Integration, Expression, and Infectivity of Exogenously Acquired Proviruses of Rous-Associated Virus-0

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We investigated the integration sites, infectivities, and expression of Rous-associated virus-0 (RAV-0) DNAs in exogenously infected turkey and chicken cells. Restriction endonuclease analyses of the DNAs of RAV-0-infected cells indicated that unique integration sites of RAV-0 DNA were detectable in clones of RAV-0-infected cells but not in mass-infected cell cultures. In addition, the sites of integration of RAV-0 DNA differed in each of the seven clones of RAV-0-infected cells examined. Thus, endogenous RAV-0 proviruses appeared to integrate at multiple sites in cellular DNA, which were distinct from the sites of integration of endogenous RAV-0 genomes. Since exogenous RAV-0 proviruses are expressed at 10^3 - to 10^4 -fold-higher levels and are 10^3 - to 10^4 -fold more infectious in transfection assays than the endogenous RAV-0 genome of uninfected V^+ chicken cells, these results are consistent with the hypothesis that transcription of the endogenous RAV-0 genome is regulated by flanking cellular DNA sequences. Although all RAV-0-infected cells contained infectious RAV-0 DNA and produced high titers of RAV-0 compared with uninfected V^+ cells, different clones of RAV-0-infected chicken cells differed by as much as 30-fold in their levels of virus production. The infectivity of the DNA of each clone of RAV-0-infected cells correlated with the amount of virus produced by that clone. However, these differences did not appear to be correlated either with the number of exogenous RAV-0 proviruses in different clones or with the infectivity of RAV-0 produced by different clones, indicating that differences either in modification of RAV-0 DNAs or in the cellular sequences flanking exogenous RAV-0 DNAs were responsible for the observed differences in expression and infectivity.

Endogenous virus-related DNA sequences homologous to the genomes of avian leukosis viruses are inherited as part of the normal genetic complement of nearly all chickens (2a, 18, 24). Most uninfected chicken cells (V^- phenotype) do not produce virus particles, but uninfected cells of a few inbred lines of chickens (V^+ phenotype) spontaneously produce a nontransforming subgroup E avian leukosis virus, Rous-associated virus-0 (RAV-0) (12, 13, 26, 27, 34).

Although RAV-0 is produced by the majority of cells in cultures of uninfected V^+ chicken embryo fibroblasts, the titer of virus produced by uninfected V^+ chicken cells is 10^3 - to 10^4 -fold lower than that produced by chicken cells which have been infected exogenously with RAV-0 (9, 31). Uninfected V^+ chicken cells also appear to contain 10^3 - to 10^4 -fold-lower amounts of RAV-0 RNA than cells which have been infected exogenously with RAV-0, suggesting that expression of the endogenous RAV-0 genome is regulated at the transcriptional level (15a, 17).

Studies of the endogenous RAV-0 genome by transfection have indicated that endogenous RAV-0 DNA of uninfected V^+ chicken cells is

approximately 10^4 -fold less infectious than the RAV-0 DNA of RAV-0-infected cells (7, 9). To account for these results, it has been proposed that expression of the endogenous RAV-0 genome is regulated by linkage to *cis*-acting regulatory sequences which inhibit transcription of the endogenous RAV-0 genome and, consequently, reduce the infectivity of endogenous RAV-0 DNA in transfection assays (9). The hypothesis that the endogenous RAV-0 genome is linked to inhibitory cellular DNA sequences has been further supported by experiments indicating that shearing the DNA of uninfected V^+ cells to the approximate size of the RAV-0 genome results in a 60-fold increase in the infectivity of endogenous RAV-0 DNA relative to the infectivity of RAV-0 DNA of exogenously infected cells (7).

According to this model for regulation of the endogenous RAV-0 genome, efficient transcription of exogenous RAV-0 proviruses of RAV-0-infected cells would result from integration of exogenous RAV-0 DNA at cellular DNA sites that are not linked to the sequences regulating transcription of the endogenous RAV-0 genome.

In the present study, we investigated the infectivities and integration sites of RAV-0 proviruses of RAV-0-infected cells to further correlate these parameters with viral gene expression. The results of restriction endonuclease analyses of DNAs of RAV-0-infected cells indicated that exogenous RAV-0 proviruses can integrate at multiple sites in the genomes of infected cells, as has been observed previously in cells infected with other avian and mammalian retroviruses (19, 21, 28, 32). In addition, we observed that the level of expression of exogenous RAV-0 proviruses in individual clones of RAV-0-infected cells was proportional to the infectivities of the RAV-0 DNAs of these cloned cells in transfection assays.

MATERIALS AND METHODS

Cells and viruses. Cells were grown in Temin modified Eagle minimal essential medium containing 20% tryptose phosphate broth (ET medium) and supplemented with calf serum or fetal bovine serum or both. Fertile chicken eggs were purchased from Hy-line, Dallas Center, Iowa. Hyline chicken embryo fibroblasts were V⁻C/O, chicken helper factor negative, and avian leukosis virus group-specific antigen negative. Fertile line 100 chicken eggs were a gift from L. B. Crittenden, U. S. Department of Agriculture, East Lansing, Mich. Line 100 chicken embryo fibroblasts were V⁺ and either C/BE or C/O in phenotype. Fertile Orlopp turkey eggs were obtained from the Wilmar Poultry Co., Wilmar, Minn. Turkey embryo fibroblasts were T/BD, virus negative, and helper factor negative.

RAV-0 was harvested from cultures of V⁺C/O line 100 chicken embryo fibroblasts. Virus production was assayed by determining the sedimentable DNA polymerase activity in culture fluids (9). Infectious RAV-0 was quantitated by endpoint dilution assays, and results were expressed as infectious units (IU) per milliliter of culture fluid.

Isolation of cell clones. Cell clones were isolated by the general procedure of Beug and Graf (3).

Line 100 V⁺C/O and V⁺C/BE chicken embryo fibroblasts were plated at a density of 100 or 1,000 cells per 100-mm culture dish in F-10 medium supplemented with 20 to 30% newborn calf serum (Microbiological Associates, Bethesda, Md.), 2.5% chicken serum (GIBCO Laboratories, Grand Island, N.Y.), 25 mM HEPES (pH 7.3), and 10% Temin modified Eagle minimal essential medium. Cell suspensions were screened microscopically before plating to ensure the absence of cell aggregates. Colonies larger than 1 mm in diameter were selected after incubation for 7 to 14 days at 37°C and were transferred to 35-mm culture dishes containing 2 ml of the medium used for cloning. After the cells reached confluency in 35-mm dishes, they were grown in ET medium supplemented with 5% calf serum and 2% fetal bovine serum.

V⁻C/O chicken embryo fibroblasts were exposed to RAV-0 at a multiplicity of infection of 0.01 in the presence of polybrene (5 µg/ml). Cells were cultured until virus production was detectable by an assay of sedimentable DNA polymerase activity (two subcul-

tures) and were cloned 10 days after exposure to RAV-0, as described above.

Extraction of DNA. High-molecular-weight DNAs were extracted as described by Copeland et al. (11).

Transfection assays. Transfection assays were performed as previously described, except that all recipient cell cultures were treated with 30% dimethyl sulfoxide after exposure to DNA (7). The infectivities of DNA were quantitated by endpoint dilution assays (8), in which recipient cell cultures were exposed to fivefold serial dilutions of DNA ranging from 0.04 to 5 µg of DNA per culture. Three to six recipient cell cultures were inoculated with each DNA concentration. Results are expressed as IU per microgram of DNA.

Preparation of [³²P]cDNA. RNA was extracted from sucrose-banded virions of RAV-2. ³²P-labeled complementary DNA (cDNA) (specific activity, 4 × 10⁸ cpm/µg) which was representative of the entire RAV-2 genome was synthesized from polyadenylic acid-containing 35S RNA by using oligonucleotides of salmon sperm DNA as primers, as described by Shank et al. (29).

Restriction endonuclease digestions, electrophoresis, transfers, and hybridizations. Samples of DNA were digested to completion with an excess of restriction endonuclease under the reaction conditions recommended by the manufacturer (New England Biolabs, Inc., Beverly, Mass.). λ DNA (2 µg) was included in all reaction mixtures to monitor the extent of digestion.

DNAs (5 µg) were electrophoresed in 0.8% agarose gels and transferred to nitrocellulose filters as previously described (10). Filters were baked, prehybridized for 2 h at 68°C, and hybridized to [³²P]cDNA overnight at 41°C; 10% dextran sulfate was included in the hybridization buffer (11).

RESULTS

Expression and infectivities of RAV-0 DNAs of uninfected and mass-infected turkey and chicken embryo fibroblasts. The integration sites of RAV-0 DNAs were investigated in turkey embryo fibroblasts (T/BD cells), V⁻ chicken embryo fibroblasts that were sensitive to exogenous RAV-0 infection (V⁻C/O cells), and V⁺ line 100 chicken embryo fibroblasts that were either sensitive (V⁺C/O cells) or resistant (V⁺C/BE cells) to exogenous RAV-0 infection. Line 100 cells of the V⁺C/O phenotype are exogenously infected with RAV-0 as a consequence of exposure to the virus which they spontaneously produce. In contrast, line 100 cells of the V⁺C/BE phenotype contain only endogenous RAV-0 genomes, since these cells are resistant to exogenous RAV-0 infection.

Uninfected T/BD, V⁻C/O and V⁺C/BE cells did not produce virus particles which were detectable by assays of the sedimentable DNA polymerase activities of culture fluids (Table 1). However, V⁺C/BE cells, but not T/BD or V⁻C/O

O cells, did produce low levels of infectious RAV-0 (1 to 10 IU/ml), which could be detected by cocultivation of V⁺C/BE cells with V⁻C/O cells or by infection of V⁻C/O cells with supernatant media of V⁺C/BE cell cultures (9, 31). In contrast, RAV-0-infected T/BD, V⁻C/O, and V⁺C/O cells produced high levels of RAV-0, which were detectable by assays of sedimentable DNA polymerase activity (Table 1) and corresponded to titers of approximately 10⁴ IU/ml of culture fluid. As previously reported (9), the DNAs of uninfected cells lacked infectivity in transfection assays, whereas the DNAs of RAV-0-infected T/BD, V⁻C/O, and V⁺C/O cells had specific infectivities of 2 to 10 IU/ μ g of DNA (Table 1).

Restriction endonuclease analyses of RAV-0 DNAs of uninfected and mass-infected cells. Astrin (1) has characterized the endogenous RAV-0-related DNAs of different phenotypes of uninfected chicken cells by the molecular weights of RAV-0 DNA-containing *Sst*I fragments. Therefore, we investigated the endogenous RAV-0-related DNAs of the cells used in the present study by digestion with *Sac*I (an isoschizomer of *Sst*I), electrophoresis in agarose gels, transfer to nitrocellulose filters, and hybridization to [³²P]cDNA representative of the 35S RNA of RAV-2, an avian leukosis virus strain that shares approximately 80% sequence homology with RAV-0 (16). No hybridization to the DNA of uninfected turkey cells was detected (Fig. 1, lane a), confirming the lack of significant endogenous avian leukosis virus sequences in turkey DNA (30). *Sac*I digestions of the DNAs of cells of the two V⁻C/O embryos used in this study yielded an RAV-0 DNA-containing fragment of 5.2 $\times$ 10⁶ daltons (Fig. 1, lanes b and c). Astrin (1) designated the endogenous virus locus defined by this fragment, which is present in most White Leghorn chickens, as *ev*-1. *Sac*I digestions of the DNAs of V⁺C/O cells (Fig. 1, lane d) and V⁺C/BE cells (Fig. 1, lane e) yielded the 5.2 $\times$ 10⁶-dalton *ev*-1 RAV-0 DNA-containing fragment and an additional RAV-0 DNA-containing fragment of 3.6 $\times$ 10⁶ daltons. The 3.6 $\times$ 10⁶-dalton *Sac*I fragment corresponds to the *ev*-2 locus, which is associated with the V⁺ phenotype in line 7 and line 100 chickens (1, 2). The observation that the *Sac*I digests of the DNAs of V⁺C/O cells are indistinguishable from those of V⁺C/BE cells is consistent with the results of Astrin (1) and suggests that exogenous RAV-0 proviruses of V⁺C/O cells may be integrated at multiple sites in an RAV-0-infected V⁺C/O cell population.

The integration sites of RAV-0 DNA in mass-infected cultures were investigated further by *Eco*RI digestions of the DNAs of RAV-0-infected T/BD, V⁻C/O, and V⁺C/O cells. *Eco*RI

TABLE 1. Virus production and infectivities of DNAs of mass cultures of uninfected and RAV-0-infected cells^a

Cells	DNA polymerase activity (cpm/h per 10 ml of culture fluid)	IU/ μ g of DNA
Uninfected		
T/BD	<2 $\times$ 10 ²	<0.2
V ⁻ C/O	<2 $\times$ 10 ²	<0.2
V ⁺ C/BE	<2 $\times$ 10 ²	<0.03
Infected		
T/BD(RAV-0)	1.8 $\times$ 10 ³	2
V ⁻ C/O(RAV-0)	3.8 $\times$ 10 ³	8
V ⁺ C/O	4 $\times$ 10 ³	10

^a Production of RAV-0 by uninfected and RAV-0-infected cells was determined by assays of the sedimentable DNA polymerase activity of culture fluids. Infectivities of uninfected and RAV-0-infected cell DNAs were determined by transfection of V⁻C/O cells. RAV-0-infected turkey cells [T/BD(RAV-0) cells] and V⁻C/O cells [V⁻C/O(RAV-0) cells] were assayed 10 to 18 days after inoculation of the donor cells with RAV-0.

FIG. 1. *Sac*I cleavage of avian cell DNAs. The DNAs of uninfected turkey cells (lane a), cells of two different uninfected V⁻C/O embryos (lanes b and c), V⁺C/O cells (lane d), and V⁺C/BE cells (lane e) were digested with *Sac*I, electrophoresed in 0.8% agarose gels, transferred to nitrocellulose filters, and hybridized to RAV-2 [³²P]cDNA. HindIII fragments of ³²P-labeled λ DNA were electrophoresed in the same gel as molecular weight markers. The molecular weights of RAV-0 DNA-containing fragments are indicated on the right ($\times 10^6$).

cleaves unintegrated linear RAV-0 DNA at two sites, yielding an internal fragment of 2.5 $\times$ 10⁶ daltons and two terminal fragments of approximately 1.5 $\times$ 10⁶ and 0.8 $\times$ 10⁶ daltons (18, 20). Therefore, *Eco*RI digestion of integrated RAV-

0 DNA should yield the 2.5×10^6 -dalton internal fragment and two junction fragments with molecular weights determined by the positions of the *Eco*RI cleavage sites in the flanking cellular DNA sequences.

*Eco*RI digestion of the DNA of mass cultures of RAV-0-infected T/BD cells yielded the 2.5×10^6 -dalton internal RAV-0 DNA-containing fragment but no detectable RAV-0 DNA-containing junction fragments (Fig. 2, lane b). Similarly, comparisons of *Eco*RI digests of the DNAs of uninfected V⁻C/O cells (Fig. 2, lane c) and RAV-0-infected V⁻C/O cells (Fig. 2, lane d) and of *Eco*RI digests of the DNAs of uninfected V⁺C/BE cells (Fig. 2, lane f) and RAV-0-infected V⁺C/O cells (Fig. 2, lane e) indicated that the DNAs of mass cultures of RAV-0 infected cells did not yield RAV-0 DNA-containing *Eco*RI junction fragments that were not present in the DNAs of the homologous uninfected cells. *Eco*RI digestions of the DNAs of both V⁻ and V⁺ cells yielded RAV-0 DNA-containing junction fragments of 5.2×10^6 and 11×10^6 daltons, which are characteristic of the *ev-1* locus (1). *Eco*RI digestions of the DNAs of V⁺ cells also yielded RAV-0 DNA-containing junction fragments of approximately 1.9×10^6 and 2.1×10^6 daltons, which are characteristic of the *ev-2* locus (1). *Eco*RI digestions of DNAs of V⁻C/O cells also yielded an RAV-0 DNA-containing fragment of approximately 0.8×10^6 daltons which has not been reported previously. The inability to detect specific RAV-0 DNA-containing junction fragments in cell cultures that were mass-infected with RAV-0 is consistent with the hypothesis that RAV-0 DNA was integrated at multiple sites in the infected cell populations.

Expression and infectivity of RAV-0 DNA in clones of RAV-0-infected chicken cells. Since junction fragments corresponding to exogenous RAV-0 proviral DNAs were not detected in mass-infected cultures, we investigated the integration sites of RAV-0 DNAs in individual clones of RAV-0-infected cells. V⁺C/BE, V⁺C/O, and V⁻C/O cells that were exposed to RAV-0 were cloned as described above, and individual clones were cultured to populations of 10^7 to 10^8 cells. All three clones of V⁺C/O cells examined produced RAV-0 and contained infectious RAV-0 DNA (Table 2). In contrast, cloned V⁺C/BE cells did not produce titers of RAV-0 that were detectable by assaying culture fluids for sedimentable DNA polymerase activity, and DNA isolated from cloned V⁺C/BE cells was not infectious (Table 2). However, production of a low titer (1 to 10 IU/ml) of infectious RAV-0 by cloned V⁺C/BE cells was demonstrated by assays of culture fluids on V⁻C/O cells (data not shown).

FIG. 2. *Eco*RI cleavage of DNAs of uninfected and RAV-0-infected cells. The DNAs of uninfected (lane a) and RAV-0-infected (lane b) turkey cells, uninfected (lane c) and RAV-0-infected (lane d) V⁻C/O cells, and V⁺C/O (lane e) and V⁺C/BE (lane f) line 100 cells were digested with *Eco*RI and analyzed as described in the legend to Fig. 1. The molecular weights of marker fragments of *Hind*III-digested ³²P-labeled λ DNA are indicated on the right ($\times 10^6$).

TABLE 2. Virus production and infectivities of DNAs of cloned chicken embryo fibroblasts^a

Cells	DNA polymerase activity (cpm/h per 10 ml of culture fluid)	IU/ μ g of DNA
V ⁺ C/O		
Clone 1	1.7×10^4	12
Clone 2	4.9×10^3	2
Clone 3	1.1×10^4	6
V ⁺ C/BE clone 1	$<2 \times 10^2$	<0.2
V ⁻ C/O(RAV-0)		
Clone 1	1.2×10^4	5
Clone 2	4.5×10^4	30
Clone 3	5.8×10^3	2
Clone 4	1.6×10^3	0.4
Clone 5	1.4×10^4	2

^a The production of RAV-0 by cloned chicken cells and the infectivities of the DNAs of cloned cells were determined as described in Table 1, footnote a.

A total of 16 clones were isolated from V⁻C/O cells 10 days after exposure to RAV-0 at a multiplicity of 0.01 IU/cell. Only five of these clones produced RAV-0, as detected by assaying culture fluids for sedimentable DNA polymerase activity or for infectious RAV-0 by inoculation of V⁻C/O cells (Table 2; data not shown). Thus,

it appeared that the population from which the V⁺C/O cell clones were isolated contained only about 30% RAV-0-infected cells, presumably due to the low efficiency of RAV-0 replication (22, 26). The DNAs of all five RAV-0-infected V⁺C/O cell clones were infectious in transfection assays (Table 2).

An examination of the data in Table 2 indicated that the amount of virus produced by different clones of RAV-0-infected V⁺C/O and V⁺C/O cells appeared to correlate with the infectivity of the RAV-0 DNA extracted from each clone of cells. This correlation is illustrated in Fig. 3, in which the amount of virus produced by each of the RAV-0-infected clones is presented as a function of the infectivity of the DNA of that clone. The amount of virus produced by each clone of RAV-0-infected cells was proportional to the infectivity of the DNA of that clone of cells (correlation coefficient, 0.96), indicating that the exogenous RAV-0 proviral DNAs were expressed to different extents in different clones of infected cells and that the levels of expression of these exogenous RAV-0 proviral DNAs correlated with their infectivities in transfection assays.

To determine whether the differences in virus production and DNA infectivity in the different clones were related to differences in their viral genomes, we investigated the specific infectivities of the viruses produced by different clones.

FIG. 3. Correlation between virus production and infectivity of DNAs of cloned chicken cells. The amounts of RAV-0 produced by individual clones of V⁺C/O cells (○), V⁺C/BE cells (×), and RAV-0-infected V⁺C/O cells (●) are shown as functions of the infectivities of the DNAs of those clones. Data are from Table 2.

Viruses produced by V⁺C/O cell clones 1, 2, and 3 and by RAV-0-infected V⁺C/O cell clones 1, 2, 3, and 4 were passaged once through V⁺C/O cells. These progeny virus stocks were then assayed by endpoint dilution on V⁺C/O cells to determine the titer of infectious virus and by sedimentable DNA polymerase activity to estimate the concentration of virus particles. The specific infectivities of the viruses produced by the different clones did not differ significantly (less than 10-fold) from an average of approximately 700 IU/cpm per h of DNA polymerase activity. Therefore, it appeared that differences in the viral genomes of the different clones did not account for the observed differences in virus production and DNA infectivity.

Restriction endonuclease analyses of DNAs of clones of RAV-0-infected chicken cells. *Eco*RI digestion of the DNA of each of the three clones of RAV-0-infected V⁺C/O cells (Fig. 4, lanes b through d) yielded specific RAV-0 DNA-containing fragments that were not detected in the DNAs of mass cultures of V⁺C/O cells (Fig. 4, lane a), uncloned V⁺C/BE cells (Fig. 4, lane e), or cloned V⁺C/BE cells (Fig. 4, lane f). The number of additional RAV-0 DNA-containing *Eco*RI fragments in the three clones of V⁺C/O cells varied from a minimum of five (Fig. 4, lanes b and c) to seven (Fig. 4, lane d), although additional fragments may not have been detected. With the exception of a 1.3×10^6 -dalton fragment which was common to V⁺C/O clones 2 and 3 (Fig. 4, lanes c and d), the RAV-0 DNA-containing fragments present in different clones had different molecular weights.

*Eco*RI digestions of the DNAs of four RAV-0-infected V⁺C/O cell clones (Fig. 4, lanes h through k) indicated that RAV-0 DNA-containing fragments with molecular weights of 2×10^6 (Fig. 4, lane h), 2.4×10^6 (lane i), 1.5×10^6 (lane j), and 4.2×10^6 (lane k) were present in the DNAs of the cloned cells but not present either in the DNAs of mass-infected V⁺C/O cells (Fig. 4, lane g) or in the DNAs of two of the non-virus-producing, apparently uninfected clones of V⁺C/O cells (Fig. 4, lanes l and m). There appeared to be only a single extra RAV-0 DNA-containing fragment in the DNA of each of the RAV-0-infected V⁺C/O cell clones, and the extra fragments appeared to hybridize less intensely than the extra RAV-0 DNA-containing fragments of the DNAs of cloned V⁺C/O cells. These results suggest the possibility that the RAV-0-infected V⁺C/O cell clones may have represented cell populations which were still somewhat heterogeneous with respect to RAV-0 DNA integration sites. This heterogeneity could have occurred if the cells did not contain stably inte-

FIG. 4. *EcoRI* cleavage of DNAs of cloned chicken embryo fibroblasts. The DNAs of mass-infected V^+C/O cells (lane a), V^+C/O cell clones 1 through 3 (lanes b through d, respectively), mass cultures of V^+C/BE cells (lane e), V^+C/BE cell clone 1 (lane f), mass-infected V^-C/O cells (lane g), RAV-0-infected V^-C/O cell clones 1 through 4 (lanes h through k, respectively), and two non-virus-producing, apparently uninfected V^-C/O cell clones (lanes l and m) were digested with *EcoRI* and analyzed as described in the legend to Fig. 1. The molecular weights of marker fragments of *HindIII*-digested ^{32}P -labeled λ DNA are indicated ($\times 10^6$). The two bands of approximately 3.5×10^6 and 4.5×10^6 daltons which were detected in lane a but not in Fig. 2, lane e, may have been due to some contamination (e.g., ribosomal cDNA) of the probe used in this experiment.

grated RAV-0 proviral DNA at the time of cloning, so that some of the cells in each clone either might have lacked RAV-0 DNA or might have contained RAV-0 DNA integrated at other sites in the cell genome. This possibility is also consistent with the detection of only a single RAV-0 DNA-containing *EcoRI* junction fragment in the DNAs of the V^-C/O cell clones. The smaller of the two terminal fragments of RAV-0 DNA (0.8×10^6 daltons) is homologous to the 3' end of RAV-0 RNA (18), which has reduced homology to the genome of RAV-2 and other exogenous avian leukosis viruses (5, 25, 35). Since [^{32}P]cDNA prepared from RAV-2 RNA was used as the probe in these experiments, junction fragments derived from the 3' terminus of the RAV-0 genome may have been undetectable in the V^-C/O cell clones. Nevertheless, the finding that clones of RAV-0-infected V^+C/O and V^-C/O cells contained unique *EcoRI* junction fragments of RAV-0 DNA with different molecular weights in different clones indicated that exogenous RAV-0 proviruses can integrate at multiple sites in the genomes of RAV-0-infected cells.

DISCUSSION

In the studies reported here, we investigated the integration sites, expression, and infectivities of exogenously acquired RAV-0 proviral DNAs of RAV-0-infected cells. Restriction endonucle-

ase analyses of the DNAs of RAV-0-infected turkey cells, V^- chicken cells, and V^+ chicken cells indicated that mass-infected cells did not contain unique junction fragments of RAV-0 DNA, suggesting that exogenous RAV-0 proviruses were integrated at multiple sites in cellular DNA. These observations, which are consistent with the results of others (1, 13, 20), were confirmed by analyses of the integration sites of RAV-0 DNA in individual clones of RAV-0-infected V^- and V^+ chicken cells. Unique RAV-0 DNA-containing junction fragments were found in the DNAs of cloned cells, indicating the presence of exogenous RAV-0 proviruses. The integration sites of exogenous RAV-0 proviral DNAs differed in each of seven clones examined, indicating that exogenous RAV-0 proviruses can integrate at multiple sites in cellular DNA, as has been reported previously for other avian and mammalian retroviruses (19, 21, 28, 32).

Exogenous RAV-0 DNAs of both mass cultures and clones of RAV-0-infected cells were 10^3 - to 10^4 -fold more infectious in transfection assays than the endogenous RAV-0 genome of uninfected V^+ chicken cells (7). The finding that exogenous RAV-0 proviruses are integrated at multiple sites, which are distinct from the site of integration of endogenous RAV-0 DNA, is thus consistent with the hypothesis that regulatory sequences flanking the endogenous RAV-0 genome of V^+ chicken cells are responsible for the

low transcriptional activity and low infectivity of the endogenous RAV-0 genome (7, 9).

Although all clones of RAV-0-infected chicken cells contained infectious RAV-0 DNA and produced high titers of RAV-0 compared with uninfected V^+ chicken cells, different infected cell clones differed in their levels of RAV-0 production. In addition, the infectivity of the DNA of each clone of RAV-0-infected chicken cells correlated with the amount of virus produced by that clone. These differences were not correlated with the number of exogenous RAV-0 proviruses present in the different clones, since some clones of V^+C/O cells containing three or four copies of exogenous RAV-0 DNA did not produce higher titers of virus than some clones of V^-C/O cells which appeared to contain only one copy of exogenous RAV-0 DNA. In addition, the specific infectivities of the viruses produced by different clones of infected cells were not significantly different, indicating that the differences in virus production and DNA infectivity of the different clones were not a consequence of differences in the viral genomes present. Therefore, these observations indicate that qualitative differences in the exogenous RAV-0 DNAs of different clones of infected cells were correlated with the extent of expression of these different RAV-0 proviruses.

Studies of DNA modification have indicated that the extent of methylation of both cellular and viral DNAs is correlated with gene expression in that inactive genes are frequently highly methylated (6, 15, 23, 33). However, it is not clear whether DNA methylation directly affects gene expression, particularly since recent studies have indicated a lack of correlation between methylation and transcriptional activity of sea urchin histone genes (4) and of human globin genes in some tissues and cell lines (33). Nevertheless, it is possible that differences in the methylation of RAV-0 proviruses could affect their expression and infectivity in transfection assays.

Alternatively, cellular sequences flanking RAV-0 proviruses could affect expression of viral DNA. In the case of the endogenous RAV-0 provirus of uninfected V^+ cells, this hypothesis was substantiated by the finding that dissociating the endogenous RAV-0 genome from its flanking cellular sequences specifically increased the infectivity of endogenous RAV-0 DNA (7). Thus, the results presented here may indicate that cellular sequences flanking exogenous RAV-0 DNAs also affect expression of RAV-0 proviruses.

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