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Segregation, viral phenotype, and proviral structure of 23 avian leukosis virus inserts in the germ line of chickens

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Summary. We have artificially introduced 23 avian leukosis virus (ALV) proviral inserts into the chicken germ line by injection of wild-type and recombinant subgroup A ALV near the blastoderm of fertile eggs just before incubation. Eight viremic males were identified as germ-line mosaics because they transmitted proviral DNA to their generation 1 (G-1) progeny at a low frequency. Eleven female and 9 male G-1 progeny carried 23 distinct proviruses that had typical major clonal proviral-host DNA junction fragments detectable after digestion of their DNA with *Sac*I, Southern blotting and hybridization with a probe representing the complete ALV genome. These proviruses, identified by their typical proviral-host DNA junction fragments, were transmitted to approximately 50% of their G-2 progeny after mating the G-1 parents to a line of chickens lacking endogenous ALV proviral inserts. One G-1 female carried 2 proviruses and another 3. The proviruses appeared to be scattered throughout the genome. One of the 14 proviruses carried by females was on the sex (Z) chromosome. Two of the 3 proviruses carried by a single G-1 female were linked with a recombination frequency of about 0.20. Twenty-one of the proviruses coded for infectious ALV. Two proviruses coded for envelope glycoprotein, and cell cultures carrying them were relatively resistant to subgroup A sarcoma virus, but failed to produce infectious ALV. One of these proviruses coded for internal *gag* proteins, had a deletion in *pol*, but produced non-infectious virus particles. The other failed to code for *gag* proteins and had no detectable internal deletions nor did it produce virus particles. Thus, we have shown that replication-competent ALV can artificially infect germ-line cells and that spontaneous defects in the inherited proviruses occur at a rather low rate.

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Introduction

In many vertebrate species DNA copies of retroviral genomes are inherited as structural host genes. These proviruses have spontaneously entered the germ line during evolution and can code for complete endogenous retroviruses, internal (*gag*) or envelope (*env*) proteins, or be unexpressed (Coffin 1982). In the mouse, under conditions where infectious endogenous virus is produced at high levels, reinfection of the germ line has been observed, sometimes at high frequencies (Jenkins and Copeland 1985; Bautsch 1986). Studies by Jaenisch et al. (1981) have shown that proviruses enter the germ line after artificial infection of early mouse embryos with murine leukemia virus (MuLV).

Retroviral proviruses that presumably enter the germ line by infection often fail to be fully expressed. Several of the avian endogenous viral (*ev*) genes have deletions in their proviral DNA that explain their inability to code for infectious virus (Hayward et al. 1980). Other *ev* genes, such as *ev*1, appear to be structurally complete but are regulated by host factors, one of which may be methylation, and they fail to produce complete virus or viral antigens (Groudine et al. 1981). Chickens that carry *ev* genes that code for infectious virus but are genetically resistant to infection with the endogenous viruses produce very low titers of virus compared to chickens that are permissive for infection (Crittenden 1974, 1979c; Smith and Crittenden 1988). Apparently the expression of the *ev* proviruses in the germ-line chromosomal location is down-regulated by cis-acting host elements, but

the proviruses are released from regulation when they are able to reintegrate in permissive somatic cells at unregulated chromosomal positions (Jenkins and Cooper 1980; Humphries et al. 1981). Rous sarcoma virus (RSV) proviral DNA can be integrated into very early chick-embryo cells but is not expressed until later in development, suggesting developmental regulation of expression (Mitrani et al. 1987). MuLV proviruses that enter the germ line after infection of early mouse embryos are often methylated and poorly expressed (Jahner et al. 1982). These observations suggest that the expression of integrated retroviral proviruses is down-regulated early in embryonic development and, in some cases, throughout the life of the animal.

The recent development of retroviruses as vectors for gene transfer to somatic or germ cells (Coffin 1985) increases the interest in developing efficient methods for introducing both replication-competent and replication-defective vectors into the germ line of domestic and experimental animals. Studies on the efficiency of insertion, the stability and integrity of the inserts and the factors regulating the expression of the viral genes and the gene of interest are crucial to the development of these methods. Our previous studies have shown that injection of live ALV, complete proviral DNA or infected cellular material into fertile eggs just before incubation leads to high rates of infection of hatched chicks. Males infected in this way, when reared to maturity and mated to uninfected hens, transmitted proviral DNA to just under 2% of their progeny. These progeny exhibited clonal junction fragments in their DNA, and some of them were shown to transmit in Mendelian fashion to their progeny (Salter et al. 1986, 1987). We now report that all 20 birds that received proviral DNA from their male parent had clonal proviral-host DNA junction fragments that were transmitted to about 50% of their progeny. The frequency of gross structural alterations and the viral phenotypes of 23 inherited proviruses are also reported.

Materials and methods

Chicken lines and experimental plan

We used line 0, a White Leghorn line that is genetically susceptible to all ALV subgroups except subgroup E and is free of all *ev* proviruses that have close homology with ALV cDNA (Astrin et al. 1979; Dunwiddie et al. 1986; L. B. Crittenden, unpublished results). Line 0 breeding stock has been maintained free of exogenous ALV infection for several generations. Salter et al. (1986) described the production of generation 0 (G-0) and G-1 chickens that were used in these studies. Briefly, high titers of wild-type or recombinant ALV, liposome-ALV preparations, infected cells or cell components were injected into the yolk of fertile line 0 eggs near the blastoderm just before incubation and the chicks hatched to produce G-0. Males infected with ALV were reared to maturity and mated with line 0 females to produce G-1 progeny. Eight of the G-0 males, after mating with line 0 females (A–H in Table 1), produced G-1 chicks that contained proviral DNA. Males A, B, E, F and G were reared from fertile eggs injected with crude nuclei from ALV-infected cells, males C and D from eggs infected with cell-free ALV, and male H from eggs injected with a liposome-ALV preparation. The positive G-1 chicks were assayed for infectious virus production at 4–6 weeks of age and again at maturity. Nine G-1 males and 11 G-1 females, positive for proviral DNA, were mated with line 0 birds to produce the G-2 progeny shown in Table 1. Selected G-2 progeny were reared to maturity and backcrossed to line 0 birds to produce G-3 progeny for virus, p27 and DNA analysis of chick-embryo fibroblasts (CEF) or intact chickens.

Sample collection

Day-old chicks were bled from the leg into heparinized capillary tubes. Approximately 80 µl of whole blood was mixed with 160 µl of phosphate buffered saline (PBS) containing 100 USP units of heparin per ml and 0.02% sodium azide, and stored in 96 well plates at 4 °C for DNA dot-blot analysis. The remaining sample was stored at −20 °C for DNA extraction and Southern-blot analysis. A second 40 µl sample was taken from each chick and stored in 160 µl of PBS with 0.25% Tween 80 for p27 assay. Heparinized whole blood or serum was collected from older chickens, rapidly cooled and stored at −70 °C until infectious ALV assays could be performed.

Virus stocks

The viruses used for infection of G-0 embryos were described previously (Salter et al. 1986). RAV-1 is a subgroup A ALV with a long terminal repeat (LTR) typical of exogenous ALV. RAV-0-A(1) is a recombinant subgroup A virus with an LTR derived from the endogenous virus RAV-0, generated by transfection of the cloned envelope gene of subgroup A into RAV-0-producing cells (Wright and Bennett 1986). 882/-16, RAV-0 was generated from molecularly cloned proviruses of the Schmidt-Ruppin strain of Rous sarcoma virus (RSV) belonging to subgroup A and from the endogenous virus, RAV-0 belonging to subgroup E. It was constructed as a replication-competent vector with a subgroup A envelope, a RAV-0 LTR and an artificial *Cla*I site for insertion of foreign genes between the envelope gene and the 3′ LTR (Hughes et al. 1986). RSV belonging to subgroups A and B were the Bryan high-titer strains with RAV-1 [BH-RSV(RAV-1)] or RAV-2 [BH-RSV(RAV-2)] as helper viruses. The virus stocks were originally obtained from P. K. Vogt and propagated on CEF that were susceptible to all subgroups except subgroup E.

Virus and p27 assay procedure

The virus assay procedure was described by Crittenden et al. (1987). One-tenth ml of thawed sample was placed on 35-mm cell-culture plates with line 0 CEF in cell-culture medium containing 2 µg per ml of DEAE-dextran. The medium was changed on days 1, 3 and 7 after infection. At day 9 after infection, the intact plates were frozen and thawed three times in the presence of 0.25% Tween-80 to release internal viral antigens. The supernatant was then assayed for the ALV *gag* protein, p27, by an enzyme-linked immunosorbant assay (ELISA). The ELISA for p27 was also used for detection of ALV antigen in heparinized whole blood stored at −20 °C or colder. The ELISA for p27 was described by Smith et al. (1979). Assays for ALV envelope expression were described by Crittenden et al. (1979a). Briefly, secondary CEF prepared from individual embryos were co-cultivated with 16Q cells, a continuous line of Coturnix quail cells infected with the envelope-defective BH-RSV (Crittenden et al. 1979a). Cell-free supernatant fluids, collected on days 3 and 5 after co-cultivation, were assayed for

Table 1. Mendelian segregation of 23 *alv* inserts and non-Mendelian transmission of ALV proviruses to G-2 backcross progeny

Virus	G-0 male	G-1 parent sex	*alv* insert no.	Molecular[a] weight	G-2[b] insert		Insert[c] minus dot-blot plus	New[d] insert
					+	−		
RAV-0-A (1)	A	M[e]	1	9.2	5	2	0	0
		M	2	12.4	15	18	0	0
		M	3	11.6	30	21	0	30
		F[f]	4	23.0	20	22	8	0
		F	5	16.0	22	19	2	1
		F	6	8.1	17	23	0	0
		F	7	23.0	12	17	5	1
RAV-0-A (1)	B	F	8	13.0	13	13	7	1
RAV-0-A (1)	C	F	9	17.5	15	12		
			10	11.1	10	17	0	0
			11	6.2	18	9		
RAV-0-A (1)	D	M	12	14.6	18	19	0	0
		F	13	7.0	19	27	7	0
		F	14	10.8	24	28	3	0
			15	9.4	24	28		
		F	16	17.1	24	18	3	0
		M	17	9.7	22	34	0	0
		M	18	10.9	19	13	0	0
882/-16, RAV-0	E	M	19	9.2	38	52	0	9
		F	20	6.9	21	11	5	0
882/-16, RAV-0	F	F	21	8.5	15	28	27	0
882/-16, RAV-0	G	M	22	10.8	25	31	1	0
RAV-1	H	M	23	20.0	13	20	0	0

[a] Approximate molecular weight (kb) of the major *Sac*I clonal junction fragment characteristic of the insert
[b] Segregation of the major *Sac*I junction fragment in the G-2 backcross progeny
[c] No. of chicks that had ALV proviral DNA but lacked any major *Sac*I junction fragment. Interpreted as congenital transmission
[d] No. of progeny with *Sac*I junction fragments that were a different molecular weight (kb) than seen in the G-1 parental DNA
[e] M – male
[f] F – female

focus formation on line 0 CEF. Assays for cellular resistance to virus infection were conducted on secondary CEF from individual embryos, by observing focus formation by ten-fold serial dilutions of pseudotypes of BH-RSV with subgroups A and B ALV.

Extraction and analysis of DNA

DNA was extracted from whole blood cells with phenol-chloroform by standard procedures (Maniatis et al. 1982). Sixty to 120 µl of blood yielded at least 100 µg of high molecular weight DNA. Restriction endonucleases were obtained from New England Biolabs, Beverley/MA. Digestions were carried out overnight under conditions recommended by the manufacturer using 3–5 units per µg of DNA. After digestion with appropriate restriction endonucleases, 5 µg of DNA were electrophoresed on 0.8% agarose gels, transferred to nylon filters (Nytran, Schliecher and Schuell, Keene/NH) and hybridized with approximately 4×10^6 cpm of nick-translated ^{32}P-labeled probe per ml of hybridization solution. The probe weas either pRAV-10, a plasmid containing the complete cDNA of RAV-1 (Sealy et al. 1983) or else a plasmid containing the complete ALV cDNA with the bacterial chloramphenicol acetyl transferase (CAT) gene inserted at the 3′ end of the *env* gene (Greenhouse et al. 1988). The conditions used were essentially those of Jenkins et al. (1982). After the filters were baked at 80 °C for 2 h, they were placed in sealable bags with 50 ml of prehybridization solution consisting of 4 × SSC (20 × SSC contains 175.2 g NaCl and 88.4 g sodium citrate per liter), 1 × Denhardt solution (100 × Denhardt solution contains 2 g ficoll, 2 g polyvinyl pyrolidone, 2 g bovine serum albumin per 100 ml), and 100 µg sonicated herring sperm DNA per ml. The bags were sealed and incubated in a shaking 65 °C water bath for 2 h. The prehybridization solution was removed and 12 ml of hybridization solution was added. The hybridization solution consisted of the prehybridization solution with 10% dextran sulfate and probe. The filters were hybridized overnight in a 65 °C shaking water bath.

The filters were rinsed once in 100 ml 3 × SSC and 0.1% SDS. They were then washed successively in a 65 °C shaking water bath for about 30 min each in 3 × SSC 1% SDS, 1 × SSC 0.1% SDS, followed by one or two washes in 0.1 × SSC, 0.1% SDS. The final wash was in 0.1 × SSC before air drying and exposure to XAR-5 X ray film (Eastman Kodak, Rochester/NY) at −70 °C with a Du Pont (Du Pont Co., Willmington/DE) Lightning Plus intensifying screen for 1–5 d.

The dot-blot procedure was modified from that published earlier (Salter et al. 1986). Eighty µl of whole blood was collected in heparinized hematocrit tubes and placed in the wells of microtiter plates in 160 µl of a solution of anticoagulant consisting of either PBS-heparin-azide or Alsevers solution. Fifty µl of a 1–60 final dilution in PBS was placed in the wells of a dot-blot apparatus and aspirated onto a nylon filter (Zetaprobe, Bio-Rad Labs, Rockville Centre/NY). The filter was then placed

on blotting paper saturated with 0.5 *M* NaOH and 1.5 *M* NaCl for 15–30 min to lyse the cells and denature the DNA, then neutralized on blotting paper saturated with 0.5 *M* TRIS pH8 and 1.5 *M* NaCl for 15–30 min, and dried and baked at 80 °C for 2 h. The filters were then hybridized to a nick-translated ^{32}P-labeled probe (1.5×10^6 cpm) under the general conditions described by Salter et al. (1986). The filters were placed in a sealable plastic bags with 20 ml of buffer per filter. The initial buffer consisted of 50 m*M* TRIS HCl, 1 *M* NaCl, 1 m*M* EDTA, 0.1% SDS and 250 µg/ml herring sperm DNA, and was incubated for 2–4 h at 42 °C. This buffer was removed and replaced with a hybridization buffer consisting of 50% formamide, 5 × Denhardt solution, 5 × SSPE (20 × SSPE contained 174 g NaCl, 27.6 g $NaH_2PO_4H_2$ and 7.4 g EDTA per liter), 0.1% SDS and 250 µg/ml herring sperm DNA. The filters were incubated for 2–4 h at 42 °C. The buffer was replaced with 5 ml per filter of fresh hybridization buffer with probe added. Hybridization took place at 42 °C for 1–2 d. The filters were washed two times in 2 × SSC, 0.5% SDS for 20 min each on a shaker and then two times in 0.1 × SSC, 0.5% SDS in a 65 °C shaking water bath for one h each. The filters were dried and exposed to film by the same procedure as the Southern blots.

Electron microscopy

Cell cultures from individual embryos were grown to confluency, scraped from the plates into phosphate buffered saline (PBS), pelleted and resuspended in cold 2.5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.2). After a buffer wash in 0.1 *M* phosphate buffer (pH 7.2), the cell pellets were embedded in agar and post-fixed in 1% osmium tetraoxide in 0.1 *M* phosphate buffer (pH 7.2) for 1 h. Following ethanol dehydration, the cell pellets were embedded in Polybed 812 (Polysciences, Warrington/PA). Thin sections were cut with an LKB Ultratome III (LKB Instruments, Gaithersburg/MD) using a Du Pont diamond knife (Du Pont, Willmington/DE) and stained with uranyl acetate and lead citrate. The sections were examined with a Philips CM-10 electron microscope (Phillips Electronic Instr., Mahwak/NJ).

Results

Mendelian segregation of ALV proviral inserts in the G-2 progeny

A summary of the segregation of 23 proviral DNA inserts in the G-2 progeny is given in Table 1. The approximate molecular weight of the major *Sac*I proviral-host DNA junction fragment for each of the 23 germ-line inserts is given in Table 1. The major *Sac*I junction fragment contains most of the viral genome including the 3′ LTR (Fig. 1).

Four RAV-0-A(1)-infected G-0 males produced 15 proviral-DNA-carrying G-1 progeny that were successfully reared to maturity and produced at least 7 G-2 progeny when mated to line 0. G-0 male C produced a single female that inherited 3 inserts (*alv*9, 10, 11). G-0 male D produced 6 G-1 progeny, 1 of which inherited 2 inserts (*alv*14 and 15).

Three 882/-16,RAV-0-infected G-0 males produced 4 G-1 progeny, each of which inherited a single insert. One RAV-1-infected G-0 male produced a single G-1 male

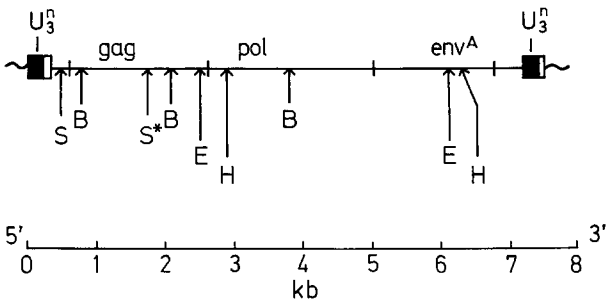

Fig. 1. Restriction enzyme map representing known cleavage sites in 882/-16, RAV-0 and those presumed to occur in the uncloned RAV-0-A(1) proviruses. The 3′ *Sac*I site designated S* is absent in 882/-16, RAV-0 and present in RAV-0-A(1). S – *Sac*I; B – *Bam*HI; E – *Eco*RI; H – *Hind*III. Adapted from Van Beveren et al. (1985)

that produced 33 G-2 progeny before dying with a massive lymphoma. This male had a single insert, but both *Sac*I proviral-host DNA junction fragments were visualized in the G-1 and G-2 progeny blots. The complete ALV proviral probe contained exogenous RAV-1 LTR sequences leading to hybridization to the homologous LTRs of the RAV-1 provirus (Salter et al. 1987). The minor *Sac*I junction fragment was not usually observed in the DNA of *alv*1 through 22 because they were generated by viruses with RAV-0 LTR sequences.

Table 1 gives a tabulation of the number of G-2 progeny from each G-1 parent that inherited or lacked the specific G-1 parental *Sac*I proviral-host DNA junction fragment. In each case the segregation approximated the 1:1 ratio expected for Mendelian segregation, assuming that each insert was hemizygous in the G-1 parent. A total of 439 G-2 progeny inherited the specific G-1 proviral-host *Sac*I DNA junction fragment and 482 lacked it. This ratio was not significantly different from 1:1 ($P \geq 0.05$) by Chi-square analysis. Even though some families had aberrant segregation ratios, the ratios showed no significant heterogeneity based on Chi-square analysis ($P \geq 0.05$).

Non-Mendelian transmission of ALV proviruses to G-2 progeny

The data summarized in Table 1 also show that some G-2 progeny lacked the G-1 parental *Sac*I junction fragments, but their DNA contained proviral sequences detected by dot-blot analysis. These progeny fell into two classes. One class contained proviral inserts that were detected by dot-blot, but no clonal junction fragment could be detected by Southern analysis after digestion with *Sac*I. In many cases these blots had a smear of DNA larger than 6 kb, that suggested insertion at many chromosomal locations in agreement with the expected integration pattern after somatic infection (Varmus and

Swanstrom 1985). All such chickens except one occurred in the progeny of G-1 females.

The other class of G-2 progeny were dot-blot positive and contained clonal *Sac*I junction fragments that were not found in their G-1 parent. These fragments occurred in about equal frequency in the same G-2 chicken with a parental junction fragment or as the only fragment. Most of the new fragments were found in the G-2 progeny of males carrying *alv*3 and 19. More than one-half of the progeny of the G-1 male carrying *alv*3 contained new provirus-host DNA junction fragments. A more detailed account of the families that acquired new inserts is published elsewhere (Crittenden and Salter 1988).

Analysis of linkage of selected inserts

Since retroviruses do not integrate at specific chromosomal sites in somatic cells or germ cells, one would expect these *alv* inserts to be widely scattered throughout the genome (Coffin 1985; Bautsch 1986). However, some of them could be genetically linked by chance.

The 14 *alv* inserts carried by G-1 females could be tested for sex-linkage in G-2 families produced by backcrossing to line 0 males, since females are the heterogametic sex in birds (Table 1). The G-1 female parent that carried *alv*4 produced 34 progeny that were classified by sex. All 16 males carried *alv*4 and all 18 females lacked it, a result compatible with sex-linkage. None of the other 13 inserts segregated with sex in G-2 backcross families, showing that *alv*4 was the only gene of this group carried on the Z chromosome.

Table 1 shows that 2 G-1 females carried more than one *alv*. Therefore, we could determine if the integration events occurred in the same chromosomal region by joint segregation analyses of backcross families. Inserts *alv*9, 10 and 11 occurred in the same G-1 female, and the joint segregations of *alv*9 and 10, 9 and 11, and 10 and 11 are given in Table 2. The 2 parental and 2 recombinant *alv* genotypes could be clearly assigned to each bird by Southern analysis of *Sac*I-digested DNA using a complete ALV proviral probe to detect the provirus-host DNA junction fragment typical for the insert. We tabulated the genotypes in the single G-2 family produced by this bird backcrossed to line 0. Since these data suggested linkage of *alv*9 and 11, we also produced G-3 backcross matings to provide more data for the joint segregation analysis presented in Table 2. Only *alv*9 and 11 were shown to be linked. There were 75 individuals that either carried or lacked both *alv*9 and 11, and 18 individuals that carried either *alv*9 or 11 alone. Inserts *alv*14 and 15 also occurred in a single G-1 female, but their joint segregation in 52 progeny did not reveal linkage (Table 2). Chi-square analysis showed that there was no significant deviation from the expected 1:1 ratio in the individual segregation of the 5 *alv* genes. Only *alv*9 and 11 showed a significant deviation from independent segregation. The estimate of recombination frequency was 0.2.

Phenotypic analysis of 23 inserts

All G-1 progeny that were dot-blot positive at 1 day of age were assayed for virus at 4–6 weeks of age and again at sexual maturity. All were positive for infectious virus except for the female parent of *alv*6 (Salter and Crittenden 1989). Blood from this bird and from all her G-2 progeny were negative for p27. We report in a compan-

Table 2. Joint segregation analysis to detect linkage among *alv* inserts that were carried by the same G-1 parent

alv pair	Generation[a]	Gametes				Total	Chi square[b]	Probability
		parental		recombinant				
		+/+	−/−	+/−	−/+			
9, 10	G-2	7	9	8	3	27		
	G-3	10	8	7	6	31		
	total	17	17	15	9	58	0.62	≥0.05
9, 11	G-2	14	8	1	4	27		
	G-3	26	27	6	7	66		
	total	40	35	7	11	93	34.93	≤0.01
10, 11	G-2	7	6	3	11	27		
	G-3	10	9	6	6	31		
	total	17	15	9	17	58	0.62	≥0.05
14, 15	G-2	8	12	16	16	52	2.76	≥0.05

[a] G-2 represents the first generation backcross of the single G-1 parent to line 0
G-3 represents the backcross of G-2 individuals carrying both *alv* inserts to line 0

[b] Chi square was calculated on the total segregation accumulated from G-2 and G-3 families. In each case the segregation of the individual *alv* insert did not differ significantly from 1:1

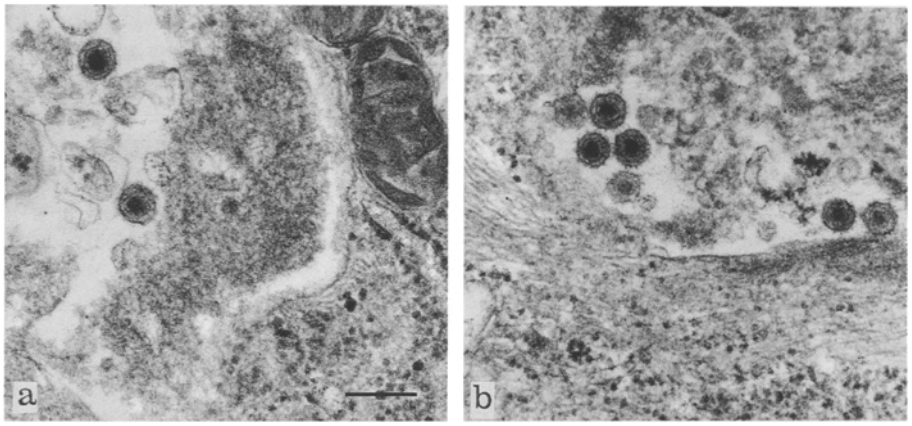

Fig. 2a and b. Electron microscopy of *alv*9 **a** and *alv*11 **b** cell cultures. Retroviral particles are clearly visible. The *bar* represents 200 nanometers

Table 3. Relative resistance of chick embryo fibroblasts (CEFs) carrying *alv* 6 and 11 that fail to produce complete ALV but express envelope protein to the subgroup A and B avian sarcoma viruses, BH-RSV (RAV-1) and BH-RSV (RAV-2)

Virus		*alv*				Mean[a]
		6	11	11	11	line 0
BH-RSV (RAV-1)	titer[b]	4.0×10^1	1.7×10^5	3.4×10^3	1.6×10^3	3.6×10^6
	relative titer[c]	0.000011	0.047222	0.000944	0.000444	1.000000
BH-RSV (RAV-2)	titer	4.9×10^5	3.1×10^6	6.6×10^5	8.1×10^5	1.6×10^6
	relative titer	0.306250	1.900000	0.412500	0.506250	1.000000

[a] Mean titer on three separate CEFs
[b] Titers per ml of virus stock were estimated by averaging the focus counts of the plates with the two highest dilutions that had foci
[c] Ratio of titer on the test embryo to the titer on line 0 CEF

ion paper that *alv*6-carrying CEF express subgroup A envelope glycoproteins and are resistant to focus formation by subgroup A but not subgroup B avian sarcoma virus, presumably due to specific interference (Salter and Crittenden 1989). Since 2 G-1 birds carried more than 1 *alv* gene, we could not evaluate the phenotypes coded by *alv*9, 10 and 11, or *alv*14 and 15 individually, until they were segregated by backcrossing these individuals to line 0 to produce G-2 or G-3 progeny carrying only 1 insert each. Assays for infectious virus were conducted on these birds, and it was found that *alv*9, 10, 14 and 15 coded for infectious ALV. Birds carrying *alv*11 failed to produce detectable ALV, but their blood cells were positive for ALV p27 by the ELISA assay.

To determine if CEF carrying *alv*11 produced envelope antigen and were resistant to subgrouip A avian sarcoma virus, we mated G-2 individuals carrying *alv*9 and 11 to line 0 and made primary CEF from individual 11-day-old embryos that were frozen in DMSO-containing medium for future use. DNA was extracted from the tissues of each embryo and analyzed for the presence of *alv*9 and 11. Individual embryo CEF that carried *alv*11 alone were thawed for assays to determine if they expressed envelope and were resistant to BH-RSV(RAV-1) (subgroup A) or BH-RSV(RAV-2) (subgroup B). Table 3 shows the embryos assayed. We used CEF carrying *alv*6, known to be virus-negative and envelope-positive, and line 0 CEF as controls. Assays for infectious ALV showed that CEF carrying *alv*6 or 11 and line 0 CEF did not produce ALV. All the CEF except line 0 produced high titers of sarcoma virus that produced foci on line 0 CEF after co-cultivation with 16Q cells (data not shown). This demonstrated that biologically active envelope glycoprotein was present in the cell membranes. The relative titers of subgroup A and subgroup B sarcoma viruses on the CEF carrying these *alv* genes are shown in Table 3. Each culture carrying 1 of the *alv* genes was relatively resistant to subgroup A, but showed no substantial resistance to subgroup B (Vogt and Ishizaki 1966). However, the CEF carrying *alv*6 were more resistant to subgroup A than those carrying *alv*11.

Electron microscopy was conducted on CEF carrying *alv*6, 9 and 11. Figure 2 shows selected micrographs. As expected, cells carrying *alv*9 that produced infectious vi-

Table 4. ALV expression phenotype and structural analysis of internal fragments generated by three restriction enzymes for 23 *alv* proviruses

alv no.	Infecting virus	Viral phenotype				Internal fragments		
		virus	p27	*env*[a]	EM[b]	*Sac*I	*Bam*HI	*Eco*RI
1	RAV-0-A (1)	+	+			−	normal	normal
2	RAV-0-A (1)	+	+			+	normal	normal
3	RAV-0-A (1)	+	+			+	normal	normal
4	RAV-0-A (1)	+	+			+	normal	normal
5	RAV-0-A (1)	+	+			+	normal	normal
6	RAV-0-A (1)	−	−	+	−	−	normal	normal
7	RAV-0-A (1)	+	+			−	normal	normal
8	RAV-0-A (1)	+	+			+	normal	normal
9	RAV-0-A (1)	+	+	+	+	+	normal	normal
10	RAV-0-A (1)	+	+			+	normal	normal
11	RAV-0-A (1)	−	+	+	+	+	altered	altered
12	RAV-0-A (1)	+	+			+	normal	normal
13	RAV-0-A (1)	+	+			+	normal	normal
14	RAV-0-A (1)	+	+			+	normal	normal
15	RAV-0-A (1)	+	+			−	normal	normal
16	RAV-0-A (1)	+	+			+	normal	normal
17	RAV-0-A (1)	+	+			+	normal	normal
18	RAV-0-A (1)	+	+			+	normal	normal
19	882/-16, RAV-0	+	+			−	normal	normal
20	882/-16, RAV-0	+	+			−	normal	normal
21	882/-16, RAV-0	+	+			−	normal	normal
22	882/-16, RAV-0	+	+			−	normal	normal
23	RAV-1	+	+			−	normal	normal

[a] Envelope assays were only conducted on CEF carrying *alv*6, 9 and 11
[b] Electron microscopy for observation of virus particles was only conducted on CEF carrying *alv*6, 9 and 11

rus had ALV particles. A similar survey of *alv*6-carrying cells failed to reveal virus particles, and *alv*11-carrying cells produced ALV particles.

A summary of the viral phenotypes coded for by all 23 *alv* genes is given in Table 4. Envelope assays were not conducted on CEF carrying each insert because it is known that envelope glycoproteins would be present in any CEF producing complete ALV.

Structural analysis of 23 inserts

In order to detect gross structural alterations that may have occurred during the process of germ-cell infection and proviral integration, we digested DNA from the G-0, G-1 and representative samples of G-2 or G-3 birds with three restriction enzymes that would detect internal alterations in the proviral DNA representing each of the 23 inserts. Figure 1 shows the restriction enzyme map of a typical ALV, with the sites for cleavage with *Sac*I, *Bam*HI, *Hin*dIII and *Eco*RI (Van Bevern et al. 1985). The *Sac*I site closest to the 3′ end of the provirus, typical of the endogenous virus RAV-0, is present only in RAV-0-A(1). *Sac*I digestion of the DNA from the 4 RAV-0-A(1) G-0 males that transmitted to G-1 showed that they all had the internal 1.4-kb fragment typical of RAV-0, but none of the G-0 males that was infected with 882/-16,RAV-0 or RAV-1 had that fragment in their DNA. Of the 15 G-1 progeny produced by the RAV-0-A(1)-infected G-0 males that carried proviral inserts, the 3 that carried *alv*1, 6 and 7 lacked the internal *Sac*I fragment. Analysis of DNA from G-2 or G-3 progeny showed that *alv*15 also lacked this fragment, but its absence could not be detected in the G-1 parent due to the presence of *alv*14; Table 4 summarizes these results. Figure 3 shows a blot of the DNA from the G-0 parent of *alv*14 and of the G-2 progeny that carried *alv*14 or 15 either alone or together. The 1.4-kb internal *Sac*I fragment is present in the G-0 and G-1 parental DNA, but missing in the DNA from the G-2 progeny carrying *alv*15. In order to determine the extent of the structural defect, we also digested the DNA with *Bam*HI and both *Sac*I and *Bam*HI. The double digestion lanes representing the G-0 and G-1 parents and the G-2 bird carrying *alv*14 and 15 showed that the smaller of the internal *Bam*HI fragments was altered, giving both a typical fragment and a smaller fragment, while no alteration was found in the sample from the G-2 progeny carrying *alv*15, and only the smaller 1.1-kb fragment was found in the *alv*14-carrying G-2 progeny. Therefore, it appears that at least some of the proviruses in this G-0 parent contained the 3′ *Sac*I site and that this site was missing in the G-2 *alv*15-carrying progeny, because the smaller

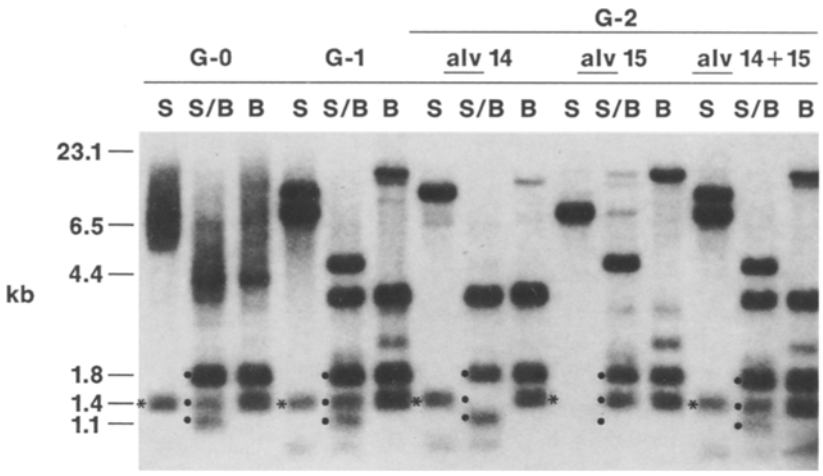

Fig. 3. Southern blot analysis of DNA from the G-0, G-1 parents, and representative G-2 progeny from chickens carrying *alv*14 and *alv*15 alone and both *alv*14 and 15. DNAs were digested with *Sac*I (S), *Bam*HI (B), or double digested with *Sac*I and *Bam*HI (S/B) and the blots were hybridized with a complete ALV cDNA probe. The *star* represents the location of the 1.4 kb-internal *Sac*I fragment. The locations of 1.8-kb and 1.4-kb internal B fragments and the 1.1-kb S/B fragment expected if the 3′ *Sac*I site is present are indicated by a *dot*

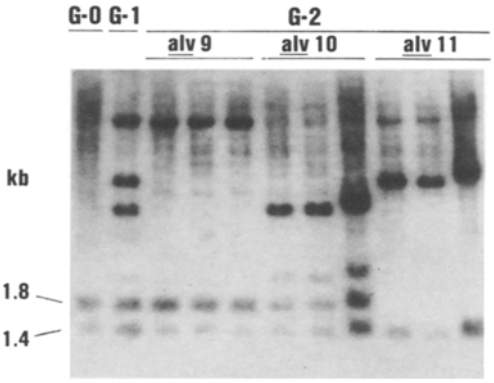

Fig. 4. Southern blot analysis of G-0, G-1, and three representative DNA samples from G-2 progeny carrying *alv*9, 10 and 11 respectively. DNA was digested with *Bam*HI and the blots were hybridized with a complete ALV cDNA probe. The locations of the 1.8- and 1.4-kb internal *Bam*HI fragments are indicated

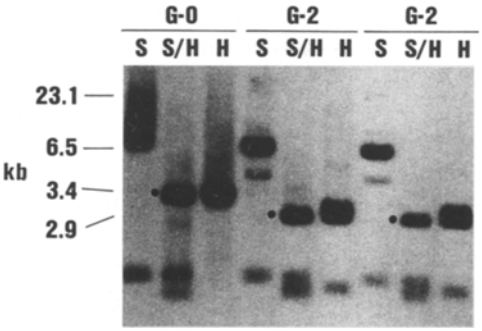

Fig. 5. Southern blot analysis of DNA from the G-0 parent and two representative G-2 progeny carrying *alv*11. DNAs were digested with *Sac*I alone (S). *Sac*I and *Hind*III (S/H), and the *Hind*III alone (H) hybridized with a complete ALV cDNA probe. The *dots* represent the internal *Hind*III fragment that is approximately 3.4 kb in the G-0 parent but 2.9 kb in the G-2 progeny

*Bam*HI fragment was unaltered with double digestion. Blots of the DNA from male A (Table 1) and from G-2 birds carrying *alv*1, 6 and 7 also showed that the 3′ *Sac*I site was missing from these inserts.

We also digested representative samples of DNA from the G-0, G-1 and G-2 of all the *alv*s with *Bam*HI and *Eco*RI, to determine if there were any gross alterations in the expected internal fragments. All the samples except *alv*11 had identical internal fragments typical of these enzymes (Table 4). The *Eco*RI digestions of *alv*23 produced the two expected additional internal fragments, because RAV-1 has one site for this enzyme in each LTR. Figure 4 shows a *Bam*HI digest of the three generations of birds carrying *alv*9, 10 and 11 that all occurred in the same female G-1 parent. The larger internal *Bam*HI fragment was missing from the *alv*11 DNAs that segregated out in G-2. This indicates that the *Bam*HI site located in the middle of *pol* is missing in *alv*11 (Fig. 1). Digestion of *alv*11 DNA with *Eco*RI showed that the large internal fragment was reduced in size. Figure 5 shows DNA digestions from the G-0 parent (C, Table 1) and *alv*11 G-2 progeny with *Hind*III, *Sac*I and both enzymes. The internal *Hind*III fragment that is best revealed by double digestion with *Hind*III and *Sac*I was clearly smaller in the G-2 DNA samples. The reduction in size of 0.5 kb is consistent with that seen in the *Eco*RI digests and suggests that *alv*11 has a deletion of about 0.5 kb in the middle of the *pol* gene.

Discussion

We have shown that infection of early chicken embryos with replication-competent wild-type and recombinant ALV can lead to germ-line insertion and Mendelian inheritance of proviral DNA. Insertion in the germ cells of the G-0 males was clearly chimeric because less than 2% of their G-1 progeny carried proviral DNA (Salter et al.

1986). The provirus-positive G-1 progeny were not chimeric and were hemizygous for each of the 23 inserts. Their diploid germ cells each carried a single copy of each insert since their progeny, after mating to line 0, gave the expected 1:1 ratio of parental provirus-positive and -negative progeny. *Sac*I digestion and Southern blotting of G-1 and G-2 DNA showed that the major provirus-host junction fragment was the same molecular weight in parents and provirus positive progeny, but varied among families (Table 1). This confirmed that each of the 23 proviruses was stably inherited at a specific chromosomal location. Stability was further confirmed for some of the inserts because the expected segregation continued into G-3.

The inserts appeared to be distributed at many chromosomal sites, as would be expected since retroviruses are not known to have specific sites of integration (Varmus and Swanstrom 1982). *alv*4 was located on the Z chromosome, the fifth largest chromosome (Somes 1984). We could only test the 14 proviruses that were carried by G-1 females for sex-linkage. Therefore, some of the nine inserts carried by G-1 males may also have been on the Z chromosome, but sex-linkage could not be detected because females are the heterogametic sex in birds. *alv*9 and 11 are linked and have a frequency of recombination of about 20%, suggesting that they are probably both on one of the larger autosomes (Table 2). Table 1 also shows that there were a number of provirus-positive G-2 progeny that could not be explained by Mendelian inheritance of proviral DNA located at specific chromosomal sites. Sixty-eight G-2 progeny were positive for proviral DNA by dot-blot but lacked clonal provirus-host DNA junction fragments, suggesting that they were somatically infected. The fact that the G-2 chicks were bled on the day of hatch for dot-blot analysis suggests that congenital rather than contact transmission accounted for the infection. Sixty-seven out of 195 clonal junction fragment negative progeny from female G-1 parents and only 1 out of 210 clonal junction fragment-negative progeny from male G-1 parents were dot-blot positive and presumably congenitally infected. This agrees with the fact that congenital transmission by males has not been observed in previous studies (Rubin et al. 1961; Spencer et al. 1980). The one infected chicken attributed to congenital transmission from its sire may have been due to an error in pedigree identification. These data support our original hypothesis that male transmission of proviral DNA is very strong evidence for genetic transmission (Salter et al. 1986).

Table 1 shows that G-1 males hemizygous for *alv*3 and 19 were clearly germ-cell chimeras for new inserts. The G-1 female parents of *alv*5, 7 and 8 each produced one new insert which could have been attributed to pedigree error. However, other studies have shown that females can also be chimeras for new inserts (Crittenden and Salter 1989). We think that new inserts are acquired by reinfection of germ cells and integration at new genomic locations. Jenkins and Copeland (1985) and Bautsch (1986) have shown that reinfection of the mouse germ line by endogenous murine viruses can take place at high rates. In contrast to our results, proviral mobility is only observed in the progeny of female mice, and Jenkins and Copeland (1985) have evidence that the ovum is infected in the ovary. It is interesting that only 2 of our 23 inserts showed a high frequency of reinsertion. Presumably the virus coded for by these inserts is germ-cell tropic or is expressed at high titers in cells adjacent to the germ cells during development. These properties could either be due to the unique structure of the insert or to its location in the host genome.

The results summarized in Table 4 clearly show that *alv*6 and 11 do not code for complete virus. The probability that chickens carrying the other inserts could have been congenitally or horizontally infected, and more of the inserts are defective, appears to be low. The G-1 progeny that were viremic at 4–6 weeks of age were also viremic as adults, suggesting that they were immunologically tolerant. Tolerance is usually induced by natural or artificial exposure of the embryo to ALV before hatching (Rubin et al. 1961; Spencer et al. 1980). Since congenital transmission from males is extremely low, it seems most likely that the G-1 embryos were infected by virus produced by the *alv* insert that they carried. The fact that 9 out of 10 of the viremic G-1 females transmitted congenitally to their progeny confirms that they carried inserts that coded for complete virus.

Extensive data have shown that *alv*6 fails to produce infectious ALV or p27, but does express envelope glycoprotein in the cell membrane that interferes with subgroup A ALV infection both in CEF and *in vivo* (Salter and Crittenden 1989). In the present study, we have shown that *alv*11 also fails to produce complete virus, but does produce p27 and envelope glycoprotein. As shown in Table 3, CEF-carrying *alv*11 are not as resistant to focus formation by subgroup A sarcoma virus as CEF-carrying *alv*6. The resistance of the single *alv*6-carrying embryo in this study was of a similar order of magnitude as that of the 5 *alv*6-carrying embryos that were evaluated in a companion paper (Salter and Crittenden 1989). One *alv*11-carrying embryo only showed 20-fold resistance, suggesting variability of expression of *alv*11. These findings parallel the studies of the defective endogenous viral (*ev*) genes *ev*3 and 6 (Robinson et al. 1981). Neither *ev* gene codes for complete virus, and both express envelope antigen. Only *ev*3 codes for p27, but *ev*6 codes for higher levels of envelope and is more resistant to infection with subgroup E viruses than is *ev*3. While the defects are different in the two systems, we have artificially duplicated the events that occurred in nature using subgroup A viruses (Hayward et al. 1980).

Lack of virus production could be due to structural alterations in the provirus or to some host-related regulatory mechanism such as position effect or methylation (Groudine et al. 1981; Jahner et al. 1982; Jenkins and Copeland 1985). We compared the internal proviral fragments, after *Sac*I, *Bam*HI and *Eco*RI digestion of DNA from the G-0 parent that was viremic with the original infecting virus to the G-1 birds, and to a representative sample of the provirus positive G-2 chickens. Such a comparison should detect gross alterations located in the coding regions of the genome. RAV-0-A(1)-infected G-0 chickens have the internal *Sac*I fragment typical of RAV-0 (Fig. 1). However, the double digestions of DNA of such birds with *Sac*I and *Bam*HI shown in Fig. 3 revealed two low molecular weight fragments: the expected 1.1-kb fragment when both *Sac*I sites are present and the uncleaved 1.4-kb internal *Bam*HI fragment expected if the 3′ *Sac*I site were absent. Only the smaller 1.1-kb *Sac*I-*Bam*HI fragment would have been expected if the original virus stock uniformly contained virions with the two *Sac*I sites found in RAV-0. Therefore, we think that the RAV-0-A(1) stock may have been heterogeneous for the 3′ *Sac*I site and that *alv*1, 6, 7 and 15 originated from germ-line infections with virions that lacked the 3′ *Sac*I site, rather than through a deletion event that occurred during the process of germ-cell infection. Only in the case of *alv*6 was the absence of the second *Sac*I associated with lack of virus production. These data, and the fact that many replication-competent viruses lack this site, suggest that the loss of the *Sac*I site alone does not account for the lack of virus production by *alv*6 and that this site is not crucial for virus replication or infectivity.

The large deletion in *pol* found in *alv*11 probably did originate during the process of germ-line infection (Fig. 4 and 5). Electron micrographs showed that *alv*11 codes for virus particles. This observation suggests that the only defect in this provirus is in its ability to code for functional reverse transcriptase. No gross structural alterations in the *alv*6 provirus were found, but electron microscopy failed to reveal ALV particles. We cannot determine if the lack of particle production by this provirus has a structural basis or if *alv*6 is down-regulated by cis-acting host elements, but the fact that no particles were observed suggests a packaging failure. The fact that only 1 out of 23 inserted proviruses had a gross structural rearrangement suggests that such rearrangements are relatively rare. We have assayed for virus production in approximately 40 more progeny that carry only newly integrated proviruses from males that carry mobile *alv* genes. We found that they all produce complete virus, suggesting that the frequency of alteration or suppression is very low (L. B. Crittenden, unpublished results).

The ease with which we were able to infect the germ line with subgroup A ALV, and the relative structural integrity of the artifically introduced ALV genes, raises several questions about the natural process of germ-line acquisition of avian retroviruses and the effect of natural and artificial selection on their observed frequency. If subgroup E viruses are also stable on initial germ-line insertion, then the low proportion of *ev* genes that are found in chicken populations that produce complete virus must be due to selection against virus-producing *ev* genes (Smith 1986). Such genes appear to be relatively innocuous, except that they are known to induce partial immunological tolerance to exogenous infection with pathogenic ALV (Crittenden et al. 1987; Smith and Fadly 1988). However, there may be subtle detrimental influences of *ev* genes producing complete virus on the fitness of chickens under intense selection for egg production (Crittenden et al. 1979b). The prevalence of subgroup A ALV in chicken populations and the fact that embryos are infected at an early age by congenital transmission increase the possibility that germ-line insertion of these pathogenic viruses does take place spontaneously in breeding populations of chickens. One would expect that insertion of a complete pathogenic virus would be severely selected against, but it is surprising that during the course of evolution, a defective gene expressing the subgroup A envelope was not produced. Therefore, either the expression of the subgroup A envelope or the germ-line insertion of the exogenous LTRs themselves may be selected against.

These studies demonstrate that replication-competent ALV vectors can infect the germ line and, therefore, may potentially be used to introduce foreign genes. They also demonstrate that rare spontaneous defects can occur during integration that prevent virus production but do not necessarily obviate gene expression. However, the defects observed in virus replication occurred by unknown mechanisms, and the chance of genetic exchange with endogenous and exogenous ALV is high. Therefore, the development of efficient replication-defective vectors and techniques for introducing them into the germ line of chickens should have a high priority.

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