV. Our mutant contains a deletion of the 3′ LTR in addition to deletion of the putative packaging signal. A packaging cell line was generated from this mutant. It produces virus at high titer following transfection by a replication-defective retrovirus vector. No helper virus was detected by our methods.

MATERIALS AND METHODS

Plasmids. The plasmid containing RAV-1 DNA was obtained from J. M. Bishop (University of California, San

Francisco). The plasmid pX343 was obtained from H. Diggelman (ISREC, Lausanne, Switzerland). It carries the bacterial gene conferring resistance to hygromycin B (HmB) driven by the simian virus 40 early promoter. Plasmid TXN3′ is a derivative of the avian erythroblastosis virus. It carries the bacterial gene conferring resistance to neomycin (Neo^r) driven by the LTR of the avian erythroblastosis virus (27). These plasmids were amplified in *Escherichia coli* C600RS or HB101.

Enzymes. Restriction enzymes, T4 polynucleotide kinase, T4 DNA polymerase, *Bal* 31 nuclease, the large fragment of *E. coli* DNA polymerase I, and calf intestinal phosphatase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), T4 DNA ligase was purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), and all linkers were purchased from New England BioLabs, Inc. (Beverly, Mass.). All enzymes were used as recommended by the suppliers.

Cells and culture conditions. Both chicken embryo fibroblasts (CEFs) and QT6 cells (25) were grown in F10 medium supplemented with 5% calf serum and 1% chicken serum. Both medium and sera were purchased from Flow Laboratories, Inc. (McLean, Va.). The QT6 cell line was a gift from C. Moscovici (Gainesville, Fla.). CEFs were prepared from C/O SPAFAS 10-day-old chicken embryos and grown as previously described (11). G418 (GIBCO Laboratories, Grand Island, N.Y.) and hygromycin B (Boehringer Mannheim) were dissolved in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.3) sterilized by filtration through 0.22-μm-pore-size filters. Concentrations of the drugs were expressed as milligrams of crude powder per milliliter and did not refer to the actual concentration of active antibiotic.

DNA transfection. Plasmid DNAs were transfected in QT6 cells as described by Kawai and Nishizawa (18). QT6 cells were plated at a density of 5×10^5 cells/60-mm dish with medium containing 2 μg of Polybrene per ml. The medium was replaced 16 h later with 1 ml of fresh medium without Polybrene containing from 0.5 to 3 μg of DNA. Six hours later, the medium was removed and replaced with 1 ml of medium containing 28.5% dimethyl sulfoxide. The cultures were kept at room temperature for 4 min, and then the medium was replaced with 4 ml of fresh medium. The cultures were further grown for 16 h, and then the cells were trypsinized and seeded into a 100-mm dish. One day later, the medium was changed for fresh medium containing drug (200 μg of G418 per ml or 30 to 80 μg of hygromycin B per ml). Thereafter, the medium was replaced every 2 days and Neo^+ or HmB^+ resistant colonies were isolated by using cloning cylinders.

Detection of viral proteins by ELISA. Production of viral proteins was monitored by an enzyme-linked immunosorbent assay (ELISA) with an anti-$p27^{gag}$ antibody (3). $p27^{gag}$ is encoded by the *gag* gene and results from posttranslational maturation of a 76-kilodalton precursor ($Pr76^{gag}$). $Pr76^{gag}$ was also detected by our anti-$p27^{gag}$ antibody.

Culture supernatant (100 μl) or 3 μg of cell proteins was used for each test. Culture supernatants were centrifugated (5 min at 10,000 × *g*) to remove cells and debris. Cell proteins were obtained as follows. Confluent cells on 60-mm dishes were trypsinized. Cells were centrifuged (5 min at 1500 × *g*), washed once in cold phosphate-buffered saline, and then suspended in 200 μl of 0.6% sodium dodecyl sulfate (SDS)–10 mM EDTA. They were lysed by one cycle of freeze-thawing. The lysate was then centrifuged (20 min at 10,000 × *g*). Supernatants were collected, and proteins were quantitated by a Bradford assay (2).

Immunoprecipitation of viral proteins. Cells (10^7) in 100-mm dishes were rinsed once with 5 ml of methionine-free minimal essential medium (GIBCO) supplemented with 10% dialyzed calf serum (GIBCO). Cells were incubated for 1 h in the same medium to which 250 μCi of L-[^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) was added. Cells were lysed with 5 ml of RIPA buffer (10 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 1% phenylmethylsulfonyl fluoride). The lysate was centrifuged at 30,000 rpm for 1 h in an SW41 rotor (Beckman Instruments, Inc., Fullerton, Calif.), the supernatant was collected, and total incorporation of [^{35}S]methionine into cellular proteins was determined by trichloroacetic acid precipitation and scintillation counting. Immunoprecipitation was done as previously described (9). An amount of each lysate equivalent to 2×10^6 cpm was incubated for 1 h with 4 to 8 μl of anti-$p27^{gag}$ antibody. The immune complexes were precipitated by the addition of 50 μl of protein-A-Sepharose (Pharmacia, Uppsala, Sweden). Immunoprecipitates were analyzed by electrophoresis on SDS-10% polyacrylamide gels, followed by fluorography.

Virus assay. For assay of helper virus, 10-day-old CEFs were seeded at 10^6/60-mm dish with medium containing 2 μg of Polybrene per ml. One day later, virus samples were added. Virus samples were collected from confluent culture dishes and then filtered through 0.22-μm-pore-size filters to remove cells and debris. The medium was replaced every 2 days thereafter, and cells were passaged for 2 weeks in culture to allow helper viruses to spread. Culture supernatants from these cells were then assayed by ELISA for $p27^{gag}$ protein.

Titers of viruses carrying the *neo* gene as a selectable marker were determined on QT6 cells by induction of G418 resistance. QT6 cells in dishes were infected with diluted virus suspensions for 8 h. Culture medium was then replaced by selective medium containing 200 μg of G418 per ml. The medium was replaced by fresh selective medium every 2 days thereafter, and resulting Neo^r colonies were scored 10 days later.

In situ detection of helper virus-infected cells by ELISA. QT6 cells were infected with virus and overlaid with agar 12 h later. Five days later, cells were washed in phosphate-buffered saline, fixed in 4% paraformaldehyde, and washed again in phosphate-buffered saline containing 0.25% Triton X-100. Polyclonal rabbit anti-$p27^{gag}$ (Life Sciences, Inc., St. Petersburg, Fla.) was added (45 min with 3% bovine serum albumin) followed by a alkaline phosphatase-conjugated goat anti-rabbit serum (Biosys, Compiègne, France) (45 min). Alkaline phosphatase activity was revealed with substrate kit III (Vector Laboratories, Inc., Burlingame, Calif.).

Cell DNA analysis. Cells were lysed in a solution containing 0.6% SDS, 10 mM EDTA, and 150 μg of proteinase K (Boehringer Mannheim) per ml for 2 h at 37°C. DNAs were then extracted with phenol-chloroform and chloroform-isoamyl alcohol successively and then precipitated at −20°C with 500 mM NaCl. They were incubated with RNase (50 μg/ml) for 30 min and then with proteinase K (100 μg/ml) for 1 h. The DNAs were then extracted with phenol-chloroform and chloroform-isoamyl alcohol, precipitated with 2 volumes of ethanol, dried, and dissolved in water.

Purified cellular DNAs were digested with several enzymes. Samples (10 μg) of digested DNAs were electrophoresed on agarose gels and transferred to cellulose-nitrate

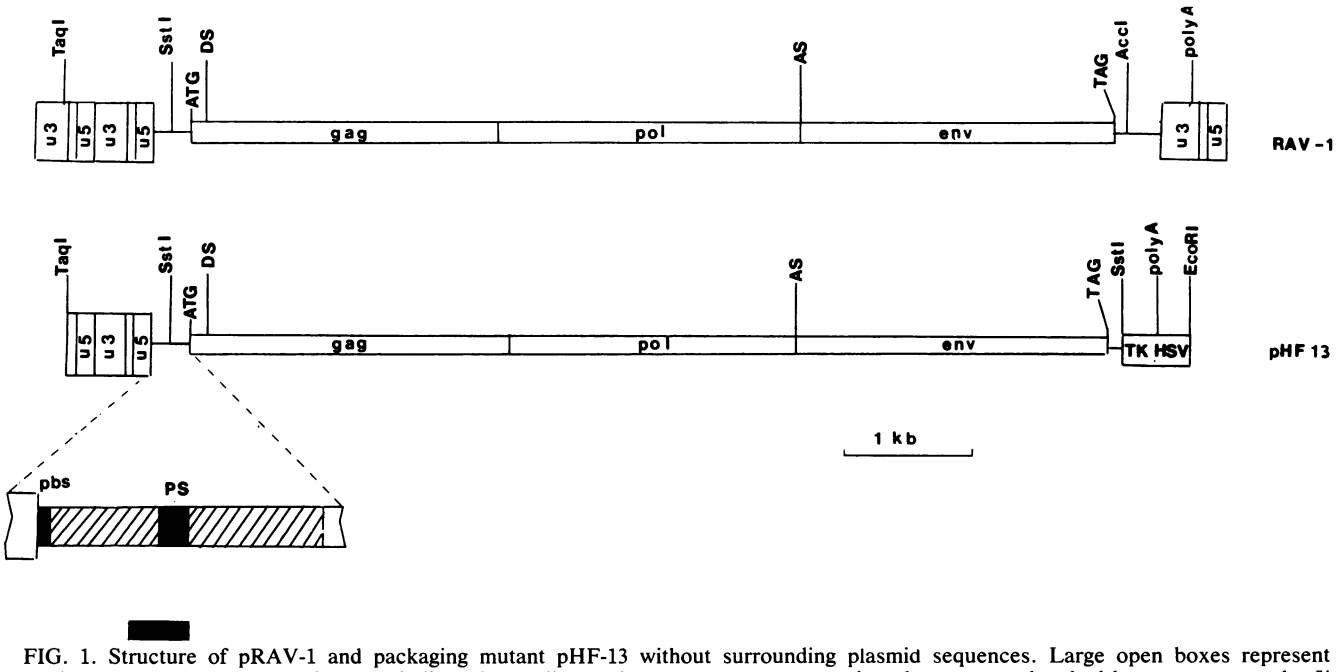


FIG. 1. Structure of pRAV-1 and packaging mutant pHF-13 without surrounding plasmid sequences. Large open boxes represent retroviral LTRs (U3 and U5 regions are indicated); small open boxes represent *gag*, *pol*, and *env* genes; hatched boxes represent the 5′ untranslated region in pHF-13 from which the putative packaging signal (PS) has been removed. The horizontal solid box represents the deletion made in pHF-13; pbs denotes primer-binding site; TK HSV denotes the polyadenylation signal of the herpes simplex virus thymidine kinase gene in pHF-13. Restriction sites used to construct pHF-13 are shown, but all the sites for a given enzyme are not necessarily shown. Important features for retrovirus replication are noted above the construct (DS, splice donor; AS, splice acceptor). The pHF-13 genome is inserted in pBR322 in place of the Amp^r gene between the *Pvu*II and *Eco*RI sites. The construct pHF-13 was made from pRAV-1, first by replacement of the 3′ LTR with the polyadenylation signal from the thymidine kinase gene of herpes simplex virus isolated as a 0.6-kilobase-pair *Sst*I-to-*Eco*RI fragment. The end of the retroviral genome was cleaved with *Acc*I, which cuts downstream of the termination codon of the *env* gene inside the 3′ untranslated sequence. Then an *Sst*I linker was inserted for the addition of a herpes simplex virus fragment containing the polyadenylation signal. The clone pRAV-1 contains two tandemly repeated 5′ LTRs. The left one was inactivated by removing the enhancer and promoter sequences in U3 upstream to a *Taq*I site. The deletion made in the 5′ untranslated region of pHF-13 was generated with *Bal* 31 nuclease starting at the *Sst*I site. This deletion is 52 base pairs long and encompasses the putative packaging signal.

filters by the procedure of Southern (42). The filters were prehybridized at 42°C for 30 min in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide, 1 h in 50% formamide–3× SSC–1× Denhardt solution (1% bovine serum albumin, 1% polyvinylpyrrolidone, 0.2% Ficoll), and 2 h in 50% formamide–3× SSC, 1× Denhardt solution–0.1% SDS–50 μg of salmon sperm DNA per ml. Hybridization was done in the same mixture containing 10^9 cpm of radioactive probe for 24 to 48 h at 42°C. Filters were then washed for 2 h at 42°C in 3× SSC–1× Denhardt solution–0.1% SDS–50 μg of salmon sperm DNA per ml and then for 2 h in 0.1× SSC–0.1% SDS–50 μg of salmon sperm DNA per ml. Filters were rinsed in 3× SSC, dried, and exposed to Kodak X-Omat AR films at −80°C with an intensifying screen.

Cellular RNA analysis. Total cellular RNAs were extracted from cells by homogenization in a solution containing 1% SDS, 200 μg of proteinase K per ml, 20 mM Tris hydrochloride (pH 7.4), 150 mM NaCl, and 200 mM EDTA. The homogenates were centrifuged overnight through a CsCl gradient at 20°C at 30,000 rpm in an SW41 rotor as described elsewhere (10). Total cellular RNAs (10 μg) were denatured (20) and then fractionated on 1.5% agarose gel containing 6% formaldehyde in MOPS (morpholinepropanesulfonic acid) buffer. Transfer to nitrocellulose filters was performed as previously described (45). Filters were prehybridized for 8 to 20 h at 42°C in 50 mM Tris hydrochloride (pH 7.0) containing 3× SSC, 20 μg of yeast tRNAs (Boehringer Mannheim) per ml, 20 μg of salmon sperm DNA per ml, 1× Denhardt solution and 50% formamide. Hybridization was done in the same mixture containing 10^9 cpm of radioactive probe for 48 h at 42°C. The filter was then washed at 42°C once in 2× SSC for 1 h, twice in 0.1% SDS–0.1× SSC for 45 min, and four times at room temperature in 0.1× SSC for 5 min. After drying, filters were exposed to X-Omat AR films at −80°C with an intensifying screen.

Preparation of labeled probes. Probes were isolated from pRAV-1. The *gag* probe was a 1.65-kilobase-pair *Bam*HI fragment. The *env* probe was a 2-kilobase-pair *Kpn*I-*Acc*I fragment. Probes were labeled by random primed synthesis (7) to specific activities higher than 10^9 dpm/μg.

RESULTS

Construction of packaging mutant. We chose the replication-competent retrovirus RAV-1 (subgroup A specificity) to produce packaging mutant virus because QT6 cells, used as the host, are highly sensitive to this subgroup. To lower the risk of generating replication-competent viruses by intermolecular recombinations, we introduced several mutations into the RAV-1 genome to prevent packaging of viral RNAs and further synthesis of proviruses. Previous studies have shown that a region between the 5′ LTR and the initiator codon of the *gag* message in ALV or avian sarcoma virus is required for efficient packaging of retroviral RNA into virions (16, 17, 19, 38). The homologous sequence in the

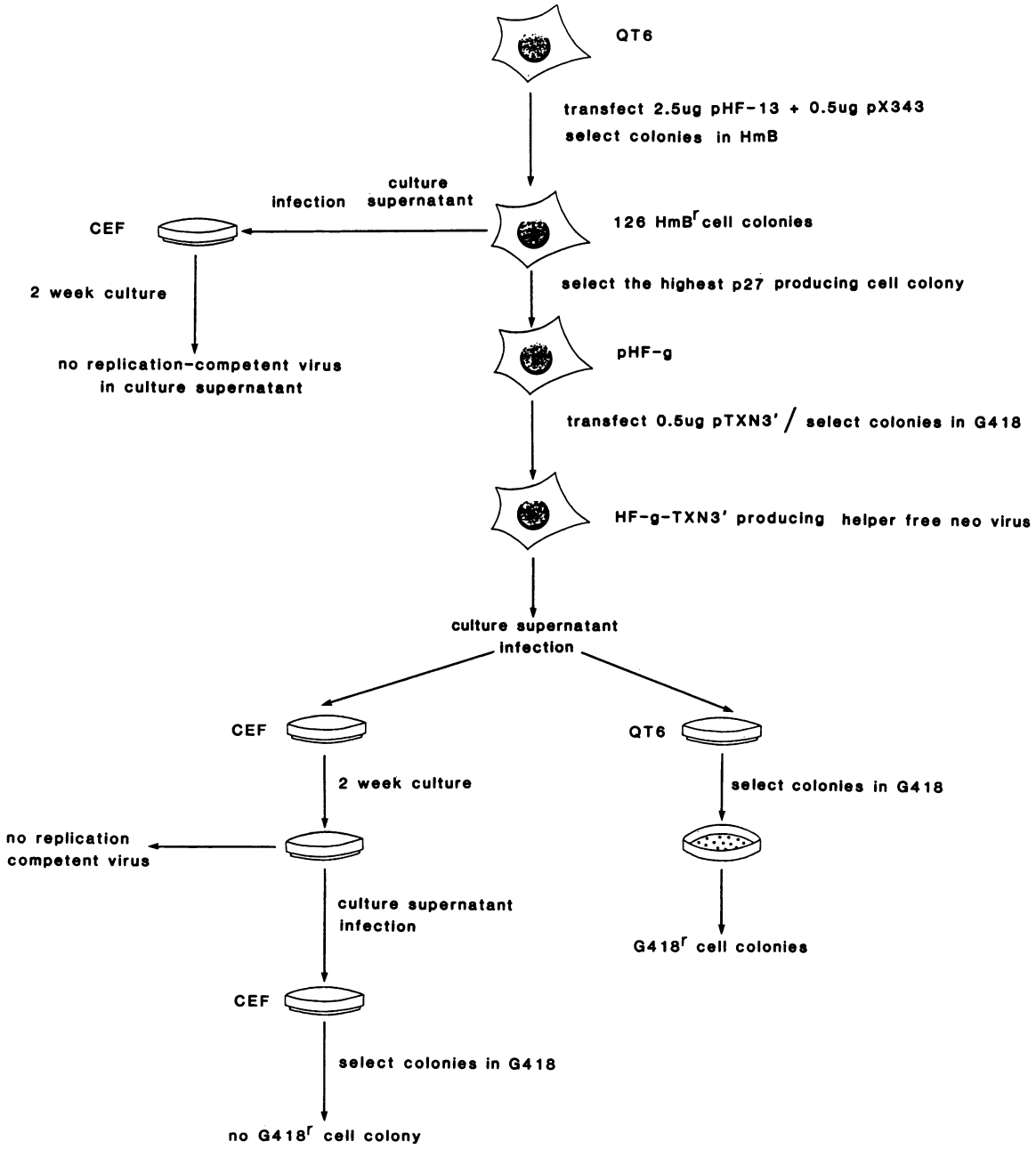


FIG. 2. Diagram of the procedure for generating a replication-defective virus and testing freedom from replication-competent virus by the double-step infection procedure. HmB^r, Resistance to hygromycin B. G418^r, resistance to G418.

genome of RAV between positions 203 and 254 from the cap site was removed from the plasmid pRAV-1 to generate the plasmid pHF-13 (Fig. 1). In this plasmid, the 3′ LTR was also removed and replaced by the polyadenylation sequence from the herpes simplex virus thymidine kinase gene, which was inserted 115 nucleotides downstream from 3′ end of the *env* gene. In RAV-1, the original polyadenylation site in the LTR is located 575 nucleotides downstream from the 3′ end of the *env* gene (Fig. 1).

Generation and testing of retrovirus-packaging cell lines. The DNA construct pHF-13 was introduced into QT6 cells by cotransfection with the plasmid pX343 carrying the hygromycin B resistance gene as a selectable marker. A total of 126 hygromycin-resistant cell colonies were isolated and screened for production of replication-competent virus (Fig. 2). Culture supernatants of these colonies were used to infect subconfluent cultures of CEFs. The CEFs were then passaged in culture continuously for 2 weeks to allow replication-competent virus to spread. Then we looked for p27^{gag} viral proteins in culture supernatants using an ELISA with an anti-p27^{gag} antibody. All CEF cultures remained RAV-1 negative. This demonstrated that none of the QT6 clones transfected with pHF-13 released replication-competent virus detectable by this method.

Among the 126 HmB^r clones analyzed, 26 were screened for production of p27^{gag} in crude cell lysates and in their

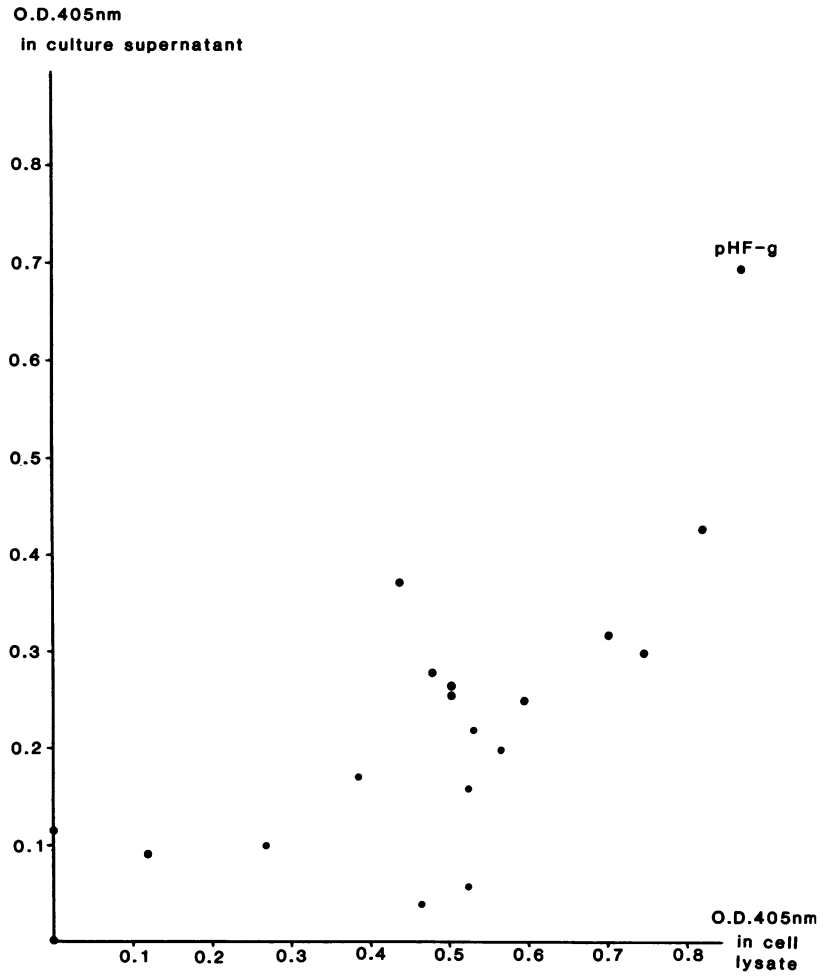


FIG. 3. $p27^{gag}$ content in culture supernatants as a function of $p27^{gag}$ content in cell lysate in 26 hygromycin B-resistant clones resulting from transfection of QT6 cells with pHF-13 and pX343. Nine clones did not produce $p27^{gag}$. They are represented as a single spot at the origin. $p27^{gag}$ content was measured by ELISA with an anti-$p27^{gag}$ antibody. $p27^{gag}$ content is expressed as the optical density at 405 nm (O.D. 405 nm). Values reported correspond to $p27^{gag}$ content in 3 μg of proteins from cell lysate and in 100 μl of culture supernatant.

culture supernatants (Fig. 3). $p27^{gag}$ contents in culture supernatants and cell lysates were positively correlated, which demonstrates that the more viral proteins are produced by the cells, the more these proteins are released in the culture supernatant. This suggests that the most efficient packaging clones should be those having the highest $p27^{gag}$ content in their culture supernatants. Thereafter, the 100 HmB^r clones that had not been analyzed in the first screening were only subjected to quantitative analysis of $p27^{gag}$ in their culture supernatant. One clone, pHF-g, was chosen because it released the highest level of $p27^{gag}$ in culture supernatant. pHF-g is shown in Fig. 2. Two other clones, pHF-d and pHF-e, with lower levels of $p27^{gag}$ were also retained.

To check whether these cells efficiently package defective retroviral genomes, we transfected pHF-g cells with DNA from the retrovirus vector TXN3′, which carries and expresses the Neo^r gene (Fig. 4). pHF-g cells transfected with TXN3′, thereafter called HF-g-TXN, were selected with G418, and a pool of 98 Neo^r colonies was further grown. Culture supernatant from these pooled clones was used to infect fresh QT6 cells, which were then selected with G418 (Fig. 2). A total of 2×10^4 Neo^r colonies were induced by 1 ml of culture supernatant from HF-g-TXN. This titer demonstrated that pHF-g cells can efficiently package defective vector genomes into infectious virions. As a preliminary test for release of replication-competent virions, culture supernatants from HF-g-TXN cells were used to infect CEFs which were then passaged in culture continuously for 3 weeks. These cultures remained $p27^{gag}$ negative, and their supernatants were unable to transmit G418 resistance to fresh CEFs. We used CEFs instead of QT6 cells in these replication-competent detection experiments because previous experiments had shown that *neo* virus titers are always higher (by a factor of two) when determined on CEFs rather

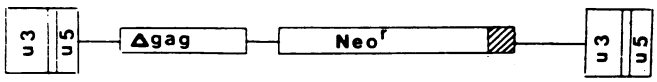


FIG. 4. Viral vector TXN3′. TXN3′ is a derivative of pXJ12 (8) from which the v-*erbA* oncogene has been deleted. The *neo* gene is inserted in place of the v-*erbB* oncogene, driven by the 5′ LTR (27). Δ*gag* indicates the 5′ end of the *gag* gene which is retained in wild-type avian erythroblastosis virus. Similarly, the striped box indicates the 3′ end of the *env* gene. Large open boxes represent retroviral LTRs (U3 and U5 regions are indicated).

TABLE 1. Test of helper cell lines

Clone	$p27^{gag}$ content (OD_{405})[a]		*neo* virus titer (G418^r CFU/ml)[b]	Production of replication-competent virus	
	Culture supernatant	Cell lysate		Double-step infection	In situ ELISA
pHF-d	0.32	0.37	2×10^3	−	ND[c]
pHF-e	0.46	0.53	4×10^4	−	ND
pHF-g	0.69	0.83	2×10^4	−	<1
TXN3′/RAV-1 QT6	14[d]	ND	3×10^5	+++	+++

[a] $p27^{gag}$ content in culture supernatants and in cell lysates of the hygromycin B-resistant clones pHF-d, pHF-e, and pHF-g as well as in TXN3′-RAV-1-infected QT6 cells. $p27^{gag}$ contents were estimated by using an ELISA with an anti-$p27^{gag}$ antibody. Values reported are optical densities at 405 nm (OD_{405}) in 100 μl of culture supernatant.

[b] Average *neo* virus titer released by a pool of about 100 G418^r clones resulting from transfection of the putative packaging clones with TXN3′ defective vector as well as from TXN3′-RAV-1-infected QT6 cells.

[c] ND, Not determined.

[d] Value estimated after dilution.

than on QT6 cells. CEFs might be more sensitive to viral infection than QT6 cells. The HF-g-TXN line was further tested for production of replication-competent virus by in situ detection of helper virus-infected cells. An ELISA with anti-$p27^{gag}$ antibodies was used on QT6 cells previously infected with large amounts of culture supernatant from HF-g-TXN cells. No $p27^{gag}$-positive cell was detected in this assay (Table 1), whereas as few as one infection event was found to be detectable when using diluted samples of culture supernatant from RAV-1-infected QT6 cells. All these data provide evidence that HF-g-TXN cells do not release any helper virus.

Similar results were obtained with clones pHF-d and pHF-e transfected with pTXN3′. As expected from quantitative analysis of $p27^{gag}$ in culture supernatants, pools of G418-resistant HF-d-TXN or HF-e-TXN cells released markedly less TXN3′ virus than HF-g-TXN cells (2×10^3 and 4×10^3 G418^r CFU/ml, respectively). None of them released detectable helper virus, as determined by the double-step infection procedure described above (Table 1). QT6 cells infected with a mixture of vector and helper viruses, TXN3′-RAV-1 QT6, were used as a control. They released TXN3′ virus with a titer of 3×10^5 G418^r CFU/ml, 15 times more than HF-g-TXN cells. Moreover, TXN3′-RAV-1 QT6 contained six times more $p27^{gag}$ protein than pHF-g cells in cell extracts and 20 times more in their culture supernatants (Table 1).

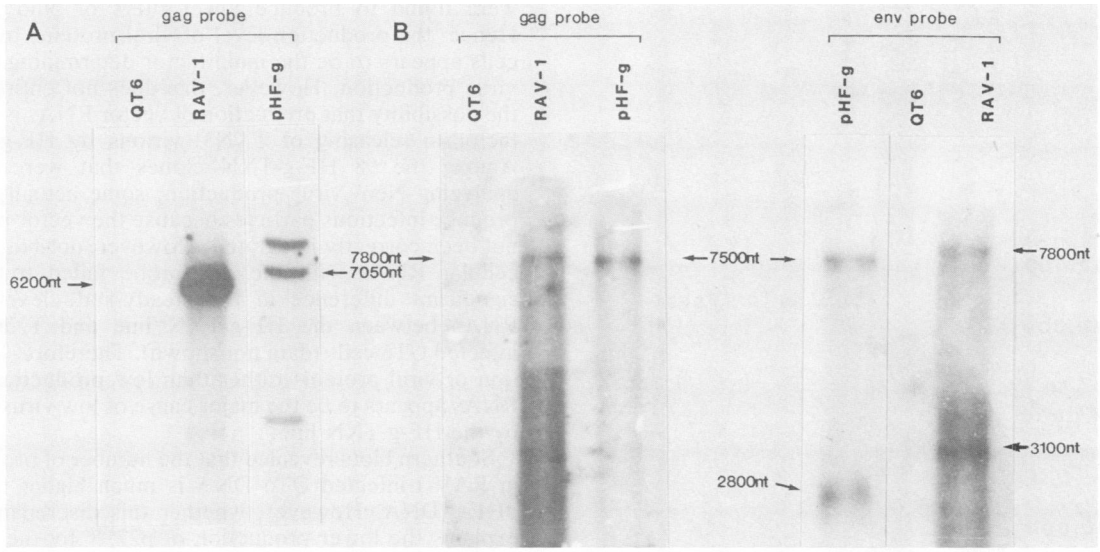
Analysis of nucleic acids and viral proteins from pHF-g cells. DNAs were extracted from pHF-g cells and from RAV-1-infected QT6 cells; they were analyzed on Southern blots by hybridization with the *gag* probe (Fig. 5A). A 7.0-kilobase restriction fragment was observed with DNA extracted from pHF-g cells after digestion with *Sst*I. It corresponded to the expected restriction fragment generated by complete digestion of the pHF-13 provirus with *Sst*I. Other bands were also observed, presumably resulting from recombinations during transfection. Therefore, at least a part of the pHF-13 provirus in pHF-g cells seems to be integrated without noticeable rearrangement. Similarly, the expected 6.2-kilobase restriction fragment generated by *Sst*I-*Sal*I digestion of RAV-1 provirus was revealed in DNA extracted from RAV-1-infected QT6 cells. However, the intensity of the 6.2-kilobase fragment in RAV-1-infected QT6 DNA was found to be well above that of the 7.0-kilobase fragment in pHF-g DNA. Multiple infections by RAV-1 could probably explain this discrepancy.

Total RNAs were extracted from the pHF-g cells as well as from RAV-1-infected QT6 cells. They were analyzed on Northern (RNA) blots by hybridization with the *gag* and *env* probes (Fig. 5B). One transcript was revealed with the *gag* probe that was similar in size to the expected genomic RNA (7,500 nt long). Two transcripts were revealed with the *env* probe. The largest one (7,500 nt long) corresponds to the genomic RNA, similar in size to that revealed with the *gag* probe. The shortest one (2,800 nt long) corresponds to the subgenomic RNA resulting from splicing of the genomic RNA. Both genomic and subgenomic RNAs produced by the pHF-13 provirus are 300 nt shorter than RNAs produced by the RAV-1 provirus (7,800 and 3,100 nt long, respectively), as expected from the changes introduced in the RAV-1 genome to produce pHF-13. However, a smear was observed in addition to the expected bands when total RNAs from RAV-1-infected QT6 cells were hybridized with the *gag* or *env* probe. This was not observed for RNAs from pHF-g cells. This smear cannot be explained exclusively by degradation during RNA extraction since it was observed several times from independent experiments. But, presently, no simple interpretation for this can be proposed.

Cellular proteins from pHF-g cells and RAV-1-infected QT6 cells were further analyzed by immunoprecipitation with an anti-$p27^{gag}$ antibody followed by one-dimensional electrophoresis (Fig. 6). *gag* proteins (the major internal structural proteins) of avian retroviruses are first synthesized as a 76-kilodalton precursor polyprotein ($Pr76^{gag}$) (46). $Pr76^{gag}$ is proteolytically cleaved via several intermediates to generate $p10^{gag}$, $p12^{gag}$, $p15^{gag}$, $p19^{gag}$, and $p27^{gag}$ proteins (47). Many bands were revealed, probably because of the polyclonal origin of the $p27^{gag}$ antibody. However, bands corresponding to $Pr76^{gag}$ and $p27^{gag}$, respectively, could be identified in pHF-g cells by comparison with RAV-1-infected QT6 cells. Therefore, in pHF-g cells, the viral proteins seem to be correctly produced although at a lower level compared with RAV-1-infected QT6 cells.

DISCUSSION

We introduced two deletions into the genome of the replication-competent retrovirus RAV-1 in an attempt to generate a nontransmissible avian provirus (pHF-13) which could still provide *trans*-acting factors required for packaging of retroviral vectors. We deleted the sequence required for efficient packaging of retroviral RNAs into virions (16, 17, 19, 32, 38) and replaced the 3′ LTR and flanking sequences by the polyadenylation sequence of the herpes simplex virus thymidine kinase gene. This pHF-13 mutant genome was introduced into QT6 cells by cotransfection with a plasmid carrying a selectable marker to generate retrovirus-packaging cell lines. The cell clone pHF-g was selected for producing the highest level of *gag* proteins in culture supernatant. It was next transfected with TXN3′, a replication-defective vector. It produced TXN3′ virus at high titer, and no replication-competent virus could be detected. Several avian packaging cell lines have recently been reported by Stoker and Bissell (43). They produce very low although detectable levels of replication-competent virus. Only the packaging signal in the leader segment has been deleted from the RAV genome to generate their packaging mutant. Most probably, deletion of the packaging signal alone is not sufficient to fully abolish RNA packaging. In contrast, the extra deletion encompassing the 3′ LTR in



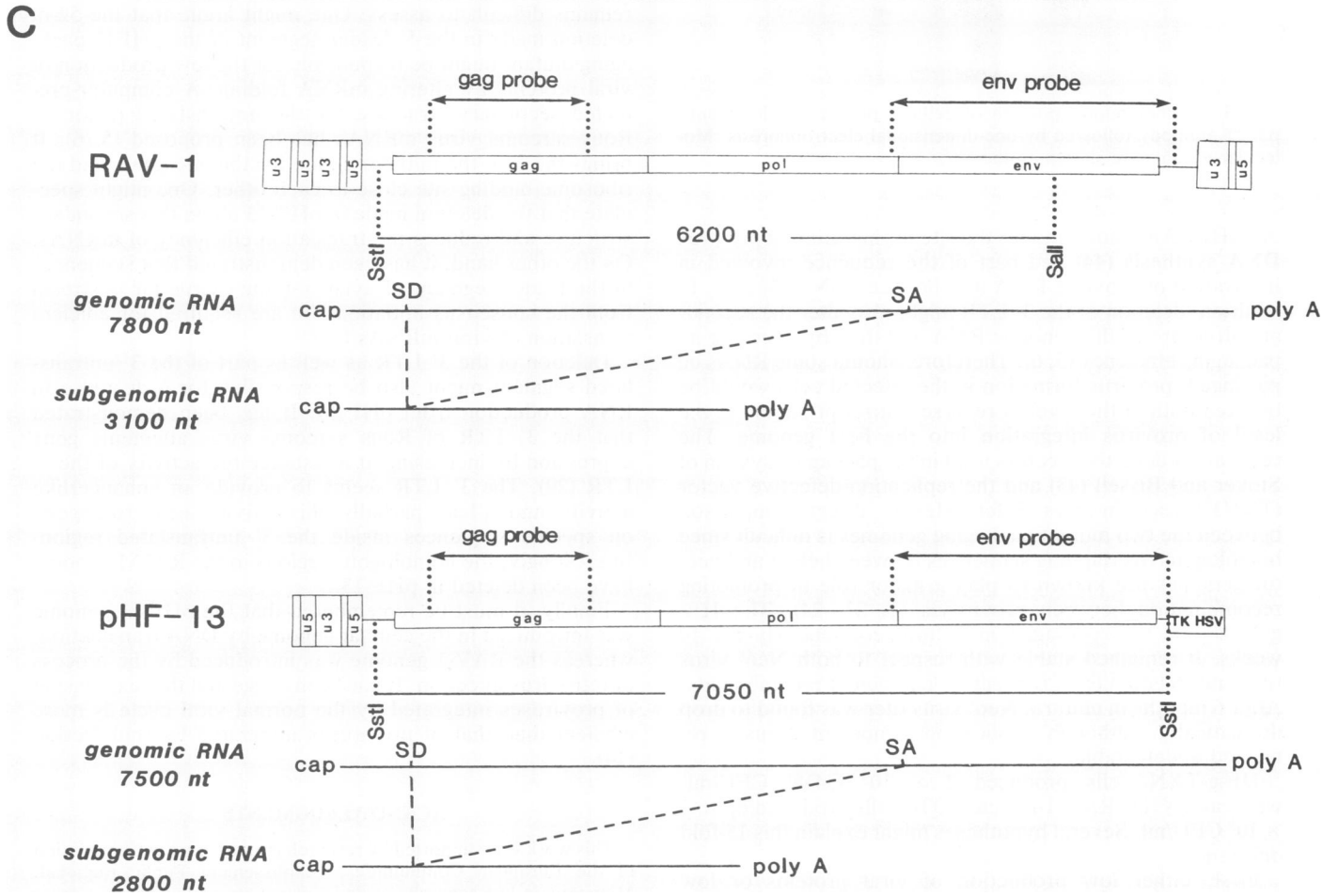


FIG. 5. (A) Southern blots of cellular DNAs extracted from QT6, RAV-1-infected QT6, and pHF-g cells, digested with *Sst*I (QT6 and pHF-g cells) or *Sst*I plus *Sal*I (RAV-1-infected QT6), and then hybridized with the *gag* probe. RAV-1 provirus was digested with *Sst*I plus *Sal*I because there is only one *Sst*I site in RAV-1 provirus. The *Sst*I site found in pHF-13 provirus at the 3′ end of the *env* gene was created for adding the polyadenylation signal of the herpes simplex virus thymidine kinase gene (see the legend to Fig. 1). (B) Northern blots of total cellular RNAs extracted from QT6, RAV-1-infected QT6, and pHF-g cells and hybridized with the *gag* and *env* probes. (C) Structure of RAV-1 and pHF-13 proviruses showing the size of the expected restriction fragments and the size of the expected genomic RNAs. SD, splice donor, SA, splice acceptor.

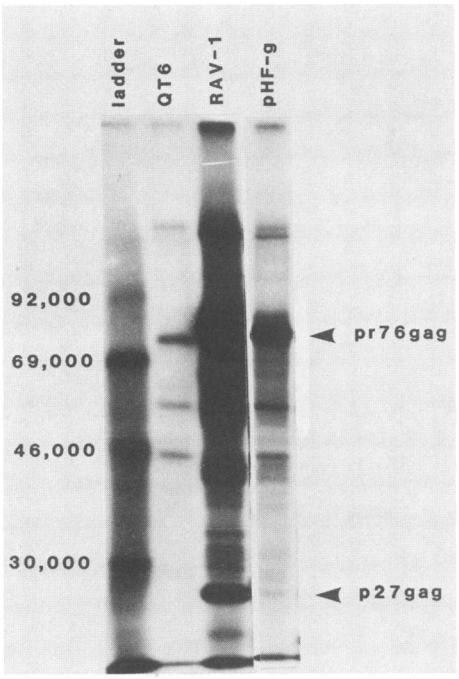


FIG. 6. Immunoprecipitation of cellular proteins with an anti-p27^{gag} antibody followed by one-dimensional electrophoresis. Molecular weights are shown on the left.

our pHF-13 mutant removes the site for initiation of provirus DNA synthesis (44) and part of the sequence involved in integration of provirus DNA into host cell DNA (30, 31). In addition, deletion of the 3′ LTR might also alter the secondary structure of the genomic RNA and thereby decrease its packaging efficiency (5, 6). Therefore, should some RNAs be packaged, provirus formation in the infected cells would be blocked both at the level of reverse transcription and at the level of provirus integration into the host genome. The replication-defective vectors used in the packaging system of Stoker and Bissell (43) and the replication-defective vector (TXN3′) used in ours differ. Hence, direct comparison between the two mutant packaging genomes is difficult since homologous overlapping sequences between helper and vector genomes are known to play a major role in promoting recombination between proviruses (1, 23, 24). The HF-g-TXN line was passaged in culture continuously for 10 weeks. It remained stable with respect to both Neo^r virus titer and freedom from replication-competent recombinants. After 6 months in culture, Neo^r virus titer was found to drop dramatically, although replication-competent viruses remained undetectable.

HF-g-TXN cells produced 2×10^4 G418^r CFU/ml, whereas TXN3′-RAV-1-infected QT6 cells produced up to 3×10^5 CFU/ml. Several hypotheses might explain this 15-fold difference.

First, either low production of viral proteins or low production of TXN3′ genomic RNAs might be responsible for lower production of TXN3′ virions by HF-g-TXN cells compared with TXN3′-RAV-1-infected QT6 cells. Both ELISA and an immunoprecipitation assay have provided evidence that production of viral proteins is significantly lower in pHF-g cells than in RAV-1-infected QT6 cells. On the other hand, packaging lines pHF-d and pHF-e, whose p27^{gag} contents in culture supernatants are significantly lower than the supernatant p27^{gag} content of the pHF-g line, were found to produce lower titers of Neo^r virus also. Hence, the production level of viral proteins by packaging cells appears to be the main factor determining the level of virus production. However, this does not entirely rule out the possibility that production of vector RNAs is the limiting factor in releasing of TXN3′ virions by HF-g-TXN line. Among the 98 HF-g-TXN clones that were pooled for analyzing Neo^r virus production, some actually might not produce infectious particles because the vector provirus has not been correctly integrated. However, dot-blot analysis of cellular RNAs with the *neo* probe failed to reveal any significant difference in the steady-state level of vector RNAs between the HF-g-TXN line and TXN3′-RAV-1-infected QT6 cells (data not shown). Therefore, low production of viral proteins rather than low production of vector RNAs appears to be the major cause of low virus production by the HF-g-TXN line.

Southern blots revealed that the number of proviral copies in RAV-1-infected QT6 DNA is much higher than that in pHF-g DNA. However, whether this discrepancy entirely explains the lower production of p27^{gag} by the pHF-g line remains difficult to assess. One might argue that the 52-nt deletion made in the 5′ leader segment of the pHF13 packaging mutant might be responsible for the low production of viral proteins by altering mRNA folding. A computer-predicted secondary structure for the untranslated region of Rous sarcoma virus mRNAs has been proposed (5, 6). It brings the cap, the initiator codon for the *gag* gene, and the ribosome-binding site close to each other. One might speculate that the deletion made in pHF-13 alters this secondary structure and reduces the translation efficiency of mRNAs. On the other hand, it has been demonstrated that sequences in the leader segment of avian sarcoma virus far upstream from the consensus initiation site are required for efficient translation of viral mRNAs (15).

Deletion of the 3′ LTR as well as part of the 3′ untranslated segment might also be responsible for a decrease in RNA production from pHF-13. It has been demonstrated that the 3′ LTR of Rous sarcoma virus augments gene expression by increasing at a distance the activity of the 5′ LTR (29). The 3′ LTR seems to provide an enhancerlike activity, and at least partially, this activity seems to depend on specific sequences inside the 3′ untranslated region. Interestingly, the homologous regions in the RAV-1 genome have been deleted in pHF-13.

Finally, it must be remembered that the pHF-13 genome was introduced in the cellular genome by DNA transfection, whereas the RAV-1 genome was introduced by the process of retrovirus infection. It has been suggested that expression of proviruses integrated by the normal viral cycle is more efficient than that of proviruses integrated by transfection (14).

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