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Selective Shedding and Congenital Transmission of Endogenous Avian Leukosis Viruses

EUGENE J. SMITH,* DONALD W. SALTER, ROBERT F. SILVA, AND LYMAN B. CRITTENDEN

Regional Poultry Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, East Lansing, Michigan 48823

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Shedding and congenital transmission of endogenous avian leukosis viruses were studied in viremic White Leghorn hens exogenously infected with viruses with endogeneous long terminal repeats (LTRs) and in four semicongenic lines of hens that naturally express infectious endogenous viruses (EVs). Relatively high titers of infectious virus EV7 (encoded at locus *ev7*), Rous-associated virus-0 (RAV-0), and recombinant 882/−16 RAV-0 were detected in blood cells and sera from exogenously infected hens, but marked differences were noted in the incidence of congenitally infected progeny. In enzyme immunoassays that detect viral group-specific antigen, little or no p27 was detected in albumens from dams infected with RAV-0. However, hatchmates infected with either EV7 or recombinant 882/−16 RAV-0, which was constructed with an RAV-0 LTR, shed high titers of p27. Similarly, semicongenic hens that expressed RAV-0 (EV2) (encoded at locus *ev2*) shed little or no p27 into albumens, but hens that harbored *ev10*, *ev11*, and *ev12* shed high titers of p27. A slower electrophoretic mobility of p27, considered to be characteristic of EVs that are restricted in congenital transmission, was not associated with low levels of shedding or congenital transmission; p27 from other EVs and p27 from an avian leukosis virus field strain, all of which are shed at high levels, had mobilities identical to that of p27 from RAV-0. Although shedding and congenital transmission appear to be controlled by the viral genome, there was no correlation between low efficiency of shedding or congenital transmission and endogenous LTR or p27 sequences.

Antigenically related avian leukosis viruses (ALVs) of exogenous and endogenous origin can coexist in chickens. In field flocks exogenously infected with ALVs of exogenous origin, virus is efficiently shed into albumens and congenitally transmitted through the egg to offspring. Immunologically tolerant progeny, in turn, perpetuate infection because they remain viremic, and females also shed high titers of virus into eggs and congenitally transmit virus throughout their lives (3, 19).

In normal chickens, endogenous viruses (EVs) are inherited as single-copy genes at loci designated *ev* (1, 18). Studies by others indicated that group-specific (gs) antigens of EV origin are found at lower levels in albumens than are antigens derived from ALVs (8, 10, 13, 15). More specifically, in semicongenic lines that carry *ev2*, relatively low levels of Rous-associated virus 0 (RAV-0) are shed (6). In another line, an absolute restriction on congenital transmission is associated with recombinant ALVs that express an altered gs antigen that is characteristic of RAV-0 and is designated $p27^0$ (17). These reports have led to the generalization that EVs have a limited or restricted ability to congenitally infect progeny. Preliminary, unpublished observations indicated, however, that EVs encoded at other *ev* loci were shed at relatively high levels. Here, we report levels of viremia and shedding of EVs in tolerant dams and congenital transmission to progeny of two subsequent backcross generations. We found that, compared with four other EVs, restricted shedding and congenital transmission were confined to RAV-0. Moreover, all EVs expressed p27 with slower electrophoretic mobilities than that of p27 from RAV-1 and RAV-2.

* Corresponding author.

MATERIALS AND METHODS

Chickens and viruses. In the first experiment, line 15B1 sires that were susceptible to all ALV subgroups and carried only *ev1*, a relatively unexpressed *ev* gene (1), were crossed with ev^- line 0 dams (2). Because this cross was susceptible to all ALV subgroups and because *ev1* was not expressed at high levels, only exogenously introduced ALVs of exogenous or endogenous origin were detected. Each of three groups of 6-day-old 15B1 × 0 embryos were inoculated in the yolk sac with 10^4 infectious units of RAV-0 (23), recombinant EV7 (5, 16), or recombinant 882/−16 RAV-0 (S. H. Hughes, E. Kosik, A. M. Fadly, D. W. Salter, and L. B. Crittenden, Poult. Sci., in press). A fourth group was an uninoculated control. The recombinant 882/−16 RAV-0 was constructed from circular DNA of the subgroup A Schmidt-Ruppin strain of Rous sarcoma virus (RSV). The *src* gene was excised, and synthetic *Cla*I linkers were ligated between the *env* gene and the long terminal repeat (LTR). RAV-0 LTR sequences were subsequently substituted between the *Cla*I and *Sst*I sites of the vector and cloned in plasmid pBR322. The *Sst*I restriction site at nucleotide 260 of the 5′ noncoding region of RAV-0 also encompassed about half of the leader sequences. Chicken embryo fibroblasts were transfected with cloned recombinant plasmid DNA, and infectious ALVs that replicated with the low efficiency characteristic of RAV-0 were recovered. Exogenously infected (G-0) dams were mated to line 15B1 sires to obtain first-generation (G-1) hens. At maturity, ALV-positive G-1 daughters were, in turn, mated to other line 15B1 sires to produce second-generation (G-2) progeny. Blood from embryos of the G-1 and G-2 generations was tested for either infectious ALVs or p27.

We also measured p27 in four semicongenic lines that harbored only one infectious EV in a line 15B background (a

parental line of 15B1 that carries *ev*1 and *ev*7) (5). Albumens, erythrocytes, and plasmas were titrated to determine levels of p27 in normal hens that carried *ev*2, *ev*10, *ev*11, or *ev*12. Loci were identified by characteristic DNA fragments that were produced after digestion with restriction endonucleases (1). These lines were maintained in an environment free of exogenous ALVs and other avian pathogens. gs antigen-negative line 15B hens of the same age and separately caged in the same building served as controls to monitor possible horizontal infection and the potential expression of EV7 from locus *ev*7 present in line 15B.

In experiment 1, EV7 was exogenously introduced as an infectious recombinant virus into susceptible embryos. Consequently, immunologically tolerant chickens expressed EV7 throughout their lives. On the other hand, *ev*7 is an unexpressed proviral locus that has the potential for activation through recombination with homologous endogenous genes (16). Previous work (5) has shown that *ev*7 may be activated after hatching, but individuals readily seroconvert.

RAV-0-A-1 is a recombinant obtained after transfection of RAV-0-producing cells with cloned polymerase and envelope sequences derived from the subgroup A Prague strain of RSV (S. E. Wright and D. D. Bennett, Virus Res., in press). This recombinant, which grows on C/E cells, contains RAV-0 LTRs but polymerase and envelope sequences from the subgroup A Prague strain of RSV.

Crosses used in these experiments were very permissive for infection by subgroup E retroviruses. Other lines, such as K-28 (17), may be less permissive for the congenital transmission of endogenous retroviruses.

ALV antigen detection. A double-antibody sandwich enzyme-labeled immunosorbent assay (ELISA) that detects the ALV major viral structural antigen (molecular weight, 27,000) (p27) was used directly with albumins, erythrocytes, plasmas, and embryo extracts (21). Rabbit antiserum prepared against avian myeloblastosis virus p27 and peroxidase-conjugated p27 antibodies were obtained from SPAFAS, Inc., Norwich, Conn. About 1 μg of trapping antibody protein was applied to each well, and after incubation of the test samples, conjugated stock anti-p27 preparations were diluted 1,000-fold. Absorbances greater than twice the average background of p27-negative control samples were scored positive.

ALV titrations. Infectious ALVs in sera were recovered from blood that was allowed to clot rapidly in the presence of inactivated chicken embryo extract (7). Blood and sera from exogenously infected groups and uninfected hatchmates were serially diluted 10-fold from 10^1 to 10^6 in cell culture medium. A 0.1-ml portion from each dilution was separately added to secondary chicken embryo fibroblasts (CEFs) from ALV-susceptible (C/O) line 15B1 and subgroup E-resistant (C/E) line 0. Growth on C/O and C/E CEFs served to distinguish between exogenous and endogenous ALVs. After two changes of medium during an incubation period of 9 days, monolayers and medium were frozen twice at −20°C, and duplicate 0.1-ml samples of culture fluids were tested for p27 by the ELISA. Previous experiments had shown that, in these cell types, the maximum titers with limiting dilutions of both exogenous and endogenous ALVs were obtained after 9 days of culturing (L. B. Crittenden, unpublished data).

Electrophoretic mobility of p27. Line 15B1 CEFs in 60-mm dishes were infected with EV stocks originally prepared from CEFs that released only EV2, EV7, EV10, EV11, EV12, or EV21 from the respective *ev* loci. RAV-0-A-1 and 882/−16 RAV-0 were also cultivated. RAV-1-infected and uninfected CEFs were concurrently treated in the same manner and served as virus-positive and -negative controls, respectively. Seven days after cultivation, monolayer cultures were incubated in methionine-free medium for 1 to 2 h. Culture medium was replaced with fresh medium containing 50 μCi of [^{35}S]methionine per ml, and the cultures were incubated an additional 4 to 6 h. The labeled cell monolayer was washed once with phosphate-buffered saline and disrupted in 150 mM NaCl–1% sodium deoxycholate–1% Triton X-100–0.1% sodium dodecyl sulfate–10 mM Tris hydrochloride (pH 7.5). For immunoprecipitation, cell lysates were clarified by 10 min of centrifugation at 15,000 × *g*. Supernatants were incubated overnight with p27 antiserum, and antigen-antibody complexes were precipitated with the Cowan 1 strain of *Staphylococcus aureus*. Proteins were separated on a discontinuous sodium dodecyl sulfate-polyacrylamide slab gel (20). Autoradiographs were developed after 3 to 4 days of exposure of Kodak X-Omat X-ray films at −70C.

RESULTS

Selective restriction of congenital transmission of ALVs. At 24 and 30 weeks of age, infectious ALV assays were done on C/O and C/E CEFs with blood and serum from three groups of hens that were infected as embryos after 6 days of incubation with RAV-0, EV7, or 882/−16 RAV-0. Among eight hens in each group, EV titers ranged from 10^2 to 10^5 infectious units per ml of blood or serum (Table 1). EV was not found in uninfected 15B1 × 0 hatchmates. In the ELISA, comparable high endpoint titers of p27 were found directly in blood cells of the three exogenously infected groups of hatchmates, but p27 was detected at relatively high titers in albumens of only EV7- and 882/−16 RAV-0-infected hens. Little or no p27 was detected in undiluted albumens from 11 RAV-0-infected hens. Because the ELISA absorbances of the two positive albumen samples were marginal, albumens from RAV-0-infected hens were not titrated.

Infectious ALV assays done with blood from 1-day-old G-1 chicks indicated that 52 and 84% of the progeny from hens infected with EV7 and 882/−16 RAV-0, respectively, were infected. We found, however, that only 12% of the progeny from viremic RAV-0-infected hens were infected. As expected, EV7 and RAV-0 grew on C/O but not on C/E CEFs, whereas 882/−16 RAV-0 grew on both cell types. Subsequent direct p27 ELISAs of blood from 19-day-old embryos of viremic G-1 dams (G-2 chicks) indicated that the incidence of RAV-0 transmission rose to 69% in the second generation. The incidences of G-2 progeny congenitally infected with EV7 and 882/−16 RAV-0 also increased to 74 and 100%, respectively (Table 1).

Consistent with marginal shedding found in parental hens, undetectable or low titers (dilution endpoints, 1:2) of p27 were also found in albumens of 14 viremic G-1 daughters of RAV-0-infected parental dams. At 128-fold dilutions, the highest dilution tested, p27 was detected in albumens of most G-1 hens congenitally infected with 882/−16 RAV-0 or EV7.

Molecular hybridizations of digested DNA confirmed that progeny from RAV-0-infected G-1 dams were indeed cogenitally infected with RAV-0. Southern blots revealed that the 1.5-kilobase-pair fragment characteristic of the internal fragment of RAV-0 was released after *Sac*I digestion (autoradiograph not shown). No progeny from uninfected hatchmates expressed infectious ALV or p27 throughout the testing period.

Levels of p27 in semicongenic hens. To determine levels of EV expression encoded at different *ev* loci, eggs were

TABLE 1. Congenital transmission of ALVs from viremic dams to progeny of the first and second generations

Virus	Viremia[a] in:		Titer of p27[b] in:			No. positive/no. tested		
	Blood	Serum	Blood cells	Plasmas	Albumens	Parental albumens (no. of hens)[c]	G-1 blood (%)[d]	G-2 blood (%)[e]
RAV-0	10^3–10^5	10^2–10^4	337	13	0	2/55 (11)	43/352 (12)	74/107 (69)
EV7	10^3	10^2–10^3	194	2	24	52/53 (11)	51/97 (52)	41/55 (74)
882/−16 RAV-0	10^4	10^3–10^5	416	34	2,195	40/40 (8)	54/64 (84)	6/6 (100)
Control	0	0	0	0	0	0/32 (8)	0/91 (0)	0/91 (0)

[a] Infectious units per milliliter. Blood and sera from eight parental hens in each group were collected at 24 and 30 weeks of age, respectively. Serially diluted samples were placed on susceptible CEFs, and monolayers were cultivated for 9 days. Virus production was measured in CEF extracts by the ELISA described in Materials and Methods.
[b] Geometric mean titers were measured directly in blood cells and plasmas from 8 hens at 30 weeks of age. One p27-positive egg from each hen was also titrated.
[c] Determined by the ELISA.
[d] Infectious ALVs in blood from G-1 chicks at 1 day of age.
[e] Direct p27 ELISA with blood from 19-day-old G-2 embryos of viremic G-1 dams.

collected from 6 to 10 hens in each group of four *ev* genotypes. Consistent with previous observations (6) on RAV-0 shedding from exogenously infected hens, we found that p27 was not detected at high concentrations in albumens from viremic EV2-infected hens (Table 2). This low titer contrasts sharply with the markedly higher titers of p27 found in albumens from hens carrying *ev*10, *ev*11, and *ev*12. Although p27 was expressed at high and comparable levels in erythrocytes from all groups, p27 was not detected in plasmas from $ev2^+$ and $ev12^+$ hens.

Electrophoretic mobilities of p27 from endogenous and exogenous ALVs. In view of the previously reported association of structurally altered p27 with restricted congenital transmission (17) and our observations on the different levels of shedding of RAV-0 and other endogenous and exogenous ALVs, we investigated the electrophoretic properties of p27. Autoradiographs of immunoprecipitates indicated that the mobilities of p27 from EV2, EV7, EV10, EV11, EV12, EV21, RAV-0-A-1, and 882/−16 RAV-0 were slower than that of p27 from RAV-1 (Fig. 1); p27 from RAV-2 also comigrated with p27 from RAV-1 (autoradiograph not shown).

DISCUSSION

Our results agree, in part, with the earlier findings of Robinson and Eisenman (17) on the restricted congenital transmission of RAV-0 in viremic hens. We found, however, that this restriction appears to be confined to RAV-0 and is not a hallmark of endogenous avian leukosis retroviruses.

In the experiments reported here, EV expression was examined both as a consequence of exogenous introduction into susceptible, gs antigen-negative embryos and as directly transcribed from *ev* loci that encode infectious virus in uninoculated hens.

In exogenously infected hens, high titers of infectious RAV-0 were found in blood cells and sera, but shedding of RAV-0 into albumens was markedly restricted. Moreover, restriction of shedding persisted because low titers of p27 were found in albumens from only 4 of 14 RAV-0-positive dams when tested 11 months later. Marginal shedding of p27 found even after exogenous infection with RAV-0 suggests that virally encoded sequences may influence transcription or translation in the oviduct but not in other tissues. The increased incidence of RAV-0 infection in G-1 and G-2 progeny may reflect selection from permanently viremic dams of variant viruses that were more efficiently transmitted but still retained seuqences that restricted shedding.

Although the possibility of germ line integration of RAV-0 cannot be ruled out, no evidence for clonal integration was found when DNAs from about 10% of the embryos of the G-2 generation were analyzed by Southern blot hybridizations with a complete proviral probe. Smears of provirus-host DNA junction fragments indicative of nonspecific integrations were observed (L. B. Crittenden and D. W. Salter, unpublished observations). Apart from junction fragments

TABLE 2. Expression of p27 in albumens, blood, and plasmas of semicongenic hens

Proviral loci	p27 in:		
	Albumens[a]	Blood cells[b]	Plasmas[b]
*ev*2, *ev*1, and *ev*7	17/25 (7) [<2; undetermined to 1:4]	79	0
*ev*10, *ev*1, and *ev*7	31/31 (10) [7; 1:4–1:32]	64	9
*ev*11, *ev*1, and *ev*7	22/22 (6) [194; 1:64–1:512]	79	6
*ev*12, *ev*1, and *ev*7	22/22 (6) [64; 1:4–1:256]	128	0
*ev*1 and *ev*7	0/6 (6) [0]	0	0

[a] Number positive/number of eggs titrated (number of hens) [geometric mean titers; range].
[b] Geometric mean titers from four hens in each group.

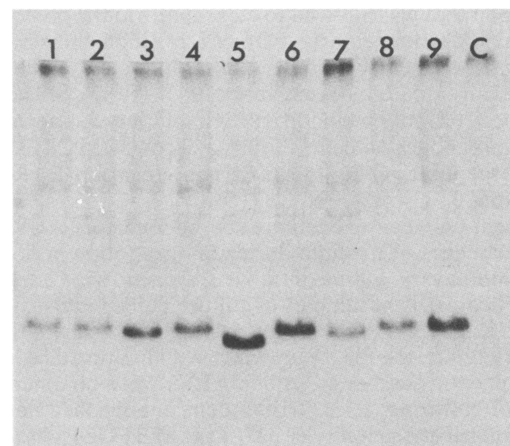

FIG. 1. Autoradiograph of [^{35}S]methionine-labeled p27 in immunoprecipitates from line 15B1 CEFs infected with RAV-0 (lane 1), EV7 (lane 2), EV10 (lane 3), EV11 (lane 4), RAV-1 (lane 5), EV12 (lane 6), EV21 (lane 7), RAV-0-A-1 (lane 8), or 882/−16 RAV-0 (lane 9), or uninfected.

characteristic of *ev*1, only the internal proviral fragment of 1.5 kilobase pairs that is unique to RAV-0 (12) was observed in G-2 progeny congenitally infected with RAV-0. These markers confirm that G-2 progeny from congenitally infected dams were indeed infected with RAV-0.

Undetectable or marginal levels of RAV-0 p27 in albumens of parental and G-1 viremic hens suggest that oviduct shedding of RAV-0 and congenital infection of embryos may operate independently. Viral sequences that negatively regulate shedding may have no influence on the mode of transmission of ALV infection. In some lines, selection for nonshedding hens may not identify all hens that congenitally infect progeny.

In contrast, EV7 and recombinant 882/−16 RAV-0, which also have RAV-0 LTR sequences, were efficiently shed and transmitted. Thus, the low growth rate attributed to the lack of strong promoter sequences in the LTRs of EVs (4) does not appear to govern levels of shedding.

The notion that capsid antigen determines congenital transmission was proposed after an association between the slower electrophoretic mobility of p27 and restricted congenital transmission was found (17). Our results confirm that p27 from RAV-1 and p27 from RAV-2 have faster mobilities than p27 from EVs, but no consistent association between mobility and restricted shedding or transmission was found. Recombinant 882/−16 RAV-0 (derived from subgroup A RSV), RAV-0-A-1, and RPL-40, a subgroup A field strain (data not shown), all of which were shed at high concentrations, expressed p27 with an electrophoretic migration pattern characteristic of that in EVs. Mobilities of p27 from RAV-0 and EV7 were identical and confirm earlier results (5). EV7, however, was shed and transmitted at higher efficiencies than was EV2 (RAV-0). Slight differences in mobilities of EV10 and EV21 suggested that alterations in p27 may be more common than hitherto recognized. The fact that $p27^0$ was not consistently associated with low levels of shedding does not rule out the possibility that the control of transmission resides elsewhere in the *gag* region of the viral genome. It does show, however, that the electrophoretic mobility of p27 is not a reliable marker for restricted shedding. The results reported by Robinson and Eisenman (17) were probably obtained through the fortuitous choice of RAV-0 recombinants made between EVs and ALVs derived from the Bryan high-titer strain of RSV (11, 22). Similar mobilities of p27 from both RAV-1 and RAV-2 may reflect their common origin. Our results suggest that p27 from both RAV-1 and RAV-2 may be a structural anomaly that arose from long-term propagation. The high degree of nucleotide sequence conservation encoding p19 in *ev*1, *ev*2, and five ALV strains indicates that the involvement of p19 in regulating transmission also appears unlikely (24). The precise location, therefore, of viral regulatory sequences that control shedding and congenital transmission is unknown.

Endogenous p27 was detected in blood cells of all semicongenic hens harboring *ev*2, *ev*10, *ev*11, and *ev*12. Although low levels of shedding were found in a few viremic RAV-0-infected hens, about 100-fold more p27 was found in albumens from hens that harbored *ev*11 or *ev*12. Intermediate levels of p27 were detected in *ev*10-bearing hens.

It is noteworthy that p27 was not detected in plasmas from $ev2^+$ and $ev12^+$ hens. Differential expression or cell-specific activation (9) of EVs or structural antigens from lymphocytes (14) may be associated with specific *ev* loci. In this context, cellular components of blood were not separated; therefore, lymphocytes may have been the source of p27 derived from EVs expressed at loci *ev*10 and *ev*11.

Because low titers of RAV-0 and high titers of field strains of oncongenic ALVs are shed, we previously suggested (6) diluting albumens to distinguish between endogenous and exogenous ALV infections in ELISAs. In view of the high levels of p27 found in albumens and the multiplicity of infectious EVs in White Leghorn chickens and other breeds, this diagnostic strategy appears invalid. Restricted shedding of RAV-0 may represent an anomaly among endogenous retroviruses.

From our observations that (i) restricted shedding of RAV-0 occurs in both exogenously infected and $ev2^+$ chickens, (ii) high titers of other EVs and recombinants with endogenous LTR sequences are shed, and (iii) altered p27 is found in EVs that are shed at both high and low levels, we conclude that restricted oviduct shedding is not controlled by p27 or LTR and adjacent sequences.

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