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Artificial insertion of a dominant gene for resistance to avian leukosis virus into the germ line of the chicken

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Summary. This report describes the unique biological properties of a transgenic chicken line that contains a defective avian leukosis virus (ALV) proviral insert that we call *alv*6. Chick embryo fibroblasts (CEF) containing this insert express subgroup A envelope glycoprotein since they yield focus-forming pseudotype virus when co-cultivated with transformed quail cells expressing envelope-defective Bryan high-titer Rous sarcoma virus (RSV). In addition, these cells display high interference to subgroup A RSV but not to subgroup B RSV infection. Chickens containing this insert are highly resistant to pathogenic subgroup A ALV infection, but show little immunological tolerance to subgroup B ALV infection. Thus we have artificially inserted a dominant gene for resistance to avian leukosis infection into the chicken germ line.

Key words: Transgenic chicken – Avian leukosis virus – Avian leukosis resistance – Defective ALV – Recombinant avian retrovirus

Introduction

The initial steps in the infection of chicken cells by avian leukosis virus (ALV) are the attachment of the retrovirus envelope glycoprotein to cell membrane receptors and the transport of virion contents to the cytoplasm. Reverse transcription and integration of the retrovirus genome into the chicken chromosomes near the c-*myc* proto-oncogene can lead to its enhanced expression (Varmus 1988) with subsequent induction of lymphoid leukosis (a B cell lymphoma usually caused by an enhanced c-*myc* expression) and other neoplasms (Fadly 1986). ALV infection also results in significant loss of productivity in mature chickens (Gavora et al. 1980; Spencer 1984).

The specificity of binding or penetration of the virion is determined by the viral envelope glycoprotein. This specificity has been used to classify ALV into different subgroups by a phenomenon called interference. Retroviruses are prevented from infecting chicken cells that were previously infected with the same subgroup by specific physical inhibition of viral adsorption to receptors or viral penetration.

Single recessive genes for resistance to infection by each subgroup of ALV exist in chickens, but the frequency of resistance to subgroup A, the most common field virus, is low in egg-producing strains, and few resistant commercial strains have been developed (Crittenden 1983). We proposed a method for providing resistance to subgroup A ALV based on an endogenous ALV model of interference (Crittenden and Salter 1986). CEF expressing subgroup E envelope glycoprotein coded for by the defective endogenous proviral genes, *ev*3 and *ev*6, are significantly more resistant to subgroup E Rous sarcoma virus (RSV) infection than CEF lacking these genes (Robinson et al. 1981). Furthermore, chickens carrying *ev*3 and *ev*6 are resistant to subgroup E ALV infection (Robinson et al. 1981). Thus, the insertion of the subgroup A ALV envelope gene into the germ line of chickens and its subsequent expression could also provide resistance to infection by ALV through interference with virus binding or penetration (Steck and Rubin 1966; Vogt and Ishizaki 1966).

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We have described the insertion of avian leukosis proviral DNA into the germ line of the chicken using recombinant and wild-type subgroup A ALV (Salter et al. 1986; Salter et al. 1987). By injecting infectious retrovirus into the yolk of fertile eggs near the developing embryo at the start of incubation and by testing the resulting viremic males for genetic transmission of proviral DNA to their progeny, we conclusively showed that proviral DNA had been inserted into the chicken germ line (Salter et al. 1987). Of 23 different germ-line inserts, two (*alv*6 and *alv*11) did not express infectious virus. Insert *alv*11 expresses the group-specific antigen (*gag* protein) and subgroup A envelope glycoprotein of ALV (Crittenden et al. 1989). We report here that CEF containing the *alv*6 proviral insert express the subgroup A envelope glycoprotein as measured by envelope expression and interference assays. Chickens containing the *alv*6 proviral insert are highly resistant to infection by pathogenic subgroup A ALV.

Materials and methods

Chicken lines

A transgenic chicken line containing the *alv*6 defective proviral insert was briefly described in the companion paper (Crittenden et al. 1989). Details on the production of transgenic chickens are given in Salter et al. (1986), Salter et al. (1987) and Crittenden et al. (1989). We used line 0, a White Leghorn line that is genetically susceptible to all ALV subgroups except subgroup E (C/E), and is free of all endogenous proviruses that have close homology with ALV proviral DNA (Astrin et al. 1979; L. B. Crittenden unpublished). Line 0 breeding stock is maintained free of ALV in the Regional Poultry Research Laboratory (RPRL) specific-pathogen-free (SPF) facility (Crittenden et al. 1979b).

Retroviruses

The source of subgroups A and B Rous sarcoma virus (RSV) was given in the companion paper (Crittenden et al. 1989). RPL-42 is a field strain of subgroup A ALV (Fadly and Okazaki 1982) used extensively as a model ALV for experimental studies in avian leukosis at RPRL. RAV-2 is a subgroup B ALV and has been described by Hanafusa (1965).

Cell culture, virus, antibody and p27 ELISA assay procedures

The procedures used for cell culture, virus and p27 (*gag*) protein ELISA assays were given in the companion paper (Crittenden et al. 1989). Antibody to ALV was determined as described by Crittenden et al. (1987).

In vitro experimental plan

Envelope expression assay. Two sires hemizygous for the *alv*6 proviral insert were mated to line 0 females and CEF were prepared from individual 11-day-old embryos. The presence of *alv*6 proviral inserts was determined by *Sac*I restriction enzyme digestion of whole embryo DNA, agarose gel electrophoresis, capillary transfer to nylon membranes and hybridization with radio labelled proviral DNA, as detailed in the accompanying paper (Crittenden et al. 1989). CEF (4×10^5 cells per 35 mm plate) were co-cultivated with 16 Q quail cells (2×10^5 cells per 35 mm plate), a cell line from *Coturnix* that is transformed by the envelope-defective Bryan high-titer Rous sarcoma virus and produces particles that lack the envelope glycoprotein (Murphy 1977). The CEF spontaneously fuse with 16Q cells and, if they are expressing envelope glycoprotein, yield focus-forming pseudotype virus that can infect susceptible CEF and form foci. Media were collected after 3 and 5 days and assayed in duplicate for focus-forming virus on C/E (line 0) CEF (Crittenden et al. 1979a).

Interference assay. A portion of the same CEF described in the last section (5×10^5 cells per 35 mm plate) was infected with ten-fold dilutions of subgroup A [BH-RSV(RAV-1)] or subgroup B [BH-RSV(RAV-2)] virus and foci were counted after 7 days.

In vivo experimental plan

Interference. Four *alv*6 hemizygous males were mated with SPF line 0 females. The presence of *alv*6 in progeny chicks was determined by a dot-blot procedure on whole blood collected from chicks at hatching (Crittenden et al. 1989). Chicks were injected intra-abdominally with 10^4 iu RPL-42 (field strain of subgroup A ALV) at hatching and then reared intermingled in two separate isolators. Sera were collected from random samplings at 2, 7, 16 and 40 weeks, and the presence of subgroup A ALV and antibody to subgroup A ALV was determined by standard procedures. Pathogenicity due to ALV was recorded from 2 through 40 weeks of age.

Tolerance. Progeny chicks from a similar mating described in the previous section were injected intra-abdominally with 10^5 iu RAV-2 (subgroup B ALV) on day 7 after hatching and then reared intermingled in two separate isolators. Sera were collected at 7 and 16 weeks from random samplings, and the presence of subgroup B ALV and antibody to subgroup B ALV was determined by standard procedures. Pathogenicity due to ALV was recorded from 2 through 30 weeks of age.

Defectiveness of proviral insert

Progeny chicks from a similar mating described above were reared intermingled in two separate isolators. Sera were collected at 40 weeks and the presence of ALV and antibody to ALV was determined by standard procedures. Pathogenicity due to ALV was recorded throughout the experiment.

Results and discussion

Description of alv6

The *alv*6 proviral locus is one of 23 recombinant ALV proviral DNA inserts that were artificially introduced into the germ line of line 0 chickens (Crittenden et al. 1989). It was originally detected as a dot-blot-positive progeny of SPF line 0 females and a viremic line 0 male made tolerant to a recombinant ALV, RAV-0-A(1) (Wright and Bennett 1986), by injection of retrovirus near the developing embryo just before the first day of incubation (Salter et al. 1986). Further research showed that, unlike the remainder of the transgenic chickens, the blood of this female progeny was negative for infectious ALV and the group-specific antigen (*gag*) protein (Salter et al. 1987). Gross structural analysis of *alv*6 proviral

DNA in the companion paper (Crittenden et al. 1989) revealed no major alterations. Both 5′ and 3′ long terminal repeats and normal size proviral internal fragments from *Bam*HI and *Eco*RI were present in the restricted DNA. However, one of the two *Sac*I restriction enzyme sites was missing, but this may be due to a heterogenous mixture of recombinant retroviruses in the virus stock used to produce the transgenic chickens (Crittenden et al. 1989).

In vitro envelope expression

Although the *gag* specific (p27) protein was not detected in the blood of chickens carrying the *alv*6 locus, we sought to determine if *env* was expressed. It is known that the *env* gene product is translated from a spliced mRNA different from the *gag* or *pol* gene products (Coffin 1985). Thus, even if there is a deletion or mutation in the *gag* or *pol* genes, transcription and translation of *env* may still occur. In the envelope expression assay shown in Table 1, only those CEF containing the proviral insert, *alv*6, complemented the *env* defect in BH-RSV to produce pseudotype focus-forming virus on C/E cells. Cocultivation of Line 0 (control) CEF and CEF lacking *alv*6 with 16Q quail cells produced little or no focus-forming virus.

In vitro interference

If subgroup A envelope is expressed, then one would expect that specific inhibition of focus formation should occur when CEF containing the *alv*6 insert are incubated with subgroup A RSV. Little inhibition should occur when similar CEF are incubated with other RSV subgroups. In the interference assay shown in Table 1, CEF carrying the *alv*6 defective proviral insert are highly resistant to focus formation by BH-RSV(RAV-1) (subgroup A) infection but not to BH-RSV(RAV-2) (subgroup B). Half-sibling CEF lacking *alv*6 and line 0 (control) CEF were highly susceptible to subgroup A and B viruses. Based on the differences in the number of foci in the interference assay in Table 1, CEF containing the *alv*6 insert are over 3,000-fold more resistant to infection by subgroup A sarcoma virus than CEF lacking *alv*6. This is about the same degree of resistance ascribed to CEF containing the *ev*6 locus that expresses subgroup E envelope glycoprotein and blocks subgroup E RSV infection (Robinson et al. 1981).

In vivo interference

We next asked if the expression of subgroup A glycoprotein could protect chickens from infection by pathogenic

Table 1. Envelope glycoprotein expression and subgroup A and B RSV interference in chicken embryo fibroblasts (CEF) carrying a defective proviral insert (*alv*6)

Sire	*alv*6 CEF	Envelope expression assay	Interference assay					
		No. of foci on C/E CEF[a]	No. of foci on C/E CEF					
			BH-RSV(RAV-1)[b]			BH-RSV(RAV-2)		
			10^{-0}	10^{-1}	10^{-2}	10^{-0}	10^{-1}	10^{-2}
857	−[c]	0[d]	C[e]	C	846	C	C	344
	+	443	28	2	0	C	C	572
	+	466	20	8	0	C	C	402
	−	0	C	C	818	C	C	526
	−	0	C	C	756	C	C	286
	−	0	C	C	1,024	C	C	446
858	−	0	C	C	768	C	C	292
	+	373	4	0	0	C	C	364
	+	414	20	4	0	C	C	486
	−	0	C	C	666	C	C	330
	+	458	28	2	0	C	C	350
	−	0	C	C	1,100	C	C	290
Control line 0		0	C	C	900	C	C	380

[a] C/E: susceptible to all ALV subgroups except subgroup E
[b] 10^{-0}, 10^{-1} ...: virus dilutions
[c] +, −: CEF that contains or lacks the *alv*6 proviral insert
[d] Average of duplicate plates
[e] C: Confluent

Table 2. Interference with subgroup A ALV infection and oncogenicity in transgenic chickens carrying the defective proviral insert (*alv6*)

Progeny	Viremia[a]				Antibody[a]			Lymphoid leukosis[a]
	Age (weeks)				Age (weeks)			
	2	7	16	40	7	16	40	
alv6 +	0/36	0/24	0/27	0/27	0/24	0/27	0/27	0/36[b]
alv6 −	39/39	20/23	23/25	0/1	1/23	3/25	1/1	22/39[c]

[a] Ratio of positive/total observed
[b] 9 out of 36 died with no evidence of neoplams
[c] 16 out 39 died with no evidence of neoplasms but 4 out of the 16 had bursa-thymus atrophy characteristic of ALV pathogenicity (Fadly 1986)

Table 3. Lack of tolerance to subgroup B ALV infection in transgenic chickens carrying a defective proviral insert (*alv6*)

Progeny	Viremia[a]		Antibody[a]		Lymphoid leukosis
	Age (weeks)		Age (weeks)		
	7	16	7	16	
alv6 +	3/16	4/28	16/16	26/28	3/30[b]
alv6 −	0/31	1/35	29/31	34/35	4/39[c]

[a] Ratio of positive/total observed
[b] 5 out of 30 died with no evidence of neoplasms
[c] 3 out of 39 died with no evidence of neoplasms and 1 out of 39 died with another neoplasm that might be caused by RAV-2 (Fadly 1986)

subgroup A retroviruses. One-week-old progeny chicks from matings of *alv6* hemizygous males and line 0 females were injected into the wing web with subgroup A BH-RSV (RAV-1) sarcoma virus. None of the 6 chicks containing the *alv6* proviral insert developed sarcomas, whereas 14 of 17 chicks lacking the *alv6* proviral insert had palpable tumors 2 weeks after injection. A similar experiment measured the long-term response of similar transgenic chickens to infection by a pathogenic field strain of subgroup A ALV (Table 2). Progeny chicks from a mating of 4 *alv6* hemizygous males with SPF line 0 females were injected with RPL-42 ALV (subgroup A) on the day of hatch. The *alv6* chickens showed no evidence of infection to 40 weeks of age as measured by sensitive virus and antibody assays, and none developed lymphoid leukosis. Infection did not occur, even though the *alv6* chickens were constantly exposed to virus shed by their infected, half-sib hatch-mates. All non-transgenic chickens became viremic, some produced antibody and many had tumors characteristic of lymphoid leukosis. The low antibody response and persistent viremia is characteristic of field strains of ALV in line 0 chickens (Crittenden et al. 1984).

Tolerance

A possible complication of the expression of the ALV envelope gene throughout the life of the chicken is the induction of immunological tolerance to glycoproteins shared by subgroups of ALV (Crittenden et al. 1987). For example, chickens made tolerant to subgroup E ALV are also more tolerant to subgroup A as well as to subgroup B ALV, a pathogenic retrovirus that has been detected in some field flocks (Calnek 1968). Thus, chickens injected with subgroup E as embryos remained viremic with subgroups A and B ALV longer, had very low humoral immunity and a much higher frequency of lymphoid leukosis (Crittenden et al. 1987). Therefore, progeny of *alv6* hemizygous males and SPF line 0 females were injected with subgroup B ALV (RAV-2) at 1 week of age. Viremia, antibody to subgroup B ALV and mortality were determined through 30 weeks. Table 3 shows that both transgenic and non-transgenic chickens responded similarly to RAV-2. Both populations of chickens developed antibody to RAV-2 and similar numbers had tumors characteristic of lymphoid leukosis. Thus, there is little evidence that expression of subgroup A envelope glycoprotein in chickens containing the *alv6* provirus induces immune tolerance to subgroup B ALV.

Defectiveness of proviral insert

The defectiveness of the defective proviral insert was monitored for 40 weeks in a separate uninfected population from similar matings. All *alv6* positive and negative chickens remained free of ALV and ALV antibodies. However, one of the *alv6* positive chickens had a bursal lymphoma at the end of the experiment even though it lacked ALV and subgroup A ALV antibodies. Thus, this may represent one of the rare spontaneous cases of lymphoid leukosis of unknown etiology that has been observed in the RPRL SPF lines of chickens maintained free of exogenous ALV (Crittenden et al. 1979b; L. B. Crittenden, unpublished results).

Production of homozygotes

One potential hazard of inserting potential beneficial foreign genes into eucaryotic germ cells is the induction of mutational changes in endogenous genes. Approximately 6% of the foreign genes inserted into the mouse germ line have caused mutations in endogenous genes. Most of the insertional mutations are in genes necessary for normal development (reviewed in Jaenisch 1988). Transgenic chickens homozygous for *alv*6 have been successfully produced. Both homozygous males and females produce semen and eggs of reasonable fertility and we have produced approximately 50 homozygous males and females for future experiments. Thus, the *alv*6 proviral insert has not disrupted genes necessary for development or reproduction. However, whether the *alv*6 proviral insert has induced mutational changes in genes involved in egg productivity traits will have to await large scale productivity trials planned for the future.

Conclusions

We have demonstrated that avian retroviruses can be used to insert foreign genes into the chicken germ line. Our earlier work showing that recombinant, replication-competent ALV vectors can infect chicken germ cells and be stably inherited (Salter et al. 1986, 1987; Crittenden et al. 1989), taken with this study, demonstrate that such vectors can be used to insert and express viral genes. This opens the way for the use of replication-competent vectors for gene insertion and selection of germ-line inserts that are replication-defective, yet express an inserted gene. The fact that expression of the artificially inserted subgroup A envelope gene is dominant for resistance to ALV suggests that inserting viral envelope genes may be a general approach for producing animals resistant to at least some classes of viruses.

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