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Research Notes

Evaluation of Japanese Quail as a Model System for Avian Transgenesis Using Avian Leukosis Viruses

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ABSTRACT Vertical transmission of avian leukosis viruses (ALV) can occur genetically through the germ-line for both male and female chickens but only nongenetically or congenitally through the female. We had previously shown that tolerantly ALV-infected males, from ALV injection into fertile chicken eggs at day of set, can transmit proviral DNA to their progeny through the germine. An attempt was made to repeat this successful retroviral germline insertion technique of chickens in Japanese quail. After an initial difficulty of infecting quail chicks in ovo at day of set with high titer nonpathogenic recombinant and pathogenic ALV, adequate numbers of tolerantly ALV-infected quail were produced by injecting ALV-infected chicken embryo fibroblasts (CEF) at day of set. Tolerantly ALV-infected male and female quail were then mated to nonviremic quail and vertical transmission of ALV to progeny chicks was determined by analyzing blood for viral antigens and proviral DNA using standard techniques. Vertical transmission of ALV was only detected in the progeny of viremic females. Thus, little or no germline transmission of ALV to progeny occurred from viremic males. Tolerantly ALV-infected males and females from congenital ALV infection, which should infect the embryo and presumably the primordial germ cells (PGC) earlier than egg injection, were mated to nonviremic quails. Vertical transmission of ALV to progeny chicks was analyzed as before. Again, vertical transmission of ALV was only detected in the progeny of viremic females. We conclude that Japanese quail will not be useful in avian transgenic studies involving ALV retroviral vectors.

(Key words: avian leukosis virus, retroviral vectors, Japanese quail, transgenic, model system)

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INTRODUCTION

Before the enormous amounts of time and expense are committed for transgenesis in larger mammals, DNA function and transgenesis are first assessed in the mouse (Pursel et al., 1989), whose small size and short generation time allows for efficient testing of germline insertion events and gene and promoter function. Currently, about 18 mo and considerable expense, space, and labor are required to fully investigate presumptive germline transmission of foreign DNA in chickens (reviewed in Bosselmann et al., 1990; Kopchick et al., 1991; Petite et al., 1993; Salter et al., 1993; Sang et al., 1993; Vick et al., 1993; Thoraval et al., 1995). Therefore, a model system for poultry was sought that could significantly decrease the expense and time required for transgenesis in chickens. Japanese quail (Coturnix coturnix japonica) have been used successfully as a model system for poultry in genetic selection and other research (reviewed in Baumgartner, 1994). Quail become sexually mature in about 6 to 7 wk as compared to 24 to 28 wk for chickens, require much less room, labor, and expense to maintain than an identical number of chickens, and are prolific layers for most of the year. We report here our attempt to reproduce our successful chicken transgenic research in Japanese quail using the same techniques proven successful and reproducible in chickens (Salter et al., 1986, 1987). These data have been previously summarized (Salter et al., 1993).
MATERIALS AND METHODS

Animal Husbandry

A randombred strain of Japanese quail maintained at the Michigan State University Poultry Research Farm was used in all studies. Quail were maintained in standard quail cages and provided ad libitum access to standard quail chow under natural light-dark cycle. Fertile eggs for injection, repopulation, or progeny testing were produced by natural matings of one male to three to five females and stored in an egg cooler until used. Quail chicks were pedigree hatched, leg- or wing-banded, and bled at 6 d posthatch from a leg vein using materials and techniques described previously for chickens (Crittenden et al., 1989). Animals were euthanatized by carbon dioxide asphyxiation.

Avian Leukosis Viruses

Wild-type and recombinant subgroup A ALV used in our successful germline work in chickens were also used in this study. Rous-associated virus (RAV)-1 is the prototype exogenous subgroup A pathogenic ALV (Crittenden, 1991) and RAV-0-A1 and RCOS(A) are recombinant ALV described fully in previous publications (Wright and Bennet, 1986; Hughes et al., 1990). Both recombinants have the nonpathogenic endogenous ALV, RAV-0, long terminal repeats (LTR) and the envelope gene of subgroup A ALV RCOS(A) and its derivatives have been designed to carry foreign genes into avian cells and the germline (Hughes et al., 1990). RPL-42, an exogenous pathogenic subgroup A field strain of ALV (Fadly and Okazaki, 1982), was also used. Stocks of wild-type and recombinant retroviruses were prepared and titered in Line 0 CEF as described previously (Crittenden et al., 1989). Virus stocks and ALV-infected CEF were stored in liquid nitrogen until used.

Egg Injection Procedure

Fertile quail eggs were injected into the yolk at day of set with 30 μL of infectious retrovirus or a suspension of ALV-infected CEF at the indicated dose using materials and techniques described previously (Salter et al., 1986).

Other Procedures

Quail chicks were determined to be infected with the various ALV by ELISA for the presence of viral p27 antigen in whole blood and by dot-blot of quail blood cells for proviral DNA using materials and techniques described previously (Crittenden et al., 1989).

RESULTS AND DISCUSSION

The success in the insertion of ALV proviral DNA into the germline of chickens was significantly enhanced by using Lane 0 chickens that are free of endogenous viral (ev) genes closely related to ALV. This procedure allowed easy detection of genetically transmitted proviral DNA and prevented recombination with the injected virus. The research effort then concentrated on testing the progeny of tolerantly infected males because it was thought that vertical transmission of retroviruses by tolerantly infected males could only occur genetically through the germline. About one-third of the viremic male Line 0 chickens that hatched from fertile eggs injected with the recombinant ALV described above at day of set transmitted proviral DNA to their progeny at frequencies ranging from about 1 to 11% (Salter et al., 1986, 1987). Further molecular and mating studies conclusively showed that all proviral positive progeny carried a proviral insert in their germline (Crittenden et al., 1989). Japanese quail seemed an ideal choice for repeating the chicken research, as they also lack endogenous retroviral genes that are closely related to chicken ALV (Frisby et al., 1979; Salter and Crittenden, unpublished results) and are susceptible to subgroup A ALV (Tooze, 1973).

Infection of Early Quail Embryos by ALV Injection

Injection of Virus. Approximately 3 × 10^3 infectious units (i.u.) of RAV-0-A1 and RCOS(A) were injected into fertile quail eggs, and blood from the hatched quail chicks were analyzed for p27 antigen by ELISA and proviral DNA by dot-blot as described above. Much to our surprise and dismay, only a few of the quail chicks from the recombinant ALV-injected fertile eggs were ELISA positive and none of the quail chicks were positive for proviral DNA in blood cell DNA (data not shown). Pathogenic ALV, which contain a more active LTR and can be grown to a higher titer, were then tested. Approximately 3 × 10^4 i.u. of RAV-1 and 3 × 10^5 i.u. RPL-42 were similarly injected into fertile quail eggs as described above. Again, the infection rate of these two pathogenic retroviruses was surprisingly low yielding only a few chicks that were ELISA-positive but still proviral DNA negative in their blood (data not shown). We have routinely hatched viremic chicks from similarly injected fertile chicken eggs at frequencies of 30 to 50% regardless of the type of ALV virus (Salter and Crittenden, unpublished data). Thus, the more efficient LTR and higher dose did not appear to be a factor in the observed infection rate. In comparison with uninjected eggs and eggs that received virus-free medium, high titer ALV supernatants appeared to have little effect on the hatchability of fertile quail eggs (data not shown).

In an effort to understand the resistance of quail to subgroup A ALV infection, 5 × 10^4 i.u. of RAV-1 was injected into the yolk sac of 1-, 5-, or 11-d-old quail embryos. All of the hatched chicks were negative for p27 antigen by ELISA or proviral DNA by dot-blot (data not shown). Routinely, chicken embryos at this age are easily infectable with RAV-1 at frequencies approaching 100%
TABLE 1. Infection of Japanese quail embryos with avian leukemia virus-infected chick embryo fibroblasts

<table>
<thead>
<tr>
<th>Infected cells injected</th>
<th>No. of chicks assayed</th>
<th>No. ELISA positive (%)</th>
<th>No. Dot-blot positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAV-0-A1</td>
<td>46</td>
<td>1 (2.2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>RCOS(A)</td>
<td>43</td>
<td>4 (9.3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>RAV-1</td>
<td>116</td>
<td>13 (11.2)</td>
<td>11 (9.5)</td>
</tr>
<tr>
<td>RPL-42</td>
<td>88</td>
<td>49 (55.7)</td>
<td>37 (42.0)</td>
</tr>
</tbody>
</table>

for p27 antigen or proviral DNA by dot-blot (Salter and Crittenden, unpublished data). Newly hatched quail chicks were injected with $10^4$ i.u. of RAV-1 and analyzed for p27 antigen, proviral DNA, and neutralizing antibody in their blood. Of 14 chicks, none had p27 antigen or proviral DNA on Day 8 after hatch and only 1 of the 14 had made detectable neutralizing antibody to RAV-1 on Day 45 after hatch (data not shown). A similar infective dose to hatched chickens produced a 80 to 100% infection rate as determined by infectious virus assay or by antibody to RAV-1 (Federspiel et al., 1991).

Although Japanese quail have been reported to be susceptible to subgroup A ALV, there was a possibility that the resistance to subgroup A infection might be due to the segregation of resistance to infection in our randomly bred breeder quails. However, all 11 3-wk-old quail developed sarcomas after injection of subgroup A Rous sarcoma virus (RAV-1) sarcoma virus into one of their wing webs (data not shown).

This lower infectivity of quail embryos and chicks by ALV appears to support results of Friis (1972), who found that quail embryo cells were infected and transformed by subgroup A Rous sarcoma virus at efficiencies comparable to chick embryo cells. However, the yield of virus from these transformed cells was much more than similarly transformed chicken cells. Presumably, little virus is made after the initial infection by virus particles to spread through the embryo to give a tolerantly infected embryo or mount an immune response.

**Infection of Virus-Infected Cells.** We have previously found that injecting approximately $1 \times 10^6$ ALV-infected CEF per fertile egg at day of set significantly increase the frequency of ALV-infected chicks (Salter and Crittenden, unpublished data). Approximately $1 \times 10^6$ ALV-infected CEF were injected into fertile quail eggs at day of set and the frequency of infected chicks for the various ALV was determined. Table 1 shows that the frequency of infected quail chicks increased significantly particularly with the pathogenic wild-type ALV. However, we still had little success in producing a significant number of virus-positive quail chicks from injected RAV-0-A1 and RCOS(A) infected cells. This difference in ELISA and dot-blot frequency probably reflects the relative sensitivity of each assay and the degree of viral infection.

Nearly all of the viremic quail chicks were from pathogenic exogenous ALV. Except for one brief report on finding tumors characteristic of lymphoid leukosis in a quail population (Wight, 1963), little information was available regarding the pathogenicity of ALV in Japanese quail. However, much to our surprise, nearly all of the quail that were viremic with the two pathogenic ALV survived through maturity and progeny testing.

**Vertical Transmission of ALV from Viremic Males and Females from Injected Eggs**

None of the few ELISA-positive quail chicks from RCOS(A) and RAV-0-A1 injected eggs survived to maturity or remained ELISA positive 1 mo later. The surviving RAV-1 and RPL-42 infected male and female quail were mated to uninfected quail, and blood from the progeny chicks were analyzed for p27 antigen and proviral DNA (Table 2). The data from both viruses have been combined for simplicity. Initially, some of the progeny of viremic males appeared to be weakly positive for p27 by ELISA and proviral DNA by dot-blot. However, in general, there was no correlation between the two assays. In addition, no more than one weakly positive progeny came from the same male. Any of the ELISA or dot-blot data that appeared to be above background were repeated by either retesting the blood sample, retesting the bird (if available), Southern analysis of enzyme-digested DNA, or by remating the viremic male to noninfected breeder females. In general, these positive progeny retested negative for viral antigen and proviral DNA by dot-blot or Southern blot. In all cases, the presumptive positive transmitting males fail to produce

<table>
<thead>
<tr>
<th>Viremic parent sex</th>
<th>Number mated</th>
<th>No. progeny tested</th>
<th>No. ELISA positive (%)</th>
<th>No. Dot-blot positive (%)</th>
<th>Retest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>34$^1$</td>
<td>2,537</td>
<td>4 (0.2)</td>
<td>13 (0.5)</td>
<td>0$^2$</td>
</tr>
<tr>
<td>Female</td>
<td>22$^3$</td>
<td>828</td>
<td>40 (4.8)</td>
<td>21 (2.5)</td>
<td>ND$^4$</td>
</tr>
</tbody>
</table>

$^1$Consisted of 9 RAV-1 and 25 RPL-42 infected males.
$^2$Presumptive ELISA-positive and dot-blot positive progeny and parents were rebled and retested. Presumptive transmitting males were remated to noninfected females and progeny analyzed.
$^3$Consisted of 3 RAV-1 and 19 RPL-42 infected females.
$^4$Not determined.
any more positive progeny even after extensive mating. We interpret these weakly positive ELISA and dot-blots to be due to a low level of false positives inherent to each assay. Thus, we concluded that all progeny from RAV-1 and RPL-42 viremic males and nonviremic females were negative for viral antigen and proviral DNA. Therefore, no infection of PGC had occurred in male quail infected with ALV at day of set.

We also measured the transmission of ALV from viremic females to progeny even though vertical transmission of ALV in viremic females occurs primarily or solely by nongenetic or congenital means (Crittenden, 1991). As shown in Table 2, viremic females from RAV-1 and RPL-42 injected eggs transmitted ALV (as detected by both ELISA and dot-blot assays) at a significant frequency to their progeny. As noted previously, the frequency of detection was higher with the ELISA assay for p27 antigen than with the dot-blot assay for proviral DNA. Consistent with these data, egg albumin from most of these viremic females were highly positive for the ALV p27 antigen. However, the frequency of congenital transmission in viremic quail is much lower than found in tolerantly ALV-infected chickens where 50 to 80% of the chicks are viremic quail is much lower than found in tolerantly ALV-infected chickens by other researchers (Kopchick et al., 1991). Male viremic males and female congenitally-infected quail, from the viremic female quail in Table 2, were mated to uninfected females and blood from the progeny quail were analyzed for p27 antigen and proviral DNA as described above (Table 3). Again, a few progeny from the congenitally infected viremic males appeared to be weakly positive by ELISA or dot-blot. Again, through procedures described previously, we interpret these weakly positive data to be due to a low level of false positives in our assays. Thus, we concluded that all progeny from RAV-1 and RPL-42 congenitally infected viremic males and nonviremic females were negative for either p27 antigen and proviral DNA. Therefore, no infection of PGC had occurred in congenitally ALV-infected male quail. As expected, a significant proportion of the progeny from congenitally infected females were positive for p27 antigen and proviral DNA (Table 3). Again, we presumed that most or all of the virus positive progeny were infected congenitally through the albumin or yolk of the egg from the viremic female.

Although not a part of this study, little or no disease characteristic of lymphoid leukemia was detected in 1-yr-old tolerantly infected male and female quail containing pathogenic ALV with titers of from $10^2$ to $10^3$ i.u./mL of blood (necropsy on over 80 viremic quail). The inherent resistance of quail to disease induction by pathogenic ALV is thus similar to the resistance of quail chicks, embryos, and PGC to ALV infection by methods proven to be successful in chickens.

In conclusion, Japanese quail will not be a useful model system for transgenic poultry using wild-type and recombinant replication competent and defective ALV that have proven successful in chickens. However, another retroviral vector, based on reticuloendotheliosis virus, has been used to produce a line of transgenic Japanese quail containing a defective reticuloendotheliosis virus vector and expressing a foreign gene (Lee and Shuman, 1990). Furthermore, methods, such as transfection of PGC, blastodermal cells, embryonic stem cells, and injection of cloned DNA into fertilized ova, now routine for mice with eventual application to large animals, will eventually prove to be useful in constructing transgenic birds (reviewed in Petitte et al., 1993; Salter et al., 1993; Sang et al., 1993), perhaps using Japanese quail as a model system.

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**TABLE 3. Vertical transmission of RAV-1 and RPL-42 avian leukosis virus in viremic Japanese Quail congenitally infected by viremic females**

<table>
<thead>
<tr>
<th>Viremic parent sex</th>
<th>Number mated</th>
<th>No. progeny tested</th>
<th>No. ELISA-positive (%)</th>
<th>No. Dot-blot-positive (%)</th>
<th>Retest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>21</td>
<td>2,136</td>
<td>2 (0.1)</td>
<td>4 (0.3)</td>
<td>0^1</td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
<td>536</td>
<td>67 (12.5)</td>
<td>21 (3.9)</td>
<td>ND^2</td>
</tr>
</tbody>
</table>

1^Presumptive ELISA-positive and dot-blot-positive progeny and parents were rebled and retested.

Presumptive transmitting males were remated to uninfected females and progeny analyzed.

2^Not determined.

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**Vertical Transmission of ALV from Congenitally Infected Viremic Males and Females**

We have previously shown that male chickens congenitally infected from tolerantly ALV-infected females are more likely to transmit genetically and at a higher frequency than males infected at day of set (Crittenden and Salter, 1998). This higher frequency presumably is due to an earlier infection of the developing embryo by the viremic female resulting in infection of more PGC. In fact, this technique has been used to produce transgenic chickens by other researchers (Kopchick et al., 1991). Male and female congenitally-infected quail, from the viremic female quail in Table 2, were mated to uninfected females and males, respectively, and blood from the progeny quail were analyzed for p27 antigen and proviral DNA as described above (Table 3). Again, a few progeny from the congenitally infected viremic males appeared to be weakly positive by ELISA or dot-blot. Again, through
REFERENCES


