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Development of Avian Sarcoma and Leukosis Virus-Based Vector-Packaging Cell Lines

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We have constructed an avian leukosis virus derivative with a 5′ deletion extending from within the tRNA primer binding site to a *Sac*I site in the leader region. Our aim was to remove *cis*-acting replicative and/or encapsidation sequences and to use this derivative, RAV-1ψ⁻, to develop vector-packaging cell lines. We show that RAV-1ψ⁻ can be stably expressed in the quail cell line QT6 and chicken embryo fibroblasts and that it is completely replication deficient in both cell types. Moreover, we have demonstrated that QT6-derived lines expressing RAV-1ψ⁻ can efficiently package four structurally different replication-defective v-*src* expression vectors into infectious virus, with very low or undetectable helper virus release. These RAV-1ψ⁻-expressing cell lines comprise the first prototype avian sarcoma and leukosis virus-based vector-packaging system. The construction of our vectors has also shown us that a sequence present within *gag*, thought to facilitate virus packaging, is not necessary for efficient vector expression and high virus production. We show that quantitation and characterization of replication-defective viruses can be achieved with a sensitive immunocytochemical procedure, presenting an alternative to internal selectable vector markers.

Retroviral vectors have proved to be highly efficient for introducing cloned genes into cultured cells and organisms. Recently developed retroviral vector packaging cell lines provide infectious, replication-defective (*rd*) viruses without helper virus contamination, thus avoiding potential problems with viral interference and helper virus-induced disease (22, 24, 41). The most extensively used system, the murine ψ2 cell line, has facilitated the transduction of genes into hemopoetic, neuronal, and embryonal carcinoma cells for studies of hemopoetic reconstitution and for chromosomal marking (7, 11, 14, 29, 32, 37, 40, 42). Such work has enhanced the understanding of differentiation, cell lineages, and the behavior of retroviral vectors in vivo.

Our studies have centred upon the interaction of Rous sarcoma virus (RSV) and the oncogene v-*src*, with both hatched and embryonic chicken tissues. We have shown that RSV-induced tumorigenesis is both inhibited in ovo (9) and dependent upon wounding in hatched birds (10). These unforseen complexities of RSV-induced disease raise a number of questions that could be addressed appropriately by using *rd* v-*src* expression vectors. Although there has been recent interest in its use, a packaging system designed specifically for avian sarcoma and leukosis virus (ASLV) vectors is not presently available. The feasibility of constructing such a system with avian leukosis virus has been in some doubt because of the poorly characterized avian leukosis virus RNA encapsidation region and the possibility that at least part of the region lies 3′ to the splice donor site within *gag* (31). The leader (L) region, however, is believed to contain at least a part of the *cis*-acting RNA encapsidation sequences (ψ), since two naturally occurring RSV packaging mutants, TK15 (27) and Se21Q1b (34), both contain deletions in L (for a review see reference 6). A recent study by Katz et al. also described a specific sequence within the L region necessary for RNA encapsidation in RSV (17). We show here that removal of a related sequence within the L region of a cloned avian leukosis virus helper is sufficient to abolish virus replication in *cis*. Using this crippled helper virus, designated RAV-1ψ⁻, we have established prototype packaging cell lines for ASLV-based vectors.

With sensitive immunocytochemical techniques (36) we show that transfected pRAV-1ψ⁻ directs the expression of viral proteins but does not support replicating virus production from either chicken embryo fibroblasts (CEF) or the quail line QT6 (25). Stable, RAV-1ψ⁻-expressing QT6 lines have remained helper virus free since their development. These same cells were shown to package three different ASLV-based *rd* vectors into infectious virions in transient assays, without significant helper virus titers. Packaging cells stably releasing vector-derived virus provide greater than 10^5 viruses per ml, with very little or no helper virus release. We have thus demonstrated both the feasibility of designing ASLV-based packaging cell lines and the construction of specific vectors for use in these cells.

MATERIALS AND METHODS

Cell culture. QT6 is a chemically derived tumor cell line of the Japanese quail, free of exogenous ASLV (kindly provided by P. Vogt, University of Southern California, School of Medicine, Los Angeles) (25). CEF were prepared from day 10 white leghorn embryos (specific pathogen free) as described previously (3). All cells were routinely grown in medium 199 (GIBCO Laboratories) supplemented with 10% tryptose phosphate broth, 4% newborn calf serum, 1% chicken serum, and 100 μg of gentamicin per ml.

Manipulation of cloned viral DNA. All recombinant DNA manipulations were performed by standard techniques (21). Proviral DNA in plasmid form is referred to with prefix p, whereas the viruses expressed have no prefix.

pRAV-1ψ⁻ (Fig. 1 and 2). The cloned helper virus genome pRAV-LTR was kindly given by B. Vennström (Differentiation Programme, European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany). This plasmid contains a cloned, permuted Rous-associated virus (RAV-1) inserted as a *Sac*I fragment 3′ to an avian erythroblastosis

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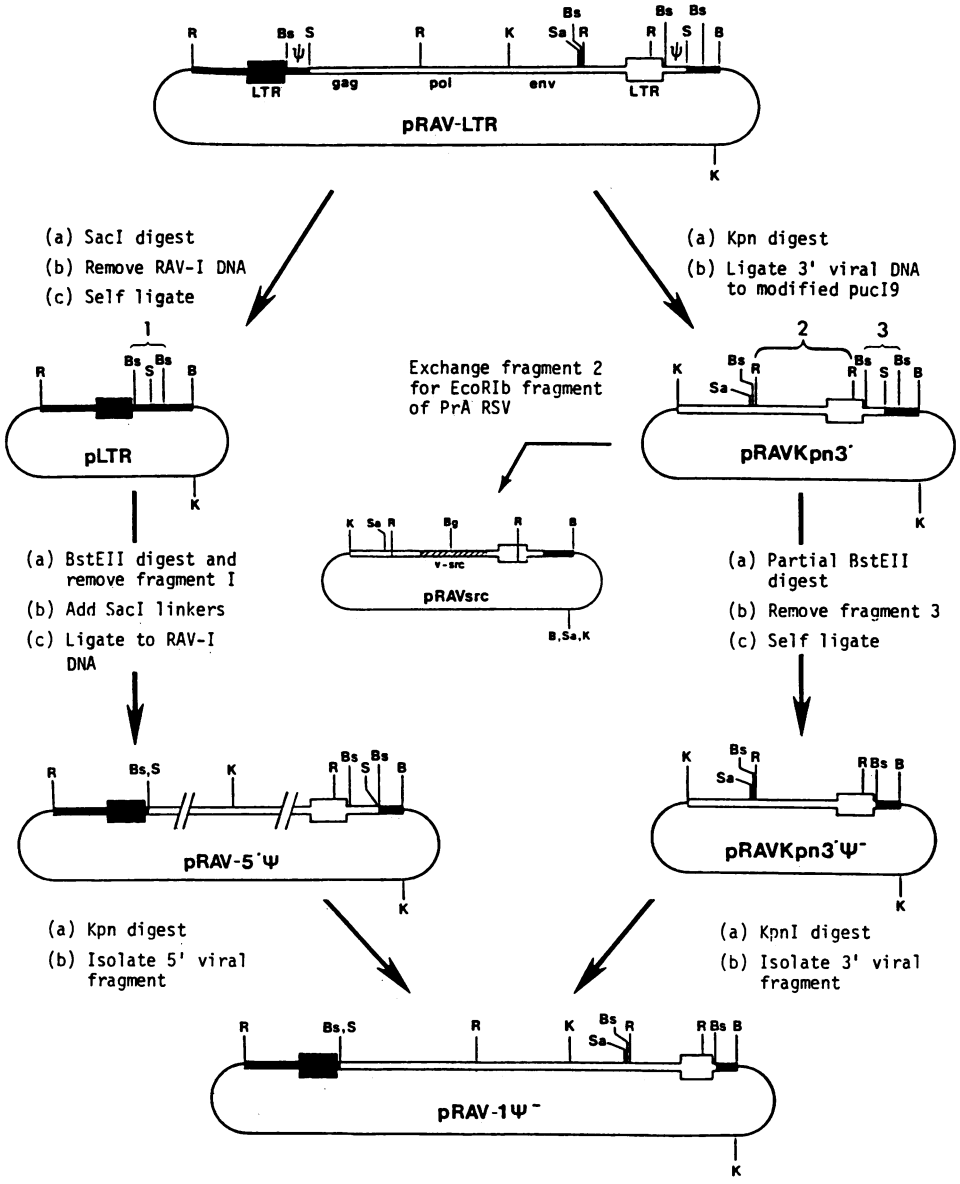

FIG. 1. Schematic representation of pRAV-1ψ⁻ construction. Boxed regions represent proviral DNA, RAV-1 (□) and AEV10 (■). Single lines represent bacterial plasmid DNA. Duplicated RNA encapsidation sequences (ψ) are indicated in pRAV-LTR. Relevant enzyme cleavage sites are indicated: B, *Bam*HI (sites within RAV-1 DNA are not shown); Bg, *Bgl*II; Bs, *Bst*EII; K, *Kpn*I; R, *Eco*RI; S, *Sac*I; Sa, *Sal*I.

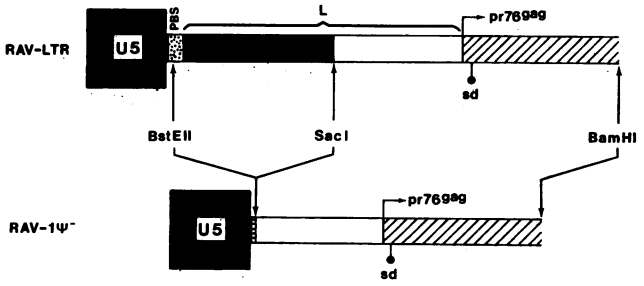

FIG. 2. Diagram representing 5' regions of pRAV-LTR and pRAV-1ψ⁻ proviruses: AEV10 DNA (▬), RAV-1 DNA (▭ and ▨). The U5 region of the AEV10 LTR is shown, with the tRNA PBS, the noncoding leader region (L), and the start of the pr76^{gag} coding region. sd, Splice donor site. The 5' region deleted from pRAV-LTR to form pRAV-1ψ⁻ is indicated.

virus (AEV10) long terminal repeat (LTR) in pSV2gpt (26, 39). Due to the permuted structure of this provirus, two similar deletions were introduced to remove putative ψ sequences. The deletion in the 5' AEV10 L region was made first by cleaving pRAV-LTR with *Sac*I to remove RAV-1 sequences. The resulting plasmid pLTR was digested with *Bst*EII to remove the L-region sequence; *Sac*I linkers were added, and the RAV-1 DNA was ligated back to create pRAV-5'ψ, containing the correct 5' deletion. Deletion of ψ sequence 3' of the RAV-1 LTR was initiated by cleaving pRAV-LTR with *Kpn*I and ligating the 3' viral DNA fragment to a modified pUC19 plasmid (lacking *Eco*RI and *Sac*I restriction sites). This plasmid, pRAVKpn3', was digested partially with *Bst*EII and self-ligated to create pRAVKpn3'ψ⁻, which now contained the 3' deletion (approximately 100 base pairs [bp] larger than the 5' deletion).

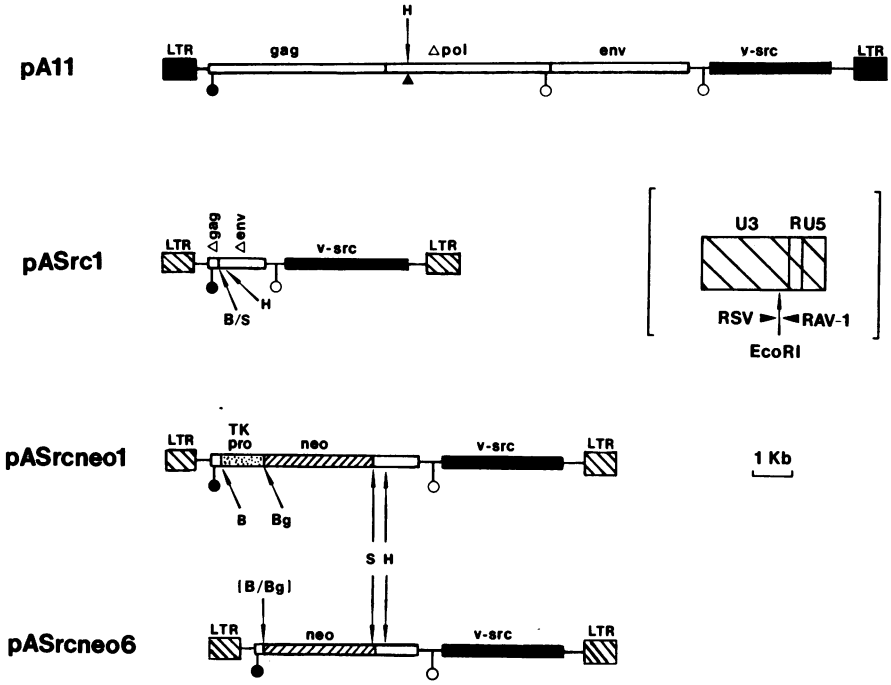

FIG. 3. Replication-defective v-*src* expression vectors. pA11 is a *pol*$^-$ B77 RSV; pASrc1 and pASrcneo1 are derived from RAV-1 and PrA RSV DNA and have chimeric LTRs (inset contains the LTRs of pASrc1 and pASrcneo1, showing boundaries of RSV and RAV-1 sequences). Δ*gag*, Δ*pol*, and Δ*env* are truncated genes. Symbols: position of deleted *Hin*dIII fragment in pA11 (▲) and splice donor (●) and acceptor (○) sites. Unique cloning sites in vectors pASrc1 and pASrcneo1 are indicated. B, *Bam*HI; S, *Sal*I; H, *Hin*dIII; TK pro, thymidine kinase gene promoter; *neo*, neomycin resistance gene.

To construct pRAV-1ψ$^-$, 5′ and 3′ proviral halves were ligated together as *Kpn*I fragments from pRAV-5′ψ and pRAVKpn3′ψ$^-$, respectively.

pA11 (Fig. 3). pA11 proviral DNA was kindly given by D. Gillespie and J. Wyke (The Beatson Institute, Glasgow, United Kingdom). The pA11 provirus is a Bratislava strain (B77) of RSV cloned from an infected rat cell line and reconstructed with a 130-bp deletion between two *Hin*dIII sites within *pol* (19). To establish long-term packaging lines expressing this vector, 10 μg of pA11 was cotransfected with 1 μg of pY3 containing the hygromycin resistance gene (*hph*^{r}) (4) onto 10^6 Q2bn cells. *hph*^{r} populations were isolated and screened for virus release.

pASrc1 (Fig. 3). A cloned *Eco*RI b fragment (containing v-*src*) from wild-type PrA RSV (kindly supplied by V. Fincham and J. Wyke [12]) was exchanged for the *Eco*RI fragment in pRAVKpn3′ containing 3′ *env* to 5′ LTR sequences. The resulting plasmid pRAV*src*1 was used to construct the complete provirus present in pASrc1. Unique, internal *Bam*HI, *Sal*I, and *Hin*dIII cloning sites are present in this vector, as are splice sites for the v-*src* mRNA (RAV-1 donor, PrA acceptor).

pASrcneo1 (Fig. 3). Provirus pASrcneo1 was derived from pASrc1. A *Bam*HI-*Sal*I fragment containing the *neo* gene and TK promoter from pIPB$_1$ (a plasmid containing the neomycin resistance (*neo*) gene of TN5 (16) preceded by the herpes simplex virus thymidine kinase gene promoter (23), kindly given by R. Sweet (College of Physicians and Surgeons, Columbia University, New York) was inserted into the *Bam*HI/*Sal*I cloning site in pASrc1 in the same transcriptional orientation as v-*src*.

pASrcneo6 (Fig. 3). Provirus pASrcneo6 was derived from pASrcneo1. The thymidine kinase promoter was removed as a *Bam*HI-*Bgl*II fragment, and the plasmid was self-ligated. The *neo* gene is expressed directly from the viral LTR.

pASrcneo7. Provirus pASrcneo7 is similar to pASrcneo6, except that the RSV *Eco*RI b fragment is derived from B77 RSV and not PrA RSV.

DNA transfection and drug selection. Calcium phosphate-mediated DNA transfection was performed essentially as described by Graham and Van der Eb (15). Precipitates were left on cells for 4 to 6 h, and the cells were then glycerol shocked (complete medium plus 15% glycerol for 100 s). To establish stable QT6 lines expressing pRAV-1ψ$^-$, the following DNAs were transfected (per 10^6 cells): 1 μg of *Bam*HI cut pIPB$_1$; 10 μg of *Sal*I-cleaved pRAV-1ψ$^-$; 10 μg of calf thymus DNA carrier. These cells were split 1 day after transfection and placed under G418 selection (200 to 400 μg/ml), and resistant colonies were isolated 2 to 3 weeks later. A second series of RAV-1ψ$^-$-expressing QT6 lines were developed in a similar manner with the *hph*^{r} expression plasmid PY3 (4) as the cotransfected marker.

Plasmids containing v-*src* expression vectors were transfected uncut (10 μg) with 10 μg of calf thymus DNA carrier per 10^6 cells.

Virus collection and characterization with expression focus unit (EFU) assay. Viruses were collected from cells in fresh medium for 2.5 h. These supernatants were cleared of cell debris by centrifugation (5 min, 600 × *g* to 1,000 × *g*) and either used directly to infect cells or stored frozen at −70°C before use. After transfection of v-*src* expression vector DNA into packaging cells, virus was collected 18 to 24 h posttransfection as described above.

An immunocytochemical assay has been specifically developed for quantitation of both replicating and *rd* infectious viruses (36). Briefly, CEF were infected with virus and

TABLE 1. pRAV-LTR- and pRAV-1ψ⁻-derived virus titers released from transfected and infected CEF and quail cell lines

Cell type	Virus expressed	Maximal virus (titer (EFU/ml)
CEF	NONE	<1
CEF	RAV-LTR[a]	1.0×10^6
CEF	RAV-1ψ⁻[a]	<1
QT6	RAV-LTR[b]	1.3×10^5
QT6	RAV-1ψ⁻[c] line Q1an	<1
QT6	RAV-1ψ⁻ line Q2an	<1
QT6	RAV-1ψ⁻ line Q2bn	<1

[a] Viral DNA was introduced by transfection, and virus titers were assayed 8 days later after one cell passage.
[b] RAV LTR was introduced by infection, and virus titers were measured after 12 days.
[c] Viral DNA was introduced by transfection, and virus titers were assayed at numerous intervals up to the present.

overlaid with agar 14 to 16 h later. The agar was removed 5 to 10 days later, and the cells were fixed in 2% paraformaldehyde. A three-layer immunodetection system was employed: layer one was an antiviral serum (either anti-p19^{gag}, two different monoclonal antibodies kindly provided by T. Pawson [Mount Sinai Hospital Research Institute, Toronto, Ontario, Canada] and D. Boettiger [University of Pennsylvania, School of Medicine, Philadelphia], or anti-pp60$^{v\text{-}src}$ [JB327] kindly supplied by J. Brugge [State University of New York at Stony Brook, School of Medicine, Stony Brook]); layer two was a biotinylated anti-mouse F(ab')$_2$ (Amersham Corp.); layer three was a streptavidin-conjugated alkaline phosphatase complex (Enzo Biochem or Zymed). After sequential binding of these three reagents, the color reaction was developed to reveal foci of antigen-expressing cells (virus titers are expressed in EFU). For characterization of nonreplicating virus, the agar overlay step was omitted, and cells were stained 3 to 5 days postinfection. This assay is highly sensitive, detecting as few as one infection event per test plate. In addition, ASrcneo1- and ASrcneo6-derived viruses were quantitated by counting the number of QT6 neomycin-resistant (*neo*^{r}) colonies remaining after virus infection and G418 selection (200 to 400 μg/ml).

RESULTS

Introduction of L-region deletion in cloned helper virus genome. Analysis of naturally occurring packaging-deficient RSV mutants suggested that critical RNA packaging sequences lie in the viral L region. We used suitable restriction sites located in a cloned helper virus genome pRAV-LTR (containing combined sequences from RAV-1 and AEV10), to remove part of L; a deletion of approximately 150 bp was introduced between the *Bst*EII site within the primer-binding site (PBS) abutting the 5′ LTR and the downstream *Sac*I site in L (Fig. 1 and 2) (see Materials and Methods). In addition to deleting a part of L, this removed 10 of the original 18 nucleotides from the PBS. Because the L region is duplicated in the downstream permuted RAV-1 sequences, this region and approximately 100 bp of 3′ adjoining sequence were also removed to avoid recombination. The final proviral construct was named pRAV-1ψ⁻.

RAV-1ψ⁻ is replication defective. To test whether the deletion made in pRAV-1ψ⁻ had removed sequences critical for virus replication, we transfected circular pRAV-1ψ⁻ and pRAV-LTR DNAs into CEF and monitored viral protein expression by immunocytochemistry and virus release. Within 8 days of transfection with pRAV-LTR, virus spread had occurred to most cells as judged by expression of p19^{gag} (data not shown). High titers of infectious virus were released from these cultured cells (Table 1). CEF transfected with pRAV-1ψ⁻, however, did not show this pattern. Rather, a minor population of CEF (<0.01%) showed p19^{gag} expression, demonstrating stable transfection of RAV-1ψ⁻ in a small number of cells without any indication of virus spread or infectious virus release (Table 1; see Fig. 5f).

To further characterize the behavior of pRAV-1ψ⁻, we transfected the cloned provirus into QT6 cells. As with quail cells in general, this tumor cell line has no endogenous viruses closely related to ASLV (13), although distantly related sequences have recently been described (5). The absence of endogenous viruses in QT6 is advantageous if recombination of introduced viral sequences (here pRAV-1ψ⁻) with cellular ψ sequences is to be avoided. The pRAV-1ψ⁻ DNA was cotransfected into QT6 cells with a selectable *neo*^{r} plasmid, and *neo*^{r} colonies were isolated, amplified, and immunocytochemically stained for p19^{gag} expression. Three positive lines, Q1an, Q2an, and Q2bn, were chosen for detailed study.

Using the EFU virus assay (see Materials and Methods), we tested whether these lines released replicating helper virus and found none (Table 1). Moreover the lines have been nonproducers for more than 9 months and are thus stable. QT6 cells infected with the pRAV-LTR-derived helper virus produced high titers of replicating virus (Table 1).

Intact proviral DNA has been detected in each line by

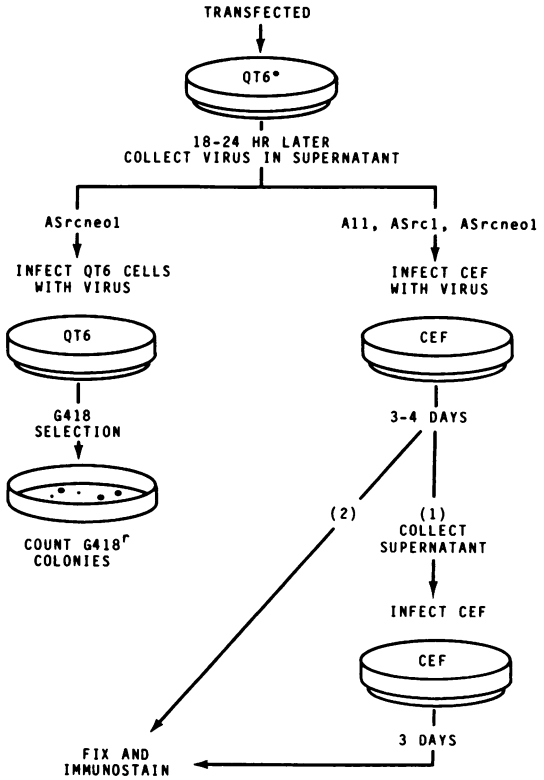

FIG. 4. Regimen for testing *rd* virus packaging in RAV-1ψ⁻-expressing QT6 cell lines. QT6* indicates QT6-derived packaging cells. Supernatants were collected from CEF (step 1) before fixation of the same cells (step 2). Methods for transfection, virus collection, and immunostaining are described in Materials and Methods.

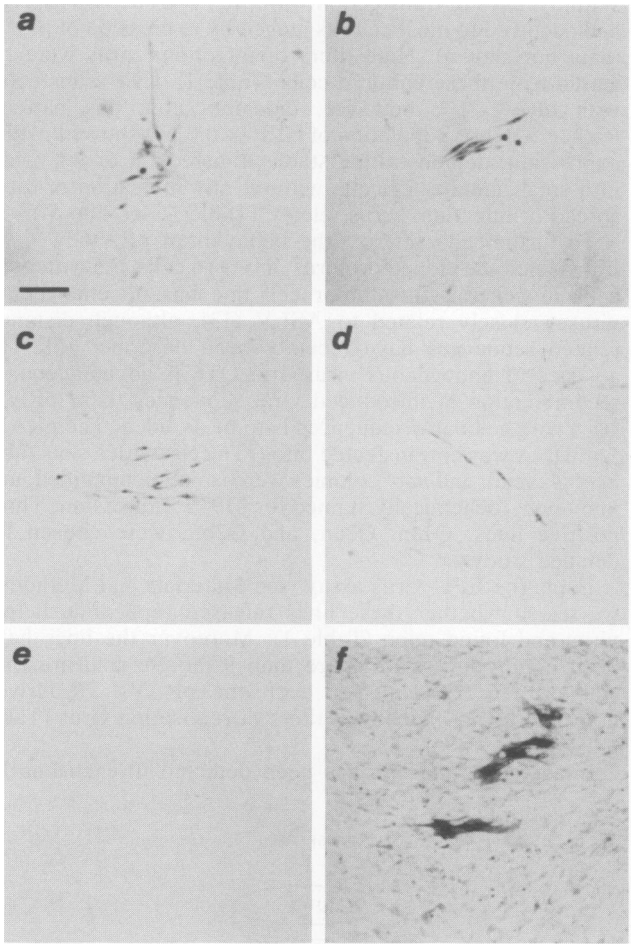

FIG. 5. Alkaline phosphatase-immunostained foci of virus-infected CEF expressing pp60$^{v\text{-}src}$ (dark cells) in an uninfected CEF background. Panels: representative foci of cells 3 to 4 days after infection with virus derived from pA11 (a and b), pASrc1 (c), and pASrcneo1 (d), uninfected CEF 4 days after inoculation with supernatant from pA11-transfected QT6 cells, stained with anti-pp60$^{v\text{-}src}$ (negative control, e); CEF expressing pRAV-1ψ^- 8 days after transfection and a single passage, stained with anti-p19^{gag} (f). Bar, 0.1 mm.

Southern analysis of restriction endonuclease-digested cellular genomic DNA (data not shown). This rules out gross proviral rearrangements as the reason for the absence of virus production by these lines. The lack of RAV-1ψ^- replication in QT6 cells corroborates the CEF transfection data discussed above, confirming that the deletion in RAV-1ψ^- eliminated its ability to release replicating virus, whereas viral p19^{gag} expression was maintained.

RAV-1ψ^--expressing cells can package replication-defective vectors. (i) Packaging of *pol*$^-$ *rd* RSV. To test whether the mutation in RAV-1ψ^- was *cis* acting, we tested whether the expressing QT6 cell lines could package *rd* viral RNA into infectious viruses without accompanying helper virus. The first *rd* vector we tested was pA11, a *pol*$^-$ Bratislava strain (B77) RSV provirus (19). Uncut pA11 DNA (10 μg) was transfected into the lines Q1an, Q2an, and Q2bn, and transient virus release into culture supernatants was tested 18 to 24 h later (Fig. 4; Fig. 5a and b). Traditional focus assays were not performed because we have shown that our immunocytochemical assay is more accurate (36; A. Stoker, unpublished observations). pA11-derived virus was released from lines Q1an and Q2bn, but none was detected from line Q2an or QT6 cells (Table 2). The fact that Q2an cells did not release virus suggested that the provirus(es) in these cells were debilitated through mutations or rearrangements not detectable by Southern analysis, and this line was therefore not studied further.

To examine whether replicating virus was concomitantly released with *rd* virus, supernatants from the infected CEF were collected before cell fixation and used to infect fresh CEF (Fig. 4). These cells were later fixed and analyzed for p19^{gag} EFU. Using RAV-LTR helper virus we confirmed the sensitivity of this procedure: single plates of CEF infected with 1 to 2 EFU of replicating helper virus released detectable progeny virus (up to 10 EFU/ml) into the supernatants 3 days after infection. In testing the pA11-derived virus, two of nine independent experiments revealed recombinant infectious virus in supernatants from infected CEF. However, virus titers were very low (12 and 1 EFU/ml, respectively), and the virus was transforming as judged by positive pp60$^{v\text{-}src}$ staining and the transformed morphology of infected CEF. These recombinant viruses appear to have arisen predominantly by recovery of the *pol* sequence by A11.

(ii) Packaging of vectors with no viral structural genes. From the above data we could conclude that the cell lines provided *pol* functions and expressed *gag* proteins. We wished to ascertain whether pRAV-1ψ^--expressing cells would provide all viral structural proteins in *trans*, and also whether by incorporating larger deletions into vectors the recombination with RAV-1ψ^- would be reduced. Two smaller v-*src* vectors were constructed which lacked all viral structural genes.

pASrc1 retains complete 5′ ψ sequences in L, the 3′ polypurine tract (35), viral splice signals for v-*src* mRNA, and the v-*src* gene of PrA RSV. Only truncated *gag* and *env* sequences remain of the viral structural genes. Transient virus release assays were performed as described above for pA11. pASrc1-derived virus was released from both cell lines tested, at titers comparable to those found with pA11 (Table 2).

pASrcneo1 expresses both v-*src* and the dominant selectable *neo* gene (under the internal control of the HSV TK gene promoter) (Fig. 3). The titer of pASrcneo1-derived virus can be estimated from the number of *neo*^{r} colonies induced in infected QT6 cells; transient expression of pASrcneo1 in Q2bn cells thus gave maximal virus titers of 5.1×10^3 EFU/ml. ASrcneo1 virus was also packaged by line Q1an, but at lower efficiency than Q2bn (Table 2). CEF infected with this *rd* virus were immunostained for pp60$^{v\text{-}src}$, and foci with similar characteristics to those induced by ASrc1 were observed (Fig. 5c and d). The virus titers estimated from *neo*^{r} colony formation were broadly comparable to the pp60$^{v\text{-}src}$ EFU, indicating that both quantitation procedures have similar accuracy.

Replicating helper virus was not detected when pASrc1 was tested in transient assays. Similarly, no replicating helper was found when pASrcneo1 was tested, with one exception: helper virus was found after one transfection of Q2bn cells, but only at the threshold of our detection technique (1 to 2 EFU/ml). Helper release was not stable, however, since a later virus collection failed to detect its presence.

Long-term virus-producing lines. The virus analyses using the transient assay demonstrated the packaging behavior of RAV-1ψ^--expressing cell lines, but the titers obtained were

TABLE 2. Transient vector-derived virus release from quail cell lines

Cell type transfected	Titer (EFU/ml) with the following *rd* vector introduced[a]:			*neo*^{r} colonies/ml[b]
	pA11	pASrc1	pASrcneo1	
QT6	<1 (<1)	<1 (<1)	<1 (<1)	<1
	<1 (<1)	<1 (<1)	<1 (<1)	<1
Q1an	3.0×10^2 (<1)	5.5×10^2 (<1)	1.8×10^2 (<1)	1.1×10^2
	4.0×10^2 (<1)	6.6×10^2 (<1)	1.4×10^2 (<1)	1.4×10^2
	7.4×10^2 (<1)	2.0×10^2 (<1)	1.2×10^2 (<1)	3.1×10^2
	1.5×10^3 (<1)			
Q2bn	9.2×10^2 (<1)	9.6×10^2 (<1)	3.8×10^2 (<1)	3.5×10^2
	1.0×10^3 (12)	1.0×10^3 (<1)	3.4×10^3 (1–2)	5.0×10^3
	1.5×10^3 (<1)	2.8×10^3 (<1)	6.0×10^2 (<1)	5.1×10^3
	1.8×10^3 (<1)			
	3.5×10^3 (1)			
Q2an	<1 (NT)			
	<1 (NT)			

[a] Titer of virus released measured with pp60v-*src* immunostaining in CEF. Figures within parentheses represent titers of replicating virus released from infected CEF, measured in p19*gag* EFU per milliliter. NT, Not tested.
[b] pASrcneo1-derived virus was analyzed.

not satisfactory for use in in vivo studies. In an attempt to obtain higher titers, stable virus-releasing clones of cells were isolated. Two approaches were used. In the first approach we cotransfected pA11 and an *hph* selectable plasmid into line Q2bn and isolated stable *hph*^{r} cells. These cells released pA11-derived virus at titers up to 30-fold greater than the comparable transient titers (Table 3). Replicating viruses (including nontransforming helper virus) were also detected at low levels with this large vector. Although certain populations released as few as 0.01% replicating viruses, we have not been able to subclone any which were completely helper free, indicating that a relatively high recombination rate was occurring between this vector and RAV-1ψ^- (either before or after virus release).

Our second approach was to establish packaging populations by direct selection for transfected *neo* vectors. For this, we first established a second series of packaging lines, using *hph* cotransfection with pRAV-1ψ^-, which behaved essentially like Q1an and Q2bn described above in transient virus release assays (data not shown). One such line, Q4dh, was transfected with pASrcneo6 and selected for *neo*^{r} (*neo* and *hph* are independently selectable [4]). pASrcneo6 expresses *neo* directly from the viral LTR (Fig. 3), since we wished to select for LTR activity and not an independent internal promoter (pASrcneo6 gave titers comparable to those of pASrcneo1 in transient assays in line Q2bn).

neo^r populations cloned in this way stably released ASrcneo6 virus at titers greater than 10^5 EFU/ml (Table 3). Virus stocks were screened for replicating viruses as described for Asrneo1 in Fig. 3. Virus screens up to 7 weeks after clone isolation did not not reveal any helper virus. Tests after 9 weeks did detect helper virus, only at the lowest threshold of our detection (Table 3). Other stable lines have been isolated with vector pASrcneo7, which is structurally similar to pASrcneo6; again, maximal *rd* virus titers were greater than 10^5 EFU/ml. The majority of these lines have remained helper free since isolation (>10 weeks) (Table 3).

DISCUSSION

Our goals in this work were twofold: (i) to design and express, in appropriate cells, a crippled ASLV-based helper virus which would complement *rd* vectors in *trans* and yet withhold its own ability to replicate, and (ii) to design specific *rd* vectors for use in these packaging cells. In accomplishing these goals, we have used novel immunocytochemical techniques to screen for replicating virus and infectious *rd* viruses.

The defective helper virus pRAV-1ψ^- was successfully expressed after transfection into QT6 cells, and these lines did not release replicating helper virus. The helper-free stability of the cells, an important consideration in their long-term use, may arise from the absence of endogenous viruses available for recombination. We have also shown that stable expression of pRAV-1ψ^- occurs in CEF after transfection, without detectable virus replication (Fig. 5f; Table 1). These latter data are at odds with those presented by Cooper and Okenquist, who suggested that stable integration of transfected *rd* viral DNA in CEF requires first virus replication and then reinfection (8). Our data with pRAV-1ψ^- argue against this interpretation as the exclusive

TABLE 3. Virus titers from stable virus-releasing packaging cells

Cell line	Vector expressed	Virus-releasing cell clones[a]	Virus titer (EFU/ml)	Replicating virus/ml
Q2bn	pA11	1	2×10^4[b]	1–5
		2	3×10^4	1–5
Q4dh	pASrcneo6	1	2×10^5[c]	1–2
		2	2×10^5	1–3
		3	2×10^5	1–3
		4	3×10^4	NT[d]
		5	3×10^4	NT
		6	6×10^3	NT
Q4dh	pASrcneo7	1	2×10^5	<1
		2	3×10^5	1–4
		3	2×10^4	<1

[a] Populations isolated from colonies after transfection of packaging line and drug selection.
[b] Virus titer measured by p19^{gag} EFU.
[c] Virus titer measured by *neo*^{r} colony formation.
[d] NT, Not tested.

route of stable expression; by immunocytochemistry we detect stable pRAV-1ψ⁻ expression in CEF populations 8 days posttransfection. This form of expression after transfection into CEF is clearly very low, however, and differential sensitivity of assay techniques may explain the differences between our data and those of Cooper and Okenquist.

Until recently the characterization of the 5′ RNA packaging signals in ASLV genomes had not been extensive. The construction of pRAV-1ψ⁻ was therefore founded upon data from two naturally occurring RSV packaging mutants (27, 34). We have shown that the deletion in pRAV-1ψ⁻, extending from within the PBS region to a *Sac*I site in L, is sufficient to prevent the release of replicating virus from avian cells and that this defect is *cis* acting. While pRAV-1ψ⁻ was being tested in our laboratory, Katz et al. presented a detailed analysis of the ψ region in RSV, concluding that a critical 30-bp packaging sequence lies upstream of the single *Sac*I site in L (17). This 30-bp sequence is absent in pRAV-1ψ⁻, and our data thus corroborate those of Katz and co-workers. When compared with TK15, however, the removal of L sequences does not satisfactorily explain the total lack of pRAV-1ψ⁻ replication. The mutant TK15 contains a deletion extending from the 3′ junction of the PBS to near the *gag* AUG start codon (28). Although this region is about 100 bp larger than that deleted in pRAV-1ψ⁻, low-titer infectious virus is released from TK15-expressing cells (18). A critical difference between TK15 and RAV-1ψ⁻ is likely to be the removal of PBS sequences in the latter. A study by Pugasch and Stacey (30) showed that removal of the entire PBS from avian leukosis virus eliminated virus replication, presumably through blocking minus-strand DNA priming (for a review, see reference 38). The pRAV-1ψ⁻ genome retains only 8 of the original 18 bases of the PBS (additional base matches may occur with the newly juxtaposed sequence), and this may also be insufficient for specific tRNA binding. Although we have no biochemical data, we believe primarily that the deletion in L severely reduces or abolishes the specific packagability of RAV-1ψ⁻ RNA, and that the PBS deletion prevents reverse transcription of any nonspecifically packaged viral RNA.

We have shown that pRAV-1ψ⁻ lines can package a *pol*⁻ B77 RSV and two specifically designed *rd* v-*src* vectors in transient assays. Significant recombination between vector pA11 and RAV-1ψ⁻ was shown to occur in these assays, and the evolution of replicating viruses in stable A11-releasing populations confirmed this finding. With vectors pASrc1 and pASrcneo1, replicating viruses were not detected in transient assays except for the single case discussed above. The stable lines releasing packaged vectors ASrcneo6 and ASrcneo7 also demonstrated that high titers of *rd* virus could be isolated from pRAV-1ψ⁻-expressing cells, with very low or undetectable release of replicating virus. Thus removal of unnecessary coding sequences in the pAS series of vectors significantly reduces recombination frequencies in comparison to pA11.

Although the removal of internal sequences also generates space for nonviral inserts, maximizing the size of the deletion must be balanced against the retention of necessary *cis*-acting sequences. Recent studies of both avian and murine retroviruses indicate that sequences in the 5′ *gag* region may augment virus expression and/or genomic RNA encapsidation and also influence the viral disease spectrum (1, 2, 33). RSV has been shown to contain a sequence in *gag* with transcriptional enhancing activity and also a sequence flanking the *Bam*HI site at position 532 (PrC RSV base equivalent) which may enhance encapsidation (32). Our pAS vectors, however, do not contain these regions. Removal of the *gag* enhancer segment appears not to hinder vector expression as judged by the ability of the vector-derived viruses to morphologically transform CEF (A. Stoker, unpublished observations). Nevertheless, we are presently examining this question in more detail. Loss of the putative encapsidation region does not significantly affect the virus titers derived from our vectors in comparison with either pA11 (in both transient and long-term virus release; Table 3), or the titer of RAV-LTR released from QT6 (Table 1). Therefore RNA packaging remains efficient after loss of this region, a finding also noted by others (20, 28).

We have shown with immunocytochemistry that accurate quantitation of vector-derived viruses is possible without using internal selectable markers. Quail cells are also resistant to infection by a number of common ASLV subgroups (25), and therefore the use of the *neo*ʳ colony-forming assay for such viruses would not be applicable. If immunocytochemical detection is a feasible alternative, selectable vector genes may be either removed or replaced by sequences of biological interest in size-constrained vectors.

In conclusion, we have constructed an ASLV-based helper virus derivative with a *cis*-acting mutation preventing the release of replicating virus. Stable quail cell lines expressing pRAV-1ψ⁻ are helper virus nonproducers but are able to package *rd* ASLV-based vectors into infectious virus. Using cell clones selected to stably express *rd* vectors, we have obtained high-titer *rd* virus with very low or undetectable helper contamination, suitable for use in vivo. The availability of this cell system will further our examination of the complex oncogene-host interactions in hatched and embryonic avian tissues. Moreover, such a system will facilitate experiments in vivo addressing broader questions of viral expression, target specificity, and cell lineage in the developing avian embryo.

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LITERATURE CITED

1. **Arrigo, S., M. Yun, and K. Beemon.** 1987. Cis-acting regulatory elements within *gag* genes of avian retroviruses. Mol. Cell. Biol. **7**:388–397.
2. **Bender, M. A., T. D. Palmer, R. E. Gelinas, and A. D. Miller.** 1987. Evidence that the packaging signal of molony murine leukemia virus extends into the *gag* region. J. Virol. **61**:1639–1646.
3. **Bissell, M. J., D. Farson, and A. S. C. Tung.** 1977. Cell shape and hexose transport in normal and virus-transformed cells in culture. J. Supramol. Struct. **6**:1–12.
4. **Blochlinger, K., and H. Diggelmann.** 1984. Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotic cells. Mol. Cell. Biol. **4**:2929–2931.
5. **Chambers, J. A., A. Cywinski, P.-J. Chen, and J. M. Taylor.** 1986. Characterization of Rous sarcoma virus-related sequences in the Japanese quail. J. Virol. **59**:354–362.
6. **Coffin, J.** 1982. Structure of the retroviral genome, p. 261–368. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

7. **Cone, R. D., and R. C. Mulligan.** 1984. High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. Proc. Natl. Acad. Sci. USA **81**:6349–6353.
8. **Cooper, G. M., and S. Okenquist.** 1978. Mechanism of transfection of chicken embryo fibroblasts by Rous sarcoma virus. J. Virol. **28**:45–52.
9. **Dolberg, D. S., and M. J. Bissell.** 1984. Inability of Rous sarcoma virus to cause sarcomas in the avian embryo. Nature (London) **309**:552–556.
10. **Dolberg, D. S., R. Hollingsworth, M. Hertle, and M. J. Bissell.** 1985. Wounding and its role in RSV-mediated tumor formation. Science **230**:676–678.
11. **Eglitis, M. A., P. Kantoff, E. Gilboa, and W. F. Anderson.** 1985. Gene expression in mice after high efficiency retroviral-mediated gene transfer. Science **230**:1395–1398.
12. **Fincham, V. J., and J. A. Wyke.** 1986. Localization of temperature-sensitive transformation mutations and back mutations in the Rous sarcoma virus *src* gene. J. Virol. **58**:694–699.
13. **Frisby, D. P., R. A. Weiss, M. Roussel, and D. Stehelin.** 1979. The distribution of endogenous retrovirus sequences in the DNA of galliform birds does not coincide with avian phylogenetic relationships. Cell **17**:623–634.
14. **Gilboa, E., M. A. Eglitis, P. W. Kantoff, and W. F. Anderson.** 1986. Transfer and expression of cloned genes using retroviral vectors. Biotechniques **4**:504–512.
15. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
16. **Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff.** 1979. Restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. Mol. Gen. Genet. **177**:65–72.
17. **Katz, R. A., R. W. Terry, and A. M. Skalka.** 1986. A conserved cis-acting sequence in the 5′ leader of avian sarcoma virus is required for packaging. J. Virol. **59**:163–167.
18. **Kawai, S., and T. Koyama.** 1984. Characterization of a Rous sarcoma virus mutant defective in packaging its own genomic RNA: biological properties of mutant TK15 and mutant-induced transformants. J. Virol. **51**:147–153.
19. **Levantis, P., D. A. F. Gillespie, K. Hart, M. J. Bissell, and J. A. Wyke.** 1986. Control of expression of an integrated Rous sarcoma provirus in rat cells: role of 5′ genomic duplications reveals unexpected patterns of gene transcription and its regulation. J. Virol. **57**:907–916.
20. **Lipsick, J. S., C. E. Ibanez, and M. A. Baluda.** 1986. Expression of molecular clones of v-*myb* in avian and mammalian cells independently of transformation. J. Virol. **59**:267–275.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1981. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **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.
23. **McNight, S., E. R. Gavis, R. Kingsbury, and R. Axel.** 1981. Analysis of transcriptional regulatory signals of the HSV thymidine kinase gene: identification of an upstream control region. Cell **25**:385–398.
24. **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.
25. **Moscovici, C., M. G. Moscovici, H. Jiminez, M. M. C. Lai, M. J. Hayman, and P. K. Vogt.** 1977. Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. Cell **11**:95–103.
26. **Mulligan, R. C., and P. Berg.** 1980. Expression of a bacterial gene in mammalian cells. Science **209**:1422–1427.
27. **Nishizawa, M., T. Koyama, and S. Kawai.** 1985. Unusual features of the leader sequence of Rous sarcoma packaging mutant TK15. J. Virol. **55**:881–885.
28. **Norton, P. A., and J. Coffin.** 1985. Bacterial β-galactosidase as a marker of Rous sarcoma virus gene expression and replication. Mol. Cell. Biol. **5**:281–290.
29. **Price, J., D. Turner, and C. Cepko.** 1987. Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. Proc. Natl. Acad. Sci. USA **84**:156–160.
30. **Pugatsch, T., and D. W. Stacey.** 1982. Analysis by microinjection of the biological effects of site-directed mutagenesis in cloned avian leukosis viral DNAs. J. Virol. **43**:503–510.
31. **Pugasch, T., and D. W. Stacey.** 1983. Identification of a sequence likely to be required for avian retroviral packaging. Virology **128**:505–511.
32. **Robertson, E., A. Bradley, M. Kuehn, and M. Evans.** 1986. Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral infection. Nature (London) **323**:445–448.
33. **Robinson, H. L., S. S. Reinsch, and P. R. Shank.** 1986. Sequences near the 5′ long terminal repeat of avian leukosis viruses determine the ability to induce osteopetrosis. J. Virol. **59**:45–49.
34. **Shank, P. R., and M. Linial.** 1980. Avian oncovirus mutant (SE21Q1b) deficient in genomic RNA: characterization of a deletion in the provirus. J. Virol. **36**:450–456.
35. **Sorge, J., and S. H. Hughes.** 1982. Polypurine tract adjacent to the U3 region of the Rous sarcoma virus genome provides a *cis*-acting function. J. Virol. **43**:482–488.
36. **Stoker, A. W., and M. J. Bissell.** 1987. Quantitative immunocytochemical assay for infectious avian retroviruses. J. Gen. Virol. **68**:2481–2485.
37. **Turner, D. L., and C. L. Cepko.** 1987. A common progenitor for neurons and glia persists in rat retina late in development. Nature (London) **328**:131–136.
38. **Varmus, H., and R. Swanstrom.** 1982. Replication of retroviruses, p. 369–512. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Vennström, B., L. Fanshier, C. Moscovici, and J. M. Bishop.** 1980. Molecular cloning of the avian erythroblastosis virus genome and recovery of oncogenic virus by transfection of chicken cells. J. Virol. **36**:575–585.
40. **Wagner, E. F., M. Vanek, and B. Vennström.** 1985. Transfer of genes into embryonal carcinoma cells by retrovirus infection: efficient expression from an internal promoter. EMBO J. **4**:663–666.
41. **Watanabe, S., and H. M. Temin.** 1983. Construction of a helper cell line for avian reticuloendotheliosis virus cloning vectors. Mol. Cell. Biol. **3**:2241–2249.
42. **Williams, D. A., S. H. Orkin, and R. C. Mulligan.** 1986. Retrovirus-mediated transfer of human adenosine deaminase gene sequences into cells in culture and into hematopoietic cells in vivo. Proc. Natl. Acad. Sci. USA **83**:2566–2570.