

Ectopic Expression of the Erythrocyte Band 3 Anion Exchange Protein, Using a New Avian Retrovirus Vector

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A retrovirus vector was constructed from the genome of avian erythroblastosis virus ES4. The *v-erba* sequences of avian erythroblastosis virus were replaced by those coding for neomycin phosphotransferase, creating a *gag-neo* fusion protein which provides G418 resistance as a selectable marker. The *v-erbB* sequences following the splice acceptor were replaced by a cloning linker allowing insertion of foreign genes. The vector has been tested in conjunction with several helper viruses for the transmission of G418 resistance, titer, stability, transcription, and the transduction and expression of foreign genes in both chicken embryo fibroblasts and the QT6 quail cell line. The results show that the vector is capable of producing high titers of Neo^r virus from stably integrated proviruses. These proviruses express a balanced ratio of genome length to spliced transcripts which are efficiently translated into protein. Using the *Escherichia coli* β -galactosidase gene cloned into the vector as a test construct, expression of enzyme activity could be detected in 90 to 95% of transfected target cells and in 80 to 85% of subsequently infected cells. In addition, a cDNA encoding the avian erythrocyte band 3 anion exchange protein has been expressed from the vector in both chicken embryo fibroblasts and QT6 cells and appears to function as an active, plasma membrane-based anion transporter. The ectopic expression of band 3 protein provides a visual marker for vector function in these cells.

Recombinant retroviruses have proven to be a highly efficient means of delivering foreign genes into cultured cells (12, 41, 42) or organisms (31, 52). The most widely used systems employ replication defective murine leukemia virus-based vectors and complementing packaging cell lines which allow transcapsidation of vector RNAs into viral particles for infecting most types of mammalian cells (9, 17, 63). In the avian system, replication-defective retrovirus vectors have mainly been constructed for specific experimental purposes (18, 43, 53), though some have been adapted for general use (6, 16). Replication-competent vectors based on Rous sarcoma virus (RSV; 29, 55) have been constructed with the viral *src* sequence replaced by a cloning site for cDNAs. These vectors, however, have a limited cloning capacity, making them unsuitable for the insertion of a selectable marker plus a gene of interest. Finally, three packaging cell lines that are based on avian leukosis virus and that provide the functions required in *trans* for the encapsidation of defective recombinant vector-derived RNAs have been reported (13, 56, 60). All harbor Rous-associated virus type 1 (RAV-1) proviruses, with partial deletions in the packaging (ψ) sequences. Though other modifications to the RAV-1 genome were made, in two of these lines (56, 60) clones which produced high titers of virus also released replication-competent helper virus as a result of recombination. The other packaging line (13) was created by separating helper functions on two independent plasmids, reducing the risk of recombinant helper virus production.

We report here the development of a flexible avian erythroblastosis virus ES4 (AEV-ES4)-based vector which contains all of the sequences known to be required for packaging of viral RNAs (11), a selectable marker (*neo*), and a large cloning capacity. The vector is capable of sustained production of high titers of recombinant virus and stable, efficient expression of cloned sequences when transfected into chicken embryo fibroblast (CEF) or QT6 cells. It can be used in conjunction with several of the avian leukosis virus helper viruses or packaging cell lines.

We have used the *Escherichia coli lacZ* (β -Gal) gene cloned into the vector pSFCV to test parameters such as transduction and expression of cloned sequences. We have shown that the recombinant β -Gal gene-carrying proviruses efficiently express β -Gal activity in up to 95% of transfected target cells and in up to 85% of infected target cells.

In addition, a eucaryotic cDNA has been inserted to test the vector. This cDNA encodes the chicken band 3 anion transport protein, which catalyzes the exchange of HCO_3^- and Cl^- at the erythrocyte plasma membrane and also functions as an anchor for the erythroid cytoskeleton. In the chicken, band 3 protein is encoded by two distinct polypeptides (30) generated by alternative initiation and splicing (34, 35). The band 3 protein transduced by our vector produces only one of these polypeptides yet appears to function as an active ion transporter correctly situated in the plasma membrane of these cells.

MATERIALS AND METHODS

Cells and culture. CEF were prepared from 11-day-old SPAFAS embryos (Lohmann GmbH, Cuxhaven, Federal

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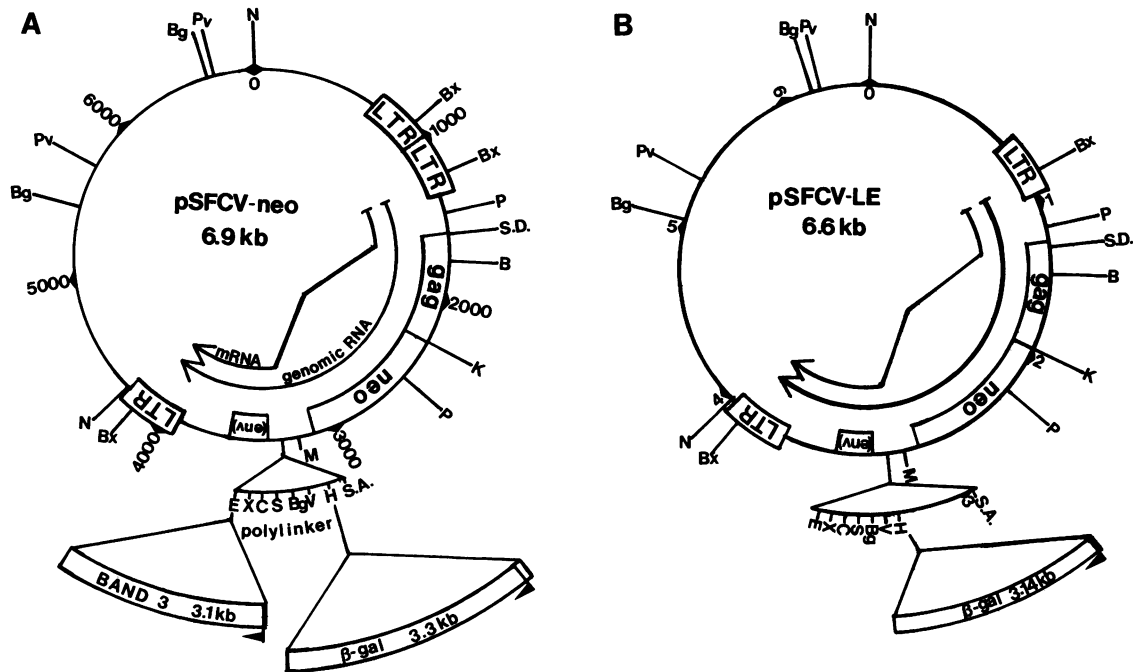


FIG. 1. Schematic representation of pSFCV-neo and pSFCV-LE vectors, showing the location of rare or unique restriction sites, nucleotide numbers (marked every 1,000 bp), locations of predicted genomic and subgenomic RNAs, location of retroviral sequences, the *gag-neo* fusion protein coding sequence (G418 resistance), splice donor and acceptor sites, and cloning polylinker. (A) pSFCV-neo, a 6,883-nt vector into which the band 3 protein coding sequence has been introduced. pSFCV-BIIIIR is a 3.1-kb band 3 protein cDNA inserted into the *EcoRI* site of pSFCV-neo. pSFCV-BIIIW is the same sequence cloned in the opposite orientation. The 3.3-kb β -Gal gene was cloned into pSFCV-neo at the *HindIII* site in the construct pSFCV- β . (B) Modified vector pSFCV-LE with a single 5' LTR and an 88-bp oligonucleotide inserted between the splice acceptor and polylinker. The vector pSFCV-LE containing a 3.14-kb β -Gal insert at the *HindIII* site is the plasmid pSFCV-Blue. S.A., splice acceptor; S.D., splice donor; B, *Bam*HI; Bg, *Bgl*I; Bx, *Bst*XI; C, *Cla*I; H, *Hind*III; K, *Kpn*I; M, *Bsm*I; N, *Not*I; P, *Pst*I; Pv, *Pvu*I; S, *Sfi*I; V, *Eco*RV; X, *Xba*I. The arrows on the inserts show the location of AUG and the direction of transcription.

Republic of Germany) as previously described (23). Both CEF and quail QT6 (44) cells were grown in Dulbecco modified Eagle medium supplemented with 4% fetal calf serum, 2% chicken serum, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.3). Selection for *Neo*^r cells was done in medium containing 0.8 mg of G418 (Geneticin; Sigma Chemical Co.) per ml (wt/vol) for CEF and 1.0 mg/ml for QT6 cells. The Q2 and Q4 packaging cell lines were maintained as described elsewhere (60) and otherwise treated as described for QT6 cells. The Isolde packaging line was maintained as recommended elsewhere (13) and otherwise treated as described for QT6 cells.

Vector construction. pSFCV-neo (Fig. 1A) was constructed from AEV clones 10 and 11 (14, 21, 64, 66), representing unintegrated circular viral DNA, and inserted into plasmid pUC19 by standard methods (40). The pUC19 polylinker was removed and replaced with *Not*I linkers. AEV-derived fragments were then cloned into the plasmid *Not*I site, and the vector was constructed with the sequences outlined in Table 1. pSFCV-LE was constructed from pSFCV-neo by a partial *Bst*XI digestion and religation to create a single, fused, upstream long terminal repeat (LTR). In addition, an 88-bp spacer sequence derived from the human epidermal growth factor (EGF) receptor (nucleotides [nt] 105 to 186) was inserted into the *Hind*III site of the polylinker, destroying the upstream site but retaining the downstream site (Fig. 1B).

DNA transfection and virus production. Transfections were performed by the method of Graham and Van der Eb as modified by Chen and Okayama (10, 24). Vector DNA was

cotransfected with RAV-1, RAV-2, or RCAN-BHpol (28, 47, 62) helper virus DNA in a 10:1 μ g (vector/helper) ratio. Selection for G418-resistant (*Neo*^r) cells was initiated 48 h after transfection by splitting the cells (1 to 3 for CEF, 1 to 10 for QT6) into G418-containing medium. Following selection of *Neo*^r cells (~6 days), cultures were maintained in normal medium. Stable clones of *Neo*^r QT6 cells producing virus were made by isolation and expansion of individual colonies following G418 selection. Virus stocks were prepared by withdrawal of G418 from the medium 24 h prior to collection of cell culture supernatants from 10-cm plates. The media supernatants were filtered through 0.45- μ m-pore-size filters (Schleicher & Schuell, Inc.) and frozen at -70°C . The Q2, Q4, and Isolde packaging cell lines were transfected according to the method of Graham and Van der Eb (24), and G418 selection and harvest of virus were done according to the procedure described above for QT6 cells.

Virus infection and titration of virus. Cells (10^6) were infected with 1 to 5 ml of culture supernatants containing 8 μ g of Polybrene (Sigma) per ml for 1 to 2 h. Normal medium was added, and the cells were grown for a further 48 h and split into medium containing G418. The medium with G418 was changed every 2 days until selection was complete, and the *Neo*^r cultures were expanded for preparation of RNA, DNA, and protein. Titration of virus produced by transfected or infected pools or clones of cells was done by infection of 5×10^5 CEF or 10^5 QT6 cells on duplicate 60-mm dishes with 0.5 ml of serial virus dilutions in medium containing 8 μ g of Polybrene per ml. The infections were done at room temperature for 15 min on a rocking platform.

TABLE 1. Location of important features in pSFCV-neo^a

Nucleotide no.	Site ^b	Source (reference)
6683	Former <i>Eco</i> RI site of pUC19	
1-8	<i>Not</i> I linker	
9	Beginning of AEV sequences	pAEV-11 (18, 59)
643-1320	5' double LTR	pAEV-11 (18, 59)
982-983	Circle junction	pAEV-11 (18, 59)
983-1210	5' U3 (promoter)	pAEV-11 (18, 59)
1220-1240	R	pAEV-11 (18, 59)
1241-1320	5' U5	pAEV-11 (18, 59)
1220	Cap site	pAEV-11 (18, 59)
1321-1335	tRNA primer binding site	pAEV-11 (18, 59)
1597	<i>gag-neo</i> initiation codon	pAEV-11 (18, 59)
1597-2243	<i>gag</i> coding sequence	pAEV-11 (18, 59)
2244-2285	Synthetic oligo	
2251-3043	<i>neo</i> coding sequence	Tn5
1614	Splice donor site	pAEV-11
3043-3291	<i>erbB</i> intron	pAEV-11
3291	Splice acceptor site (<i>v-erbB</i>)	pAEV-11
3302-3370	Cloning polylinker	Synthetic oligo
3320	Stop codon (spliced mRNA)	Synthetic oligo
3303-3356	Cloning sites	Synthetic oligo
3357, 3362, 3366	Stop codons (all frames)	Synthetic oligo
3369-3603	AEV <i>env</i> (noncoding)	pAEV-10 (18, 59)
3604-3896	AEV 3' noncoding region	pAEV-10 (18, 59)
3897-4135	3' U3	pAEV-10 (18, 59)
4135-4155	R	pAEV-10 (18, 59)
4156-4235	3' U5	pAEV-10 (18, 59)
4129-4134	Polyadenylation signal	pAEV-10 (18, 59)
4156	Polyadenylation site	pAEV-10 (18, 59)
4242	End of AEV sequence	pAEV-10 (18, 59)
4243-4251	<i>Not</i> I linker	
4252	Former <i>Hind</i> III site of pUC19	

^a Details of the construction and compiled sequence of pSFCV-neo can be obtained upon request over computer network (BVE@Kitomo.KI.SE) or by sending an address-labeled Macintosh-formatted diskette. Sequences derived from AEV-ES4 are present at nt 1 to 4242 and make up the retroviral segment, including the bacterial neomycin phosphotransferase gene and a 68-bp synthetic cloning linker following the splice acceptor. All cloned sequences are intended to be expressed from the spliced subgenomic transcript, must supply their own AUG, and are cloned in the same orientation as viral sequences. The selective marker, G418 resistance, is provided by a *gag-neo* fusion protein. A synthetic linker joins the two reading frames to create the p55^{gag-neo} hybrid protein. Nt 4243 to 6683 represent the pUC19 plasmid backbone. In pSFCV-LE, modifications which leave a single 5' LTR and extend the distance from the splice acceptor to the cloning linker have been introduced.

^b U3, U5, R, Unique 3', 5', and repeated sequences of the LTR, respectively; *env*, retroviral envelope gene.

Five milliliters of normal medium was then added, and the cells were returned to the incubator. The cells were washed and refed with fresh medium 24 h later and refed with medium containing G418 48 h after the time of infection. Thereafter, the selective medium was changed every day until easily visible Neo^r colonies appeared (~12 days), at which time the cells were fixed in methanol-acetone (2:1) and stained with gentian violet to count colonies. Only colonies of average size or larger were counted as resulting from the initial virus infection; they were used to calculate titers as infectious Neo^r units per milliliter.

Supernatants from the Q2 and Q4 packaging lines were assayed for titer on QT6 cells as described above. Virus production by Isolde lines were similarly assayed for titer on CEF cells or by dot blot (see below). To test for the production of replication-competent recombinant helper virus, fresh QT6 cells were infected with culture supernatants from SFCV-neo-transfected Q2 or Q4 cells, or CEF cells were infected with culture supernatants from SFCV-Blue-transfected Isolde cells and then selected for outgrowth of

G418-resistant colonies. The G418 was withdrawn from the medium, and the culture supernatants were once again harvested to infect fresh QT6 or CEF cells, which were selected on G418 for outgrowth of resistant colonies. Any Neo^r colonies appearing in this final infection were assumed to be due to recombination of the input SFCV-neo or SFCV-Blue vector sequences with the resident RAV-1 genome of the Q2, Q4, or Isolde packaging line.

Estimation of virus titer by using RNA dot blot. Virus titers from cells harboring SFCV-Blue constructs were estimated by dot blot hybridization. Virus was pelleted from supernatants of SFCV-Blue-transfected Isolde clones or from helper virus-cotransfected CEF pools and used to prepare RNA for dot blotting as previously described (33). Serial threefold dilutions of RNA were applied to nitrocellulose by using a dot blot manifold (40) and hybridized by using a ³²P-labeled 910-bp *neo* probe. Conventionally assayed SFCV-Blue supernatants (see Virus infection and titration of virus above) were used as controls for comparative estimation of virus titers by scanning the autoradiograph with an integrating laser densitometer. These values were converted to estimated virus titer, expressed as units^c per ml (see Table 3).

Southern and Northern (RNA) analyses. Genomic DNA was prepared from 10-cm plates of confluent cells, digested with *Bst*XI or *Bgl*III, subjected to electrophoresis on 0.8% agarose gels, and transferred to nylon membranes (Hybond N; Amersham Corp.). Southern blots were probed with a ³²P-labeled *neo* fragment prepared by random hexamer labeling (19) (Multiprime Labeling System; Amersham). Poly(A)⁺ RNA for Northern analysis was prepared as previously described (65) from 15-cm plates of semiconfluent cells. Poly(A)⁺ RNA (1 to 2 μg) was electrophoretically separated on 1% agarose-formaldehyde gels containing MOPS (morpholinepropanesulfonic acid, pH 7.0) at 140 V for 4 to 5 h. The RNA was transferred to membranes and hybridized with ³²P-labeled *neo*, *env*, or chicken band 3 protein fragments derived from pSFCV-BIIIR.

β-Gal assays. In situ assays for β-Gal production were performed on glutaraldehyde-fixed subconfluent plates of SFCV-Blue-transfected or infected CEF or Isolde cells according to a protocol described by Dannenberg (15, 50). Blue colonies were counted after 6 to 10 h. To determine the percentage of colonies expressing β-Gal, these plates were rinsed in phosphate-buffered saline, treated with acetone-methanol, and stained with gentian violet to assess the total number of Neo^r colonies. Only blue colonies of uniform size (β-Gal) were scored.

Protein analyses. Immunoprecipitation of ³⁵S-labeled cell extracts with *gag* and band 3 protein antibodies was done as previously described (3), using protein A-Sepharose (Pharmacia) and monoclonal antibodies MCA2E (67) to chicken band 3 protein, MC4.2.A5 (25) to chicken hemoglobin, and M3CZC (26) to viral *gag* (p19) polypeptides. Labeled extract (10⁷ cpm) was used for each immunoprecipitation.

For immunofluorescence studies, fibroblasts were detached from the culture plastic with EDTA and fixed in paraformaldehyde. Anti-chicken band 3 protein monoclonal antibody coupled to fluorescein isothiocyanate-goat anti-mouse immunoglobulin was used to visualize the location of the band 3 protein, as described elsewhere (68).

Assay for anion transporter function. Anion transporter activity was tested in two ways. First, SFCV-BIIIR- and SFCV-BIIIW-infected (Neo^r) cells were cultured for 24 h in the presence or absence of 0.4 mM DIDS (diisothiocyanodihydrostilbene-2,2' disulfonic acid; Sigma), a specific inhibitor of band 3 protein activity. The presence of vacuoles was

monitored under a phase-contrast microscope. Second, cells infected with SFCV-BIIIR or SFCV-BIIIW were grown in parallel in normal media adjusted to pH 7.2 or 8.0 by the addition of bicarbonate and allowed to grow for 48 h. The presence of vacuoles was monitored under the microscope.

RESULTS

Strategy for pSFCV-neo vector construction. We chose the AEV-ES4 genome as the basis for the construction of pSFCV-neo, since the AEV LTRs are active in many types of avian cells, including fibroblasts, erythroblasts, myeloblasts, and blastoderm cells (4, 20, 22). Also, the virus is efficiently packaged when pseudotyped with a variety of helper viruses, yielding titers of 10^5 to 10^6 infectious particles per ml (20, 22). In pSFCV-neo, the LTRs and *gag* sequences originate from the cloned AEV genome (66; Fig. 1). Because packaging sequences, a splice donor site, and other functions (11) are in part located in the *gag* regions of avian sarcoma and leukosis viruses, we chose to retain 646 nucleotides of *gag* coding sequence (*NaeI* site). To be able to accommodate a selectable marker (Neo^r) as well as one or several additional genes in the vector genome, we fused the necessary parts of *gag* in frame with the bacterial neomycin phosphotransferase gene (*neo*). In this way, transcription, packaging, and selectable marker functions were confined to a minimal genetic unit. The *gag-neo* hybrid protein has an estimated molecular size of 53,000 daltons and is expressed from genome-length RNA at easily detectable levels.

The Neo^r gene is followed in pSFCV-neo by a short intervening sequence and a splice acceptor site, both derived from AEV. In spliced subgenomic RNAs from avian retroviruses, the splice donor site is preceded by the six initial codons of *gag*. To allow the expression of unmodified proteins from subgenomic mRNAs, we inserted after the splice acceptor site a polylinker containing a terminator codon in frame with the upstream AUG. Consequently, cloned sequences had to supply their own start codon. The restriction sites in the polylinker are followed by stop codons in all three reading frames, and mRNAs utilize polyadenylation signals in the 3' LTR. The *HindIII* site at the start of the polylinker and the *EcoRI* site at the end facilitate the insertion of genes from pUC vectors. The *Clal* site in the SFCV-neo polylinker allows the insertion of an additional gene by the use of adaptor plasmids (28), which carry their own splice acceptor site, initiator AUG, and cloning polylinker. All polylinker sites except that for *BglI*, which is also present in the plasmid vector, are unique in pSFCV-neo. The *BstXI* sites in the LTRs are unique and can be used to excise the integrated provirus. *NotI* linkers separate SFCV sequences from the pUC backbone in the plasmid pSFCV-neo. The genomic transcript has a calculated length of 2,963 nt, and the spliced mRNA is 1,250 nt, excluding the insert and poly(A) tail. The estimated cloning capacity based on RSV-derived vectors (48, 58) is greater than 6.5 kb.

pSFCV-BIIIR (band 3, Fig. 1A) represents the vector pSFCV-neo containing an *EcoRI* insert of 3.1 kb containing the cDNA for the avian erythrocyte band 3 protein (68) in the same orientation as the viral sequences. The insert harbors its own ATG initiating codon and stop codons. This particular construct was designed to express band 3 protein as a spliced viral transcript via the promoter and polyadenylation site in the retroviral LTRs. pSFCV-BIIIW contains the same *EcoRI* fragment in the opposite orientation.

pSFCV- β (β -gal, Fig. 1A) contains 3.3 kb of bacterial β -Gal sequence cloned at the *HindIII* site of the SFCV-neo

polylinker. The expression of the spliced β -Gal mRNA from SFCV- β was easily detectable by Northern analysis (data not shown), but transfected cells scored weakly or unevenly positive when in situ assays were used to determine β -Gal activity. Since the construct produced only weak enzyme activity, it was used for quantitation of virus production and Southern blot analysis only.

pSFCV-LE (Fig. 1B) was constructed from pSFCV-neo by partial *BstXI* digestion and religation to create a single upstream LTR. In addition, a modification was made to ensure the efficient translation of inserted sequences. In pSFCV-neo, the presence of a stop codon following the splice acceptor prevents the fusion of *gag* sequences to cloned genes. To provide a proper distance (37) between this stop codon and the AUG of cloned sequences, we inserted a synthetic 88-bp fragment representing a modified form of the human EGF receptor 5' untranslated region (nt 105 to 186) into the *HindIII* site of the pSFCV-neo polylinker. This version of the vector efficiently allowed production of β -Gal (assayed in situ for enzyme activity) as a *HindIII* insert in pSFCV-LE.

pSFCV-Blue (β -gal, Fig. 1B) represents a 3.14-kb *E. coli* β -Gal (50) fragment cloned into the *HindIII* site of pSFCV-LE in the same orientation as viral sequences. This construct was used for in situ quantitation of β -Gal activity and virus production (Tables 2 and 3).

Expression of neomycin resistance by SFCV-neo. To test the ability of SFCV-derived *gag-neo* hybrid protein to provide G418 resistance to CEF and QT6 cells, pSFCV-neo was compared with the plasmid pSV2neo (45) in parallel transfections. Equal amounts of plasmids were transfected onto CEF cells, and the resulting sizes and numbers of Neo^r colonies were noted. pSFCV-neo gave between 50 and 70, pSFCV- β gave 80 to 100, and pSFCV-Blue gave 90 to 100 Neo^r colonies per μ g of DNA, comparable with that observed using pSV2neo (25 to 30 Neo^r colonies per μ g) at 0.8 mg of G418 per ml. At G418 concentrations of 2.0 mg/ml, all plasmids gave 10 to 15 Neo^r colonies per μ g of DNA. The size of the Neo^r colonies from SFCV transfectants were comparable with those of pSV2neo transfectants.

Virus production by pSFCV-neo-helper-transfected cells. To estimate the amount of virus released by cells harboring the SFCV-neo provirus, constructs were cotransfected into CEF or QT6 cells with several helper virus DNAs such as RAV-1, RAV-2, and RCAN-BHpol. RCAN-BHpol is a construct based on RSV-Schmidt-Ruppin A, which lacks *v-src*, contains the *pol* region of the Bryan high-titer strain (RSV), and was designed to produce higher titers of virus (28, 47, 62). Culture supernatants were harvested from pools of CEF cells or clones of QT6 cells at different times after transfection, and titers were then determined on equivalent populations of CEF or QT6 cells. The titrations were judged by the outgrowth of G418-resistant colonies and expressed as infectious Neo^r units per milliliter of culture supernatant. The production of recombinant virus was greater than 10^5 Neo^r units per ml by all Neo^r -selected groups of cells, regardless of the cell type, helper virus, or SFCV provirus present, with the exception of RAV-1 transfectants (Table 2). In general, RAV-1-transfected cells produced lower levels of virus with any SFCV construct than did RAV-2- or RCAN-BHpol-transfected cells. In addition, virus production by QT6 cells (clones) tended to be more uneven than that by CEF (pools), which was not unexpected. The results also show that relatively high virus production (1×10^4 to 2×10^4 Neo^r units per ml) can be detected in unselected cells

TABLE 2. Production of recombinant virus

Provirus	Cell type	Helper	Titer (Neo ^r units/ml) ^a	
			48 h	G418 selected
SFCV-neo	CEF	RAV-1	<1 × 10 ³	4 × 10 ⁶
		RAV-2	ND	1 × 10 ⁶
		RCAN-BHpol	2 × 10 ⁴	5 × 10 ⁶
	QT6	RAV-1	>1 × 10 ³	1 × 10 ⁵
		RAV-2	>1 × 10 ³	1 × 10 ⁶ -8 × 10 ⁶
SFCV-β	QT6	RCAN-BHpol	1 × 10 ⁴	5 × 10 ⁶
		RAV-1	<1 × 10 ³	<1 × 10 ³
		RCAN-BHpol	1 × 10 ⁴	1 × 10 ⁶ -10 × 10 ⁶
SFCV-BIIIR	CEF	RCAN-BHpol	ND	1 × 10 ⁶ - 8 × 10 ⁶
SFCV-BIIIW	CEF	RCAN-BHpol	ND	2 × 10 ⁵ -10 × 10 ⁵
SFCV-Blue ^b	CEF	RCAN-BHpol	ND	2 × 10 ⁶ - 4 × 10 ^{6c}

^a Titers of less than 10³ were not scored. ND, Not done. Results are tabulated from several pSFCV variants and helper viruses. CEF titers are taken from pools of cells, and QT6 titers are averages of individual clones.

^b Includes the percentage of cells scoring positive in situ β-Gal assays.

^c Of neo^r colonies, >89% were β-Gal positive.

harboring pSFCV-neo and RCAN-BHpol as early as 48 h posttransfection.

The production of virus by the SFCV-neo-transfected packaging lines Q2 and Q4 (Table 3) was lower than that by helper-cotransfected cells, which was expected. Half of the Q2 transfectants produced titers of between 10² and 10³ infectious Neo^r units per ml, while the other half produced virus at levels too low to detect (<20 Neo^r units per ml). The Q4 packaging line produced consistently higher titers of around 2 × 10⁴ Neo^r units per ml, though again from only half of the clones selected. No recombinant helper virus was detected from any of the clones when the techniques described in Materials and Methods were used. However, when SFCV-neo-transfected Q2 or Q4 cells were maintained as uncloned pools of G418-resistant cells, even for short periods, recombinant helper could be detected in the medium of QT6 cells infected with culture supernatants from those pools. This meant that recombination of SFCV-neo with the resident RAV-1 genome of Q2 or Q4 cells was frequent enough to detect in any transfection but could be avoided by selection of individual clones of virus-producing cells.

Production of virus by pSFCV-Blue-transfected Isolde packaging cell pools or clones was consistently between 1 × 10⁴ and 5 × 10⁴ Neo^r units^c per ml of cell culture supernatant (Table 3). This was equal to those values observed with packaging line Q4. However, unlike the situation with Q2 or Q4 transfectants, the presence of recombinant helper virus

could not be detected (see Materials and Methods) in cloned or uncloned pools of Isolde transfectants. Of 22 Neo^r clones tested, all scored positive for β-Gal production, with 18 clones at least 90% β-Gal positive. When assayed for virus production by dot blot, these 18 cloned lines all produced about 10⁴ virus units per ml (Table 3). A total of 80 to 85% of cells infected with these Isolde supernatants also scored positive by in situ β-Gal assays.

Integration and maintenance of SFCV-neo provirus. To examine the status of the SFCV provirus, virus supernatants were used to infect CEF or QT6 cells, and Neo^r pools (CEF) or clones (QT6) of cells were isolated and expanded for Southern analysis. *Bst*XI, which cuts in the retroviral LTRs, and *Bgl*II, which cuts once in SFCV, were used to determine integration sites and copy number, respectively, of the SFCV provirus in infected cells. The SFCV-neo provirus was integrated and stably maintained at one to three copies per cell in all cell populations (Fig. 2). The probe used for this purpose was a 910-bp *neo* fragment. In some lanes (SFCV-β*), 10 pg (~1 copy equivalent) of pSFCV-neo plasmid DNA was included in the restriction digests for comparison. The first lane contains *Bst*XI-cleaved pSFCV-neo DNA alone.

Expression of β-Gal activity by pSFCV-β and pSFCV-Blue. Transfected Neo^r populations of CEF cells expressing SFCV-β were assayed in situ for β-Gal activity. In general, 1 to 10% of the cells expressed enzyme activity, and clones of cells remained uneven, with a fraction losing or gaining

TABLE 3. Virus titers for transfected cell lines^a

Packaging line	Transfecting agent	Cell clones ^b	Titer ^c	Presence of helper	β-Gal positive (%)
Q2	SFCV-neo	1-4	7 × 10 ¹ -8 × 10 ²	-	
		5-10	<1 × 10 ¹	-	
		Pool	2 × 10 ²	+	
Q4	SFCV-neo	1-5	2 × 10 ⁴ -5 × 10 ⁴	-	
		6-10	<1 × 10 ¹	-	
		Pool	3 × 10 ⁴	+	
Isolde	SFCV-Blue	1-18	2 × 10 ⁴ -5 × 10 ^{4d}	-	>90
		19-22	1 × 10 ³ -5 × 10 ^{3d}	-	20-50
		Pool	1 × 10 ^{4d}	-	>90

^a Packaging cells were transfected with DNA from pSFCV variants, and individual Neo^r clones were isolated and assayed for titer. The clones are grouped according to relative titers.

^b Pools were of uncloned Neo^r cells.

^c Titers of less than 2 × 10¹ were not scored in this assay.

^d Estimated virus titer from dot blot assays expressed as Neo^r units^c per milliliter.

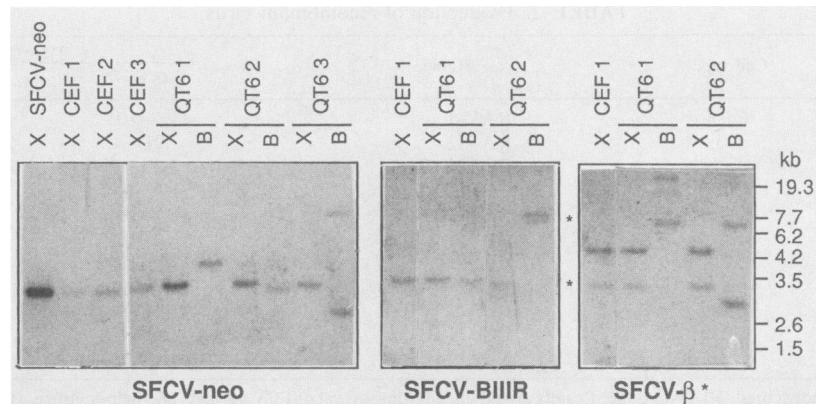


FIG. 2. Southern blot of SFCV provirus in SFCV-neo- or SFCV-BIIIIR-infected QT6 or CEF cells. High-molecular-weight DNA (10 μ g) from infected cells was cleaved with *Bgl*II (lanes B) to analyze integration sites or with *Bst*XI (lanes X) to determine copy number of proviruses. SFCV-neo, DNA from SFCV-neo-infected pools of CEF cells (pools 1 to 3) or isolated clones of QT6 cells (clones 1 to 3); SFCV-BIIIIR, DNA from SFCV-BIIIIR-infected pools of CEF cells (pools 1 and 2) or isolated clones of QT6 cells (clones 1 and 2); SFCV- β^* , DNA from SFCV- β -infected pools of CEF cells (pool 1) or clones of QT6 cells (clones 1 and 2) along with 10 pg (\sim 1 copy equivalent) of pSFCV-neo DNA as an internal control for comparison. The asterisks represent the migration of the pSFCV-neo internal plasmid controls in SFCV- β^* lanes. Numbers on the right indicate the sizes of fragments of the *Sty*I-cleaved λ DNA marker. The first lane in panel SFCV-neo contains a pSFCV-neo plasmid control. The probe used for this purpose was a 32 P-labeled 910-bp *neo* fragment.

expression as the cells were passaged for short periods. In an attempt to remedy the failure of pSFCV- β to efficiently express β -Gal activity, the vector was modified as described above. The new version, pSFCV-Blue, was then assayed in two sets of experiments for its ability to transduce β -Gal activity to transfected or infected cells (Tables 2 and 3). CEF or Isolde cells were transfected with pSFCV-Blue and plated sparsely enough to distinguish individual colonies following G418 selection. These were assayed in situ for β -Gal production and later stained with gentian violet to count all Neo^r colonies. In two separate transfections, 106 of 117 (90%) and 50 of 56 (89%) of Neo^r CEF colonies were positive for β -Gal activity. Of 22 isolated Isolde clones, all were positive for β -Gal activity, with 18 clones being >90% positive. To assess the ability of SFCV-Blue viruses to transduce β -Gal activity, supernatants of the 18 transfected Isolde cell clones were used to infect fresh CEF cells. Of the clones tested, all infected CEF pools scored positive by in situ staining. About 90% of the infected cells in any pool were β -Gal positive. When these CEF infectants are grown sparsely to isolate colonies, >80% of the Neo^r colonies were β -Gal positive, depending on the virus stock. These results are in stark contrast to the weak β -Gal activity transduced by our previous construct, pSFCV- β .

Expression of RNAs from SFCV-neo LTR. Northern analysis of SFCV-neo-infected CEF or QT6 cells was used to determine the relative amounts of SFCV genomic and spliced RNAs present in infected cells. A *neo* probe detected the full-length genomic RNA in the SFCV-neo- and SFCV-BIIIIR-infected cells (Fig. 3). An *env*-specific probe detected all RNAs of vector and helper virus origin. Cells containing SFCV-neo proviruses without inserts produced the 3.0-kb full-length SFCV-neo transcript, but they all failed to produce the expected spliced transcript of 1.25 kb. Instead, a 2-kb cryptically spliced RNA was observed. In cells harboring SFCV proviruses containing foreign sequences (Fig. 3, band 3), the relative amount of this cryptically spliced RNA was lower than in SFCV-neo-infected cells, but an additional cryptically spliced mRNA was seen at 1.8 kb. In cells harboring the SFCV-BIIIIR construct, the probe for band 3 detected mainly the 6.1-kb full-length RNA, the 4.35-kb spliced subgenomic RNA, and the cryptic 1.8-kb RNA. The

SFCV-neo 2-kb cryptic splice could result from the utilization of a potential cryptic acceptor site created at the *gag-neo* junction. The 1.8-kb cryptic splice could result from splicing within the band 3 protein coding sequences. The presence of these alternatively spliced mRNAs did not seem to interfere with the production of sufficient *neo* to provide G418 resistance or the production of band 3 protein from the correctly spliced mRNA. Experiments to determine the origin of these cryptic splice points are currently under way as part of an attempt to eliminate the points from the SFCV constructs.

Expression of SFCV *gag-neo* protein. Immunoprecipitation of *gag*-related proteins from SFCV-BIIIIR-infected cells (Fig. 4) showed two specific products. The pr76^{gag} protein represents the precursor *gag* protein expressed by the helper virus, whereas the smaller *gag* polypeptide is the vector-derived *gag-neo* fusion protein of 55,000 M_r . The observed size of the p55^{gag-neo} protein is in agreement with the estimated size of a 482-amino-acid *gag-neo* fusion protein of 53,000 daltons. Though the amount of p55^{gag-neo} protein was low compared with that of pr76^{gag} of the helper virus, it was sufficient to provide Neo^r, even at concentrations of 3 mg of G418 per ml, which is necessary for the selection of erythroblast cultures (data not shown).

Band 3 protein expression from SFCV in fibroblasts. The cDNA encoding chicken erythrocyte band 3 protein was successfully expressed in fibroblasts using the pSFCV-neo vector. Immunoprecipitation of band 3 protein (Fig. 4) in CEF cells transfected with SFCV-BIIIIR and RCAN-BHpol showed a strong band in the 95-kDa molecular size range which is absent from SFCV-BIIIW (opposite orientation)-transfected cells and similar to the band for the native protein immunoprecipitated from EGF receptor-transformed erythroblasts (33). This size is in agreement with the established molecular weights of erythrocyte band 3 polypeptides of 100,000 and 105,000 (doublet). The cDNA used to express band 3 protein in the SFCV-BIIIIR construct represents only one of these two polypeptides, which are generated by alternative initiation and splicing of mRNAs (34, 35).

Indirect immunofluorescence of CEF expressing the SFCV-BIIIIR constructs (Fig. 5A) using a band 3-specific monoclonal antibody showed a bright ring of fluorescence

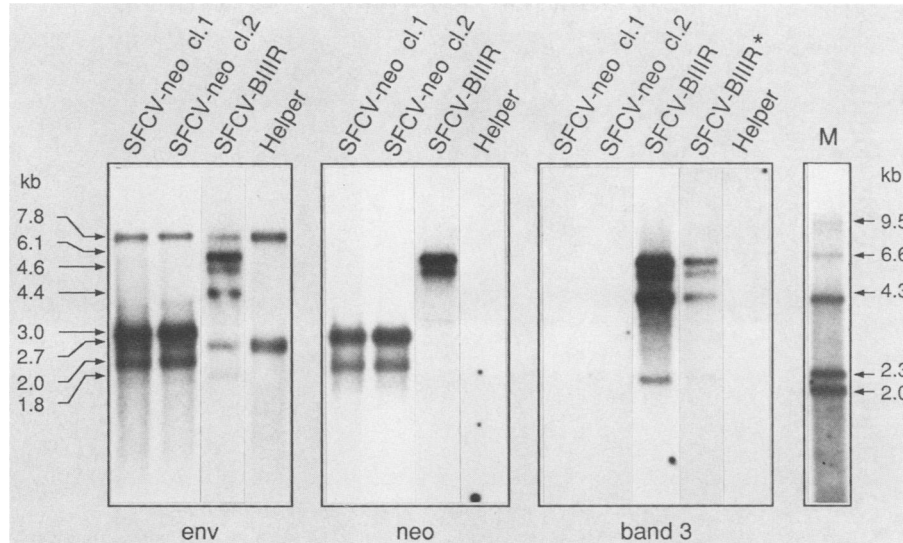


FIG. 3. Northern blot of RNAs expressed from SFCV proviruses in SFCV-neo- and SFCV-BIIIR-infected cells. Poly(A)⁺ RNA (2 μ g) from SFCV-neo-infected QT6 cell clones (cl.), an SFCV-BIIIR-infected QT6 cell clone, or helper virus-infected QT6 cells was analyzed with a battery of probes. An *env* probe was used to detect all viral transcripts, a *neo* probe was used to detect SFCV-specific transcripts, or a chicken band 3 probe was used to detect band 3 transcripts. The marker (lane M) was an end-labeled *Hind*III-*Eco*RI digest of λ DNA. The arrows on the left indicate the migration of the various RNAs: helper virus genomic and subgenomic transcripts, 7.8 and 2.7 kb; SFCV-neo-derived genomic RNA, 3.0 kb; cryptically spliced RNA, 2.0 kb; band 3 protein-derived genomic RNA, 6.1 kb; subgenomic RNA, 4.4 kb; cryptically spliced RNAs, 4.6 and 1.8 kb. Lane SFCV-BIIIR* represents a shorter exposure of the SFCV-BIIIR lane to its left. The ³²P-labeled probes used for this purpose were a 580-bp *env* fragment, a 910-bp *neo* fragment, and a 3.0-kb band 3 protein fragment, all derived from SFCV-BIIIR.

located at the periphery of cells transfected with SFCV-BIIIR DNA and absent in SFCV-BIIIW counterparts. This is consistent with the plasma membrane localization of band 3 protein in erythrocytes and shows that the vector-encoded protein behaves in a manner consistent with its role in vivo. All Neo^r clones or pools of band 3 protein-transfected or infected cells thus far analyzed have produced band 3 protein by one or more of the three criteria we use, i.e., immunoprecipitation, immunofluorescence, or vacuolization.

CEFs infected with SFCV-BIIIR, but not SFCV-BIIIW, viruses become highly vacuolated. In order to assess whether this was due to the ion exchange activity of the ectopically expressed band 3 protein, two experiments were performed. The first was to alter the bicarbonate content of the cell culture medium, which can be used to modulate anion transport activity. After 48 h in normal pH 7.2 medium, the vacuoles remained (Fig. 5B), but when the same cells were grown in pH 8.0 medium in a parallel experiment, the vacuoles disappeared. There was no change in SFCV-BIIIW-transfected cells under the same conditions. All cells grew normally at both pH values.

Second, we used the compound DIDS (a stilbene disulfonate derivative), which functions as an inhibitor of band 3 protein by irreversibly binding to the extracellular domain of the protein and blocking anion transport. The addition of 0.4 mM DIDS to the cell culture medium caused, within 24 h, the elimination of vacuoles from SFCV-BIIIR-transfected cells while having no apparent effect on their growth (Fig. 5C). There was no effect on SFCV-BIIIW-transfected cells under the same conditions (data not shown). These results also indicated that the ectopically expressed band 3 protein was located in the plasma membrane in the correct orientation and mediated anion transport, as does the endogenous erythrocyte protein. Each cell expressing the band 3 protein

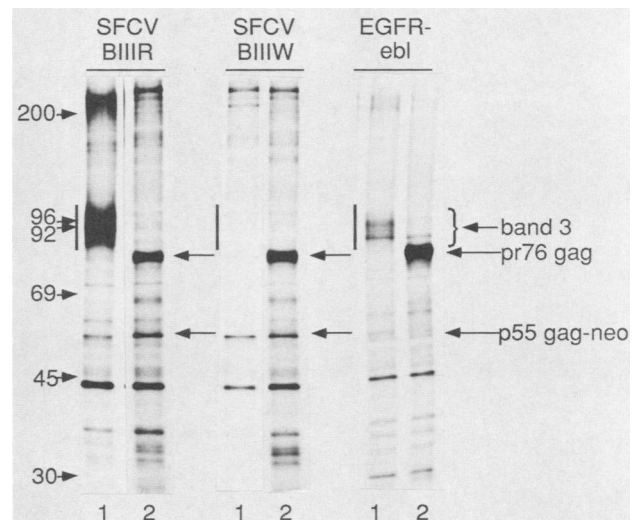


FIG. 4. Immunoprecipitation of p19 *gag*-related polypeptides or band 3 proteins from SFCV-BIIIR-infected cells. ³⁵S-labeled proteins from infected cells were immunoprecipitated with anti-band 3 protein (lane 1) or anti-p19^{gag} (lane 2) antibodies. The proteins were prepared from SFCV-BIIIR (left)- or SFCV-BIIIW (center)-infected CEF cells. The right-hand panel contains these proteins immunoprecipitated from human EGF receptor-transformed erythroblasts (EGFR-eb1). Arrows at the extreme right correspond to the anticipated migration of band 3- and p19^{gag}-related proteins; pr76 *gag*, helper virus *gag* protein precursor; p55 *gag-neo*, *gag-neo* fusion protein encoded by SFCV-neo. The vertical bars to the left of lanes 1 represent the anticipated migration of band 3 polypeptides. The arrows to the right of lanes 2 indicate the anticipated migration of *gag* proteins. Numbers on the left correspond to the migration of molecular size standards in kilodaltons.

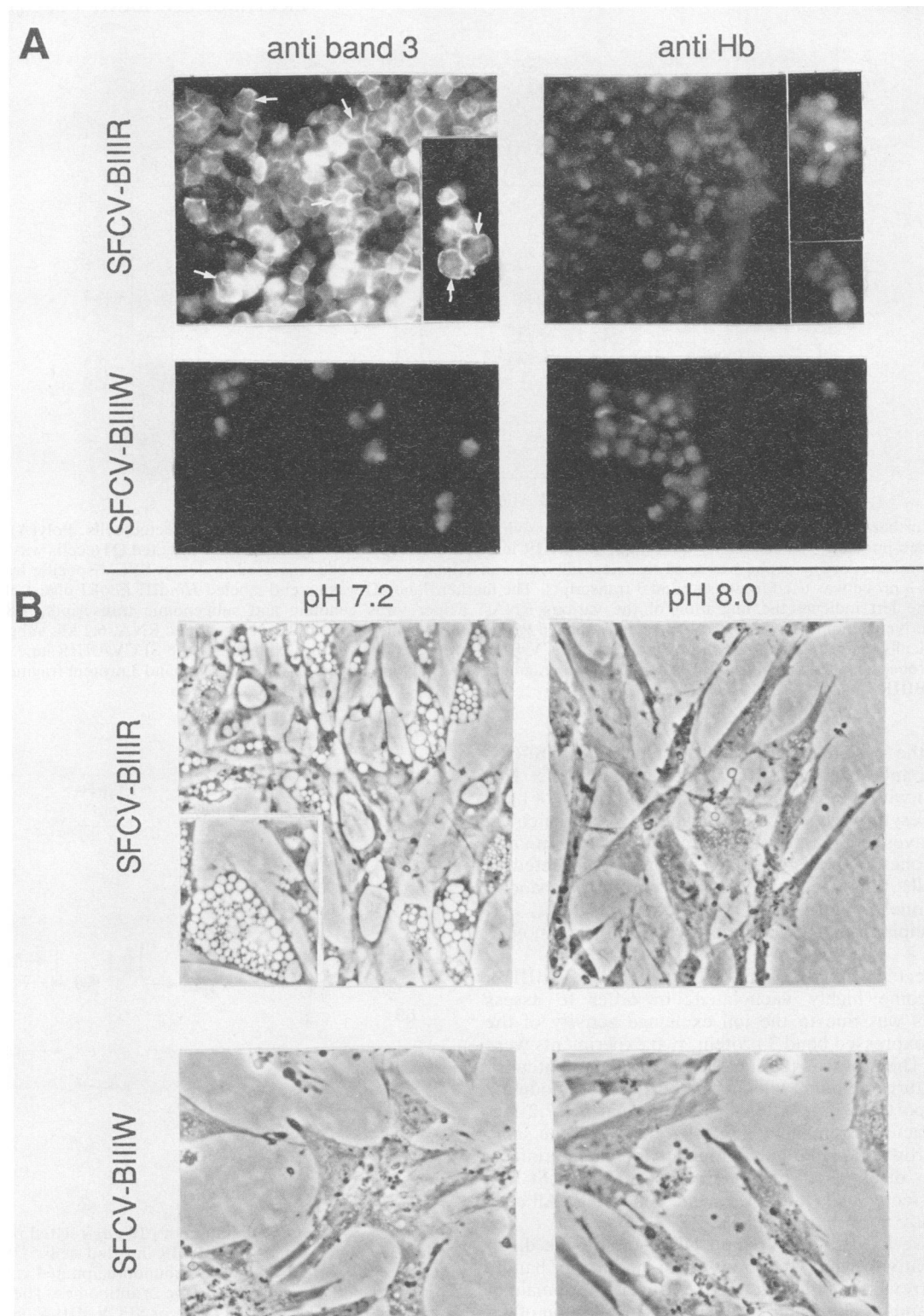


FIG. 5. (A) Band 3 protein-specific immunofluorescence of intact CEF cells containing SFCV-BIII proviruses. Indirect immunofluorescence of CEF cells expressing SFCV-BIIIR and SFCV-BIIIW proviruses. The left half of the panel shows cells stained for expression of band 3 protein, and the insert shows an enlargement of the same cells. The right half of the panel shows the same cells stained with antibodies to hemoglobin (Hb; negative control). Notice the intense peripheral staining of SFCV-BIIIR cells when the band 3 protein antibody is used (arrows). (B) Effect of pH on the vacuolization of SFCV-BIIIR-containing fibroblasts. The left half of the panel shows CEF cells grown for 48 h at the normal pH of 7.2. The right half of the panel shows the same pool of cells grown for 48 h in pH 8.0 medium. Notice the multiple, large vacuoles in SFCV-BIII-containing cells at the normal pH. (C) Effect of DIDS on band 3 protein-expressing fibroblasts. CEF cells harboring SFCV-BIIIR proviruses were cultivated for 24 h at pH 7.2 in medium containing DIDS (right) (see Materials and Methods), an inhibitor of band 3 protein ion exchange function. The figure on the left shows the same cells under normal conditions (pH 7.2, no DIDS).

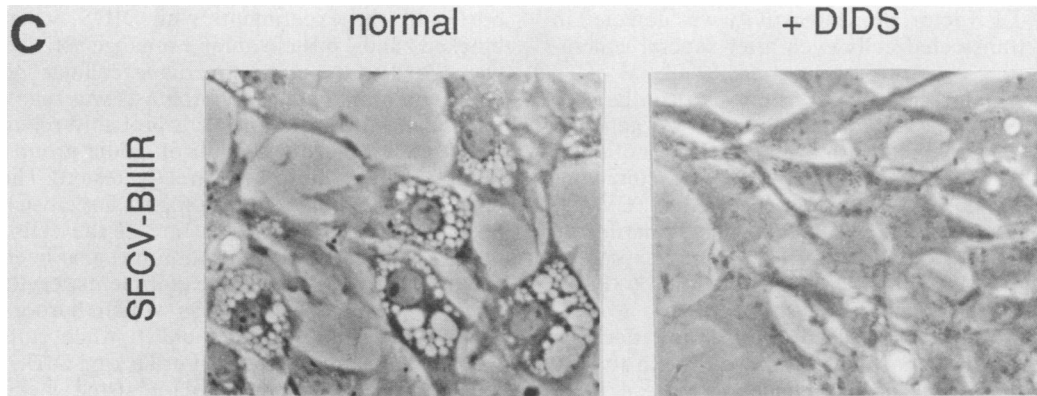


FIG. 5—Continued

acquired vacuoles, which served as an excellent visual marker for vector function in this cell type.

DISCUSSION

The SFCV-neo vector was adapted from AEV-ES4 with the object of retaining several features important for viral replication, such as the high activity of the viral LTRs in many types of avian cells and the ability of AEV to produce large amounts of virus with functions provided in *trans* by several different helper viruses. The LTRs contained the viral promoter, cap site, polyadenylation signals, proviral integration sites, and one of the important enhancer sequences, located in the R-U3 region (38, 54). In addition, several *cis*-acting elements outside of the viral LTRs were also retained, as discussed below.

Packaging sequences (ψ) of the avian retroviruses are found at several points on the viral genomic RNA. An important ψ sequence lies in the untranslated leader region between U5 and the start of *gag* (32). This ~30-bp sequence is located ~250 bp from the 5' end of the genomic RNA and constitutes a major part of the sequences required for efficient encapsidation of viral RNAs; it overlaps in part with sequences affecting viral replication and protein translation (5, 46, 49). In addition, another important packaging signal lies within the structural gene for *gag* (51), about 150 bp downstream of the splice donor site. Also, some of the sequences directly involved in the genome dimerization step necessary for encapsidation are located in this region (5). Finally, noncoding sequences 3' of *env* are thought to be involved in viral genome assembly (27, 58) for packaging. SFCV-neo was designed to encompass all of the sequences mentioned above.

In the avian sarcoma and leukosis viruses, other *cis*-acting sequences overlap the *gag* coding sequences, and they are retained in SFCV-neo. These sequences affect splicing (39, 57, 61), contain determinants of viral tropism (53), and also make up a second important enhancer region (1, 8, 61). Recent studies using *in vitro* chloramphenicol acetyltransferase assays show that a series of deletions in *gag*, extending from the 3' end, result in a continual reduction in transcriptional activity compared with that of the intact sequence (1). In mutations extending beyond a critical region, transcription is markedly reduced. This enhancer region, required for efficient viral transcription in some cell types (1, 61), is located just upstream of the *gag-neo* junction in pSFCV-neo (nt 2243).

To avoid promoter interference by competing transcrip-

tional regulatory domains (18), we chose to express inserted sequences from spliced, subgenomic RNAs in SFCV-neo. RSV efficiently utilizes multiple splice acceptors in this context (*env* and *src*). The splice donor site in SFCV is located after the sixth codon in *gag*, and the splice acceptor site is that of the *v-erbB* gene, both derived from AEV. In the context of SFCV, these sequences functioned efficiently to direct the splicing of subgenomic mRNAs when cloned inserts were present in the SFCV-neo polylinker. In the absence of cloned sequences, the expected subgenomic mRNA could not be detected, and cryptically spliced RNAs were present in all SFCV-neo variants. These cryptic splices probably result from the introduction of foreign sequences, such as *neo*, into the retroviral genome and do not seem to interfere with splicing or translation of the correct mRNAs in pSFCV-transfected or infected cells. Cryptic splicing has also been noted in murine retrovirus vectors (7; personal communication); the aberrant splicing seems to have no effect on expression of correctly spliced RNAs. However, experiments are currently under way to determine the sites of these aberrant splice points and remove them from pSFCV.

The SFCV polylinker is immediately downstream of the SFCV splice acceptor and contains first a stop codon, closing the reading frame initiated in the RNA leader; then restriction sites for *HindIII*, *EcoRV*, *BglII*, *SfiI*, *ClaI*, *XbaI*, and *EcoRI*; and then stop codons in all frames. By using adaptor plasmids which carry the RSV *src* splice acceptor, leader sequence, and an adjoining polylinker on a *ClaI* fragment (25), a second spliced mRNA could potentially be produced by SFCV-neo proviruses.

In pSFCV-LE, a further modification has been made in the region near the splice acceptor. We have inserted a modified, synthetic 88-bp human EGF receptor fragment (nt 105 to 186) to provide a spacer between the stop codon and the beginning of the polylinker. This sequence, in an unmodified form, has been previously used by our lab to produce about 10^6 EGF receptor molecules per cell from an AEV vector in a similar context (33). It has been shown that a minimum sequence is required between a stop codon and an in-frame AUG to allow efficient ribosomal reinitiation (37). The original SFCV- β -Gal construct (SFCV- β) was a 3.3-kb fragment cloned into the first (*HindIII*) site of the polylinker. SFCV- β produced weak, erratic β -Gal activity in transfected and infected cells, although β -Gal-specific subgenomic RNA was abundant in these cells (data not shown). However, when the β -Gal gene was cloned into the *HindIII* site of the

modified SFCV-LE vector, enzyme activity was detected in >90% of Neo^r transfected cells even after several generations in culture.

The potential cloning capacity of SFCV-neo can be estimated from RSV. This virus encodes a 9.3-kb genomic RNA, and infectious mutants with a genome length of up to 10.1 kb exist (48). This would allow, by analogy, cloning of between 6.4 and 7 kb of foreign sequence into SFCV-neo without impairing the efficiency of packaging. Experiments to test this are currently under way: two genes expressed from separate splice acceptors will be inserted into pSFCV-LE. The total insert size will be 6.5 kb.

The fusion of *gag* to *neo* in SFCV-neo did not affect the ability of the neomycin phosphotransferase protein to provide Neo^r in the presence of high concentrations of G418, and the *cis*- and *trans*-acting activities present in *gag* appeared to be undisturbed. Consequently, all of the important regulatory signals encoding splicing, enhancer, and ψ sequences were retained in the *gag* region, while the *neo* portion efficiently provided G418 resistance to infected cells, as shown by titration experiments. In SFCV-neo, both the unspliced and the spliced mRNAs were efficiently translated into protein, as shown by immunoprecipitation of the *gag-neo* and band 3 proteins.

We have used the *E. coli lacZ* (β -Gal) gene to test the features of vector function. Using two separate β -Gal constructs, we have tested expression of β -Gal protein by in situ analysis and analyzed virus titers from SFCV- β -gal-transfected cells. Using the SFCV-Blue construct in transfection experiments with both QT6 and CEF cells, we have shown that enzyme activity is efficiently expressed in >90% of G418-selected clones or pools of cells. When supernatants from three of these cell clones were used to infect CEF cells, >80% of colonies were β -Gal positive Neo^r. SFCV-Blue has also been used to express β -Gal in erythroblasts (data not shown), and >95% of the infected Neo^r erythroblasts expressed enzyme activity by in situ analysis. SFCV- β -RCAN-BHpol-cotransfected QT6 cells efficiently produced 10⁶ Neo^r units per ml of virus, and SFCV-Blue-RCAN-BHpol-cotransfected CEF cells yielded similar titers of 2 \times 10⁶ to 4 \times 10⁶ Neo^r units per ml.

We chose the cDNA for the erythrocyte anion transporter to analyze the expression of a eucaryotic gene from the SFCV-neo vector. We are also interested in the role of band 3 protein repression by *v-erbA* in the AEV transformation of erythroblasts (68). The SFCV viruses are intended for use in erythroblasts as well as fibroblasts, and it was anticipated that the ectopic expression of band 3 protein in fibroblasts could be readily monitored. The expression of the anion transporter protein from the SFCV-BIIIR provirus, in fact, caused vacuolization of the infected fibroblasts. Recently published data from studies using a neuronal homolog of murine band 3 protein expressed in COS cells (36) showed that ectopic anion transporter expression caused intracellular acidification. This was also seen when murine erythroid band 3 protein was expressed in *Xenopus* oocytes (2). Thus, under normal culture conditions, the anion transporter exchanges intracellular HCO₃⁻ for extracellular Cl⁻ in a manner dependent on the bicarbonate and chloride content of the medium. In fibroblasts infected with SFCV-BIIIR viruses, vacuolization was presumably due to intracellular acidification, since this phenotype could be abolished by growing the cells in alkaline medium. An interpretation of this is that the addition of extra bicarbonate to increase the pH of the medium altered the anion exchange activity, thereby relieving the vacuolization. This hypothesis is sup-

ported by the experiments with DIDS, which irreversibly blocked band 3 protein anion exchange activity. When DIDS was added to the culture medium, cellular vacuolization in band 3 protein-containing fibroblasts was relieved. Effects of intracellular acidification were probably relieved in SFCV-BIIIR cells by sequestration of acidic groups in vacuoles, though this has not been directly tested. The presence of band 3 protein and the multiple, large vacuoles did not appear to affect the growth rate of the cells. This band 3 protein-associated vacuolization has also been reported for erythroblasts (68). In these experiments, erythroblasts were infected with an AEV virus which harbors a defective *v-erbA*; the cells were vacuolated when cultivated in medium of the inappropriate pH or lacking DIDS, whereas this phenotype was absent in pH-adjusted medium. Furthermore, the vacuolization in erythroblasts could be abolished by expression of the normal *v-erbA* oncogene, which completely repressed band 3 protein gene expression.

The phenotype induced by the ectopic expression of band 3 protein activity at the plasma membrane of infected fibroblasts provided a visual marker for the correct transcription, translation, transport, and stability of sequences transduced by SFCV-neo. Though the band 3 anion exchange protein is encoded by two polypeptides in avian cells, we have shown that the expression of one of these proteins is sufficient for the proper transport, plasma membrane localization, and expression of anion exchange activity in SFCV-BIIIR-infected CEFs. We estimate that the amount of band 3 protein expressed in these fibroblasts is similar to the abundance of native band 3 in mature erythrocytes (data not shown), judged by immunoprecipitation experiments.

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