Clonal Analysis of the Integration and Expression of Endogenous Avian Retroviral DNA Acquired by Exogenous Viral Infection

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Rous-associated virus-0 is one of several endogenous avian retroviruses that are transmitted vertically and that can be isolated from different inbred lines of chickens. These viruses, referred to here as induced-leukosis viruses bearing a subgroup E glycoprotein (ILV-E), are all closely related. Clonal populations of fibroblasts from line 15g and line 100 inbred chickens have been examined for the presence and expression of exogenously acquired ILV-E sequences. Restriction enzyme analysis of uniform populations of line 15g fibroblasts, prepared by cloning cells either before or after infection with ILV-E, indicates that viral sequences were inserted at multiple sites within the cell genome. Analysis of 49 clonal populations of line 100 fibroblasts containing between one and five copies of exogenous ILV-E sequences demonstrated that each clone was characterized by a unique set of viral DNA insertions within the cell genome. The expression of the exogenous ILV-E sequences within these fibroblast clones was examined by using reverse transcriptase activity as a measure of virus production. Some clones produced an amount of virus equivalent to that produced by an equal number of the uncloned ILV-E-infected parental fibroblasts. Other clones produced 5- to 10-fold less virus. Still other clones produced no detectable virus at all. Among nine clones derived from cells containing a single copy of the ILV-E provirus, the level of virus production differed more than 100-fold. DNA from these clones was analyzed with several different restriction endonucleases to characterize the location and arrangement of the ILV-E sequences. All nine clones consisted of cells that appeared to contain a complete provirus inserted (i) in a different site within the cellular DNA and (ii) in an orientation that was colinear with the viral genomic RNA. It was observed that several cleavage sites potentially affected by methylation were equally available for cleavage in all clones regardless of the level of viral production.

Infection of a cell by a retrovirus results in the formation of a DNA copy of the viral genome. This viral DNA intermediate is inserted into host cell DNA to form a structure referred to as the provirus (33). Analysis of integrated retroviral DNA sequences, present in both mammalian and avian cellular DNA, indicates that the provirus is terminally redundant and colinear with unintegrated linear viral DNA (14, 19). Several investigations have demonstrated a similarity between the provirus and the bacterial transposon (9, 18, 29). Analysis of one of the endogenous retroviral loci in the chicken, ev-1, has revealed that this locus resembles the generalized structure of the transposon (13). This generalized structure is characterized by a large direct repeat of several hundred base pairs at its termini and by a few base pairs present as inverted repeats distal to the direct repeats. The structure of the provirus has been designated as cell DNA—3'-5' virus DNA 3'-5'—cell DNA (14). The results of several studies have uniformly suggested that the viral sequences are inserted into many sites within the host chromosome. Whether integration into different sites within the host DNA influences expression of the inserted viral sequences is unknown.

Several endogenous avian retroviruses transmitted through the germ line tissue have been identified (8, 24, 27). Some of these viruses, such as ILV-15 and ILV-E7, have been associated with specific endogenous loci characteristic of different inbred lines of chickens (2, 7, 25). In general, endogenous viral sequences are expressed inefficiently and produce very low titers of virus. These viruses can, however, establish an infection in a permissive cell in which an exogenously acquired provirus is inserted into
the host cell genome. Several studies have demonstrated that cells containing exogenously acquired ILV-E proviruses are associated with increased virus production (6, 16, 30). Two experimental systems have been established in which fibroblasts from an inbred line of chickens can be obtained that contain either endogenous or exogenous and exogenous ILV-E sequences. Uninfected line 15B fibroblasts contain two endogenous loci, ev-1 and ev-7. These cells can be exogenously infected by ILV-E15, an endogenous virus derived at least in part from ev-7 (26). Line 100 fibroblasts, containing the endogenous loci ev-1 and ev-2, are V+ (8) and produce low levels of ILV-E7, an endogenous virus derived from ev-2 (2). Fibroblasts from some line 100 chickens are resistant to infection by ILV-E7 and contain only the endogenous loci. Other line 100 chickens are sensitive to ILV-E7 infection so that fibroblasts from these chickens acquire exogenous copies of ILV-E7 (8).

The amount of virus produced from an uncloned population of cells exogenously infected by one of the endogenous avian viruses, such as ILV-E15-infected line 15 cells or ILV-E7-infected line 100 cells, is 109 to 104-fold greater than that produced from an equivalent population of cells containing only the endogenous sequences (30). Restriction enzyme analysis of the DNA from the ILV-E-infected line 15 and line 100 fibroblasts suggested that the exogenous viral sequences were inserted in many sites within the cell DNA (16, 17). Further, several restriction endonuclease cleavage sites present in DNA from cells containing the exogenous ILV sequences were absent from or unavailable for cleavage in the DNA containing only the endogenous sequences (16). In the absence of an analysis of virus production from clones containing a single complete exogenous provirus, it was not known from these studies whether all exogenously introduced ILV sequences (despite possible insertion at many locations) produced virus at equal rates. Further, it was not known whether any exogenously acquired proviruses were expressed as inefficiently as the endogenous sequences.

To examine the integration and expression of individual exogenous ILV-E sequences, we prepared clonal populations of ILV-E-infected line 15B and line 100 fibroblasts. We have examined these clones both for the presence and arrangement of ILV-E sequences and for the production of virus. The results indicate that two exogenous avian retroviruses, ILV-E7 and ILV-E15, integrated into multiple sites within the host genome. Our analysis of nine individual proviruses, all of which appeared complete, showed that they produced virus at rates that varied by more than 100-fold.

**MATERIALS AND METHODS**

**Cells and viruses.** All cells were cultured in plastic dishes (Nunc, Denmark) with Dulbecco modified Eagle medium (Flow Laboratories, Bethesda, Md.) containing 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) supplemented with calf serum (Sterile Systems, Logan, Utah) and newborn calf serum (Microbiological Associates, Walkersville, Md.) in a humidified atmosphere at 38°C containing 5 to 10% CO2.

Chicken embryo fibroblasts (CEF) prepared from line 15B and line 100 chickens have been described previously (16). The line 15B fibroblasts were V+, C/C, chicken-helper factor negative, avian leukosis group-specific antigen negative, and free of avian leukosis virus. The line 15B fibroblasts used in this study were prepared from a single embryo and contained the endogenous viral loci 1 and 7 (ev-1 and ev-7) as defined by SacI restriction endonuclease analysis (1). Line 100 chickens are V+, producing an endogenous virus with a subgroup E glycoprotein (ILV-E7) and segregating for susceptibility to infection by subgroup B and E avian leukosis virus. The line 100 fibroblasts used in this study were derived from two individual embryos, one C/BE and one C/O. Both embryos were V+ and contained the endogenous viral loci 1 and 2 (ev-1 and ev-2) (1).

Fibroblasts prepared from one line 15B embryo, previously described (16), spontaneously produced an endogenous virus with a subgroup E glycoprotein. This virus has been designated induced leukosis virus-15B (ILV-E15) and, as previously suggested (26), appears to be derived, at least in part, from ev-7. Because line 15B fibroblasts are Gr+ (23), high titers of this virus were prepared from fibroblasts of this embryo after spontaneous exogenous ILV-E15 infection.

**Preparation and characterization of cloned CEF.** CEF were cloned by the procedure of Beug and Graf (3). The cells clones were usually second or third passage after preparation of primary cultures. Cells were cloned in Ham F-10 (Microbiological Associates) supplemented with 20% fetal calf serum (Reheis, Phoenix, Ariz.), 0.5% beef embryo extract (GIBCO Laboratories, Grand Island, N.Y.), 2.5% chicken serum (GIBCO) and 0.025 M N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (pH 7.3). Approximately 100 cells were plated per 90-mm dish and incubated at 38°C for 9 to 10 days. Individual colonies were removed by using trypsin and cloning rings and transferred to 17-mm wells in Dulbecco modified Eagle medium supplemented with 10% tryptose phosphate broth, 3% fetal calf serum (Sterile Systems), 3% calf serum, and 1% chicken serum. Plating efficiency, as defined by the number of colonies establishing a diameter of 5 to 6 mm, was approximately 20 to 30%. The cloning efficiency, as defined by the number of colonies that grew to populations greater than 5 x 107 cells, was 5 to 6%.

During the course of this study, several cloning experiments were conducted. Although uninfected CEF from several different line 15B embryos were...
cloned (all with similar efficiency), results from only one embryo are presented here. Some clones of uninfected line 15w CEF were infected with ILV-E15 after the original isolation of the clone. After transfer of the clone to a 17-mm well, approximately $5 \times 10^5$ to $10 \times 10^5$ cells, the cells were exposed three times, each time for 60 min at 38°C, to freshly harvested ILV-E15. By an identical procedure, line 15s CEF were also infected with ILV-E15 before cloning. The infected cells were then cultured for four to six generations before cloning.

Clones of line 15 or line 100 CEF containing exogenously introduced DNA copies of the endogenous viruses ILV-E15 and ILV-E7, will be referred to, respectively, as line 15(+) and line 100(+) clones. Both line 15(+) and line 100(+) clones were characterized for the production of virus by measurement of the RNA-directed DNA transcriptase activity in the culture fluid. A 16-h culture fluid from a single 60-mm dish was clarified by centrifugation for 10 min at 3,000 × g. The sedimentable transcriptase activity was then concentrated by centrifugation for 1 h at 60,000 × g, suspended in 50 μl of 50 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA and stored at −20°C. Enzyme activity was measured per 5 μl of the concentrated culture fluid. After disruption of the sample in a final concentration of 20 mM Tris-hydrochloride (pH 8.0), 1 mM EDTA, 0.25% Nonidet P-40 and 12 mM dithiothreitol, enzyme activity was measured by a reaction containing final concentrations of 20 mM Tris-hydrochloride (pH 8.0), 1 mM EDTA, 100 μCi of [methyl-3H]dATP per ml (specific activity, 80 Ci/mmOL), and 0.5 μg of polyadenylic acid-deoxythymidylic acid. Under these conditions, enzyme activity was linear for 20 to 30 min.

The RNA-directed DNA transcriptase activity was determined for a single 60-mm dish on 2 consecutive days containing approximately $2 \times 10^7$ and $4 \times 10^7$ cells, respectively. Using enzyme activities obtained from uncloned line 100 C/O fibroblasts, we have determined (i) that the transcriptase activity accumulates in cultures in a linear fashion for 16 to 20 h, (ii) that the amount of activity detected is proportional to the cell density of a given culture up to at least $6 \times 10^5$ cells per 60-mm plate, and (iii) that sample error due to the mechanics of the assay averaged 20%. The results of the transcriptase assay are presented as the number of counts per minute incorporated for a 30-min reaction by the virus produced from $4 \times 10^7$ cells during a 16-h interval.

Preparation of viral and cellular DNA. ILV-E15 and ILV-E7 DNA intermediates were prepared by a modification of the procedure described previously (16). Uninfected line 15w CEF were plated at 4 x 10^7 cells per 90-mm plate. At 2 h after plating they were exposed to either ILV-E15 or ILV-E7. The virus was prepared from cultures of line 15(+) and line 100(+) CEF, respectively, by harvesting the growth medium every 2 h from producing cultures and adding it immediately to the line 15w CEF. The infection, 2 h at 38°C, was repeated for a total of four times, and then growth medium with 10% calf serum was added. The cells were prepared for a Hirt extraction 24 h after the last infection. The Hirt supernatant was extracted with phenol and chloroform and precipitated with ethanol. After centrifugation, the precipitate was resuspended in 10 mM Tris-hydrochloride (pH 8.0)–10 mM EDTA and treated with 100 μg of RNase A per ml for 30 min in 0.5% sodium dodecyl sulfate for 2 h at 37°C. The DNA was further treated with 100 μg of proteinase K per ml in 0.5% sodium dodecyl sulfate for 2 h at 37°C, extracted with phenol and chloroform, and precipitated with ethanol. After centrifugation, the DNA was dissolved in 10 mM Tris-hydrochloride (pH 8.0)–10 mM NaCl–10 mM EDTA and reprecipitated with 200 mM sodium acetate and ethanol. After two or three additional precipitations with 200 mM sodium acetate and ethanol, the DNA was dissolved in 10 mM Tris-hydrochloride (pH 8.0)–10 mM NaCl–1 mM EDTA and stored at 4°C. The viral DNA prepared in this manner appeared to contain only the linear form of the intermediate as described previously (16).

Cellular DNA was prepared using a modification of the procedure described earlier (16). The cells were collected by centrifugation after trypsinization and washed in phosphate-buffered saline (10 mM PO4, [pH 7.5], 150 mM NaCl). The cells were swollen in 10 mM Tris-hydrochloride (pH 8.0)–10 mM NaCl–10 mM EDTA for 15 min on ice, adjusted to 1% Triton X-100, and incubated at 37°C for 15 min, and the nuclei were collected by centrifugation at 4,000 × g for 10 min. The nuclei were resuspended in 10 mM Tris-hydrochloride (pH 8.0)–10 mM NaCl–10 mM EDTA, adjusted to 0.025% sodium dodecyl sulfate and 100 μg of RNase A per ml, and incubated for 2 h at 37°C. The preparation was then adjusted to 0.5% sodium dodecyl sulfate and 100 μg of proteinase K per ml and rocked gently overnight at 37°C. The preparation was extracted twice with phenol and twice with chloroform and dialyzed against 10 mM Tris-hydrochloride (pH 8.0)–10 mM NaCl–1 mM EDTA at room temperature and stored at 4°C.

Restriction endonuclease analysis, DNA electrophoresis, and hybridization. Restriction endonucleases HhaI, KpnI, SacI, SalI, XbaI, and XhoI were purchased from New England Biolabs (Cambridge, Mass.). Restriction endonucleases BglII, BglIII, BamHI, EcoRI, and HincII were purified as described earlier (16). Restriction endonuclease HindIII was purchased from Boehringer Mannheim (Germany). All nuclease digestions were carried out using the conditions as defined by New England Biolabs. Viral linear DNA intermediates were mapped for the location of specific restriction endonuclease cleavage sites using standard methods. Since preparations of both ILV-E15 and ILV-E7 DNA contained very little DNA (less than 1 or 2 μg per five digestions), restriction endonuclease reactions were carried out based upon added carrier λ DNA, usually 1 μg per reaction. When two different restriction endonucleases were used to digest a single DNA sample, the reaction was carried out first for the enzyme requiring the lowest concentration of salt. After the first reaction was completed, the salt was adjusted for the second reaction, and the second enzyme was added. After endonuclease digestion, viral DNA samples were adjusted to 15% sucrose and 0.02% bromophenol blue and were loaded directly on 1.4% agarose gels for electrophoresis and hybridization analysis as described below. Some experiments required the isolation of specific viral DNA fragments produced by one restriction endonuclease before digestion with the second
endonuclease. Such DNA fragments were isolated after electrophoresis on 1% low-melt agarose gels (Bethesda Research Laboratories). DNA fragments were recovered by slicing the gel, dialyzing the gel slices against 10 mM Tris-hydrochloride (pH 8.0)-10 mM MgCl₂ at room temperature (twice for 15 min), melting the gel at 68°C for 10 min, and equilibrating the sample at 37°C. After the addition of carrier λ DNA, viral DNA fragments could be isolated directly after extraction with phenol and precipitation with 1% potassium acetate and ethanol. Alternatively, the fragments could be digested directly with a second endonuclease and then isolated. Analysis was continued after electrophoresis on a 1.4% agarose gel as described below.

Cellular DNA digested with restriction endonucleases was extracted with phenol and precipitated with 1% potassium acetate and ethanol overnight at −20°C. The DNA was collected by centrifugation (25,000 × g for 10 min), resuspended in 10 mM Tris-hydrochloride (pH 8.0)-10 mM NaCl-10 mM EDTA-15% sucrose-0.02% bromophenol blue and electrophoresed on 1 or 1.4% agarose gels.

Electrophoretic analysis used 1 or 1.4% agarose gels (Sea Kem; Marine Colloids, Rockland, Maine) in 60 mM Tris-acetate (pH 8.0)-60 mM NaCl-6 mM EDTA at approximately 1 V/cm. Agarose gels were prepared for DNA transfer to BA85 nitrocellulose filter paper (Schleicher & Schull Co., Keene, N.H.) as previously described (16). DNA transfer was carried out using either 6× or 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as described by Southern (31).

DNA bound to nitrocellulose filter paper was analyzed for the presence of virus-specific DNA sequences by hybridization to tdPRB [³²P]RNA, 0.5 × 10⁶ to 1 × 10⁸ cpm/µg, using conditions described previously (16). tdPRB RNA was labeled at 5' OH ends by using [γ-³²P]ATP and polynucleotide kinase. Control experiments showed that hybridization analysis of ev-1, ev-2, and ev-7 (1) with either Rous-associated virus-0 [³²P]-RNA or tdPRB [³²P]-RNA provided identical results. Molecular weight determinants were based upon molecular weights of DNA fragments produced by EcoRI endonuclease digestion of (i) line 7 control DNA containing ev-1 and ev-2 (2, 16) and (ii) the ILV-E7 linear DNA. Electrophoresis and transfer of these two DNA samples provided molecular weight markers of (i) 10 × 10⁶, 4.8 × 10⁶, 2.5 × 10⁶, 2.15 × 10⁶, and 2.0 × 10⁶ daltons and (ii) 2.5 × 10⁶, 1.65 × 10⁶, and 0.85 × 10⁶ daltons, respectively. Fugl RX film and Du Pont Cronex Lightning Plus intensifiers were used for autoradiography at −80°C.

RESULTS

Restriction enzyme characterization of two different ILV-Es and two populations of ILV-E-infected cells. The linear DNA intermediates of two endogenous viruses, ILV-E7 and ILV-E15, were prepared and characterized with site-specific restriction endonucleases. The results are presented in Fig. 1 in the form of

![ILV-E7 and ILV-E15 restriction maps](http://jvi.asm.org/)

**Fig. 1. Location of site-specific endonuclease cleavage sites on the ILV-E7 and ILV-E15 linear DNA molecules.** Site-specific endonuclease cleavage sites for 13 different enzymes are located on two different linear maps. The specific sites account for production of the DNA fragments listed in Table 1. The ILV-E15 map includes all the sites located in these experiments. The ILV-E7 map includes only those enzyme sites that distinguish the ILV-E7 and ILV-E15 DNA molecules. The three Hha I-specific fragments common to both linear DNAs are located by the crossed bars between the maps.
linear maps locating different cleavage sites on each of the two DNA molecules and in Table 1 as a list of the molecular weights of the specific fragments produced by the different restriction endonucleases. Our results generally agree with those of a previous study that characterized the ILV-E7 linear intermediate (28). Minor differences in the BgIII analyses are unexplained. The two endogenous viruses can be distinguished by using either HincII or SacI to analyze the two linear DNA intermediates. The absence of a SacI cleavage site on the ILV-E15 linear DNA has made it possible to enumerate the number of integrated ILV-E15 proviruses present in cellular DNA from a cloned population of cells. The single XbaI site in the ILV-E7 linear DNA

<table>
<thead>
<tr>
<th>Endonuclease</th>
<th>DNA fragment mass ((\times 10^{-6} \text{ M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>0.4 0.9 1.1 2.6</td>
</tr>
<tr>
<td>BglII</td>
<td>3.2 1.8</td>
</tr>
<tr>
<td>BglIII</td>
<td>1.1 1.7 2.2</td>
</tr>
<tr>
<td>EcoRI</td>
<td>1.65 2.5 0.85</td>
</tr>
<tr>
<td>HhaI</td>
<td>0.38 0.9 0.43</td>
</tr>
<tr>
<td>HincII</td>
<td>1.9 2.2 0.9</td>
</tr>
<tr>
<td>HincII*</td>
<td>1.9 2.2 0.3 0.6</td>
</tr>
<tr>
<td>HindIII</td>
<td>2.0 2.3 0.7</td>
</tr>
<tr>
<td>KpnI</td>
<td>3.5 1.5</td>
</tr>
<tr>
<td>PstI</td>
<td>1.7 2.75 0.55</td>
</tr>
<tr>
<td>SacI</td>
<td>5.0</td>
</tr>
<tr>
<td>SacI*</td>
<td>0.3 1.0 3.7</td>
</tr>
<tr>
<td>SaI</td>
<td>4.05 0.95</td>
</tr>
<tr>
<td>XbaI</td>
<td>2.35 2.65</td>
</tr>
<tr>
<td>Xhol</td>
<td>0.5 3.1 1.4</td>
</tr>
</tbody>
</table>

* Digestion of ILV-E7 and ILV-E15 linear DNA was performed with different site-specific endonucleases. The products of digestion were analyzed by hybridization to tdPRB \(^{[32P]}\)RNA after electrophoresis on a 1.4% agarose gel and transfer of DNA fragments to nitrocellulose paper with 20× SSC. Molecular masses were calculated from DNA fragments of 12.6×10^6, 6.3 \(\times 10^6\), 4.4 \(\times 10^5\), 2.8 \(\times 10^5\), 2.5 \(\times 10^5\), and 2.3 \(\times 10^6\) daltons produced by HindIII digestion of \(\lambda\) DNA.

* Fragments are listed in order of their location on the linear viral DNA intermediate proceeding from the 5' to the 3' end of the molecule. Except for HincII and SacI analysis, both ILV-E7 and ILV-E15 have apparently identical site-specific endonuclease digestion products.

* Only three specific fragments have been characterized and assigned to specific regions of the ILV-E7 and ILV-E15 DNA molecules. At least four other fragments smaller than those listed have been observed.

* HincII cuts the ILV-E7 linear DNA at 6.6 kbp to produce 0.3 \(\times 10^6\) and 0.6 \(\times 10^6\)-dalton fragments instead of a 0.9 \(\times 10^6\)-dalton fragment derived from the ILV-E15 linear DNA.

* SacI does not cut the ILV-E15 linear DNA, whereas the ILV-E7 linear DNA is cut twice, at 0.45 and 1.95 kbp. has been used to enumerate the number of exogenous ILV-E7 proviruses inserted into the DNA of clonal populations of line 100 cells. Both the HincII and HhaI endonucleases have been used to identify the presence of specific portions of the viral genome within newly integrated exogenous sequences. This is possible because at least six HhaI sites (at approximately 3.0, 3.6, 4.8, 5.9, 6.5, and 7.2 kilobase pairs [kbp] in both ILV-E15 and ILV-E7) and two of the HincII sites (at 2.7 kbp in ILV-E15 and at 2.7 and 6.6 kbp in ILV-E7) are not available for cleavage in the endogenous sequences from which these viruses are derived. Consistent with our earlier observations, these sites are cleaved only in the unintegrated viral DNA or in the exogenously acquired provirus (16). Apparently, these sites are modified in the endogenous sequences though we have evidence supporting this explanation for only the HincII sites (data not shown).

Figure 2 presents the results of an analysis of DNA from both line 15h and line 100 cells with the HincII and HhaI endonucleases. Line 15h fibroblasts containing exogenous ILV-E15 sequences and line 100 fibroblasts containing exogenous ILV-E7 sequences will be referred to as line 15(+) and line 100(+) cells. Their uninfected counterparts will be designated as line 15(−) and line 100(−) cells. Four endonuclease-specific DNA fragments characteristic of both the line 15(+) and line 100(+) cells are absent from line 15(−) or line 100(−) cellular DNA. The HincII-specific 2.2 \(\times 10^6\)-dalton fragment, and the HhaI-specific 0.38 \(\times 10^6\), 0.43 \(\times 10^6\), and 0.9 \(\times 10^6\)-dalton fragments derived from the DNA of exogenously infected cells correspond in size with fragments derived from the ILV-E7 and ILV-E15 linear DNA intermediates consistent with their location within the internal portion of the virus (data not shown). The 2.2 \(\times 10^6\)-dalton HincII-specific fragment has been located between 2.7 and 6.15 kbp on both linear maps (Fig. 1). HhaI analysis of ILV-E15 and ILV-E7 has located three fragments of 0.38 \(\times 10^6\), 0.43 \(\times 10^6\), and 0.9 \(\times 10^6\) daltons as indicated in Fig. 1. Other smaller fragments are identified but poorly resolved with our present approach and it is clear that only a small number of the HhaI cleavage sites have been identified.

In contrast to the HincII and HhaI analysis of line 15(+) and line 100(+) cellular DNA, BamHI, BglII, BglIII, EcoRI, HindIII, KpnI, SacI, SaI, XbaI, and Xhol do not reveal specific new viral DNA fragments associated with the presence of exogenous sequences in these cells (Fig. 2, reference 16, and data not shown). This observation has been explained by cleavage of the newly acquired exogenous sequences into two types of fragments: (i) fragments that are
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Fig. 2. HhaI, HincII, HindIII, and SacI restriction endonuclease analysis of DNA from line 15 and line 100 fibroblasts. DNA prepared from line 15(+), line 15(−), line 100(+), and line 100(−) cells was digested to completion with one of the site-specific endonucleases HhaI, HincII, HindIII, or SacI. The DNA fragments produced by digestion were analyzed by electrophoresis on 1% agarose gels and hybridization to tdPRB[rPJRNA after transfer of the DNA to nitrocellulose filters with 6×SSC. The DNA fragments of 10×10⁶, 4.8×10⁶, 2.5×10⁶, 2.15×10⁶, and 2×10⁶ daltons used as markers, as described earlier, migrated to positions as indicated by the lines at the left of each pair of gels.

derived from the internal portion of the virus which comigrate with identical fragments derived from the cleavage of the endogenous viral sequences and (ii) fragments that are derived from the ends of the integrated virus which contain the ends of the provirus in association with different lengths of cellular DNA. These fragments are not observed in this analysis which is consistent with multiple sites of integration. To be able to examine both the insertion and expression of exogenous viral DNA sequences after ILV-E infection of the chicken cell, we prepared cell clones infected with either ILV-E7 or ILV-E15 and analyzed them with several restriction endonucleases.

Restriction enzyme analysis of clonal populations of line 15B CEF. Two approaches have been used to characterize clonal populations of cells containing exogenously introduced endogenous viruses. Firstly, uninfected line 15B fibroblasts were cloned and then infected with ILV-E15 so that the infection of a clonal population could be examined. Secondly, uninfected line 15B fibroblasts were infected and then cloned so that any insertion events within a population of cells derived from a single cell could be analyzed. In no instance was infection of a clone associated with the appearance of any identifiable potential virus-cell junction fragment. The results of these analyses are consistent with insertion of ILV-E15 DNA into multiple sites within each of the clones analyzed.

Using the first approach, 17 clones of uninfected line 15B fibroblasts were prepared and infected with ILV-E15. The clones were analyzed with HincII- and HindIII-specific endonucleases both before and after infection. Two types of infected clones were observed. Results typical of each of these clones are presented in Fig. 3. Twelve of the ILV-E-infected clones, typified by the clone designated no. 1, yielded no DNA fragments specific for exogenous se-
sequences after HindIII analysis. All of the fragments identified were identical to those detected after a similar analysis of DNA prepared either from the uninfected parental clone or from an uncloned population of uninfected or ILV-E15-infected 15(-) fibroblasts (Fig. 2). However, the 2.2 x 10^6-dalton fragment specific for exogenous ILV-E sequences was detected after HindII analysis (Fig. 3). Therefore, whereas the HindII-specific internal DNA was present demonstrating that the cells were infected, no DNA fragments representing potential junctions between viral and cellular DNA were detected.

The second type of clone, of which five were isolated, was typified by the clone designated no. 2. These clones contained unintegrated viral linear DNA molecules. HindIII and HindII analyses revealed the two ends of the linear DNA (Fig. 3 and Table 1), whereas HindII analysis demonstrated the distinctive 2.2 x 10^6-dalton internal DNA that results from the acquired HindII sites present in exogenous sequences. DNA from two of the clones was fractionated to remove unintegrated viral DNA by using agarose gel electrophoresis, and the high-molecular-weight cell DNA was recovered and analyzed with HindII-specific endonuclease. Both clones contained integrated viral sequences as revealed by the presence of the HindII-specific, 2.2 x 10^6-dalton DNA fragment (data not shown). The presence of large quantities of unintegrated linear DNA 1 week after infection has been reported previously (10). Whether the two types of clones observed differ significantly in the synthesis and integration of viral linear molecules is unknown. The results of the HindIII and HindII analyses of these ILV-E-infected line 15(-) fibroblast clones support the hypothesis that viral DNA is integrated at multiple sites within the DNA of different cells after ILV-E infection of a clonal population.

Results obtained from restriction enzyme analysis of ILV-E-infected cells cloned after viral DNA integration further support this hypothesis. Line 15(+) fibroblasts were infected with ILV-E15 and cloned four to six generations later as described above. Fourteen line 15(+) clones were isolated and characterized by using the SacI and HindIII site-specific endonucleases. SacI does not cut the ILV-E15 viral DNA (Fig. 1) and so can be used to enumerate the number of individual viral DNA proviruses resident in the DNA of a clonal population. SacI analysis of 10 of the clones indicates that the cellular DNA associated with each of the inserted viral sequences results in SacI specific "viral-cellular" DNA fragments that form a unique pattern for each clone (Fig. 4). Each clone contained the two endogenous loci, ev-1 and ev-7 (5.8 x 10^6 and 8.0 x 10^6 daltons, respectively), as defined by SacI analysis (1). Of 14 clones examined, 11 contained three or more additional DNA fragments containing virus-specific sequences, indicating that three or more viral DNA insertion events had occurred within these clones (Fig. 4 and data not shown).
Two clones contained two additional viral DNA fragments (clones no. 2 and 3), and one clone contained only one additional viral DNA fragment (clone no. 4). Based upon DNA fragments of known molecular mass, including ev-1 and ev-7 as defined by SacI analysis, the viral-cellular DNA fragments ranged in size from approximately 5 x 10^6 to 12 x 10^6 daltons. Of 58 individual exogenously acquired viral-cellular DNA fragments examined, 53 were greater than 5.5 x 10^6 daltons, whereas five were between 5.0 x 10^6 and 5.5 x 10^6 daltons. No fragments less than 5 x 10^6 daltons were observed. All of the clones produced infectious virus as detected by the reverse transcriptase assay (data not shown).

The DNA from clones no. 2, 3, and 4 was further analyzed by using HindIII-specific endonuclease. Since the endogenous locus ev-1 was present in all three clones, it was not possible to determine whether the additional exogenously integrated sequences contained the HindIII-specific, 2.3 x 10^6-dalton internal DNA fragment (Fig. 1 and 2 and Table 1). HindIII analysis of the clones containing one and two copies of exogenously acquired ILV-E15 did, however, reveal the presence of two and four additional viral DNA fragments, presumably viral-cellular junction fragments, not observed after a similar analysis of DNA from line 15(-) cells (data not shown). Our results are consistent with each SacI-specific viral-cellular fragment containing a complete copy of the ILV-E15 genome. Based upon the size of the SacI fragments, our results suggest that integration of ILV-E15 sequences leading to less than a complete provirus is a relatively uncommon event. A more definitive analysis of the viral DNA sequences was hindered due to the presence of more than one copy of ILV-E15 per clone and the limited amount of DNA available. However, clone no. 4, containing only a single copy of ILV-E15, was analyzed further as presented below.

Restriction enzyme analysis of clonal populations of line 100 CEF. We prepared single cell clones from a line 100(+) chicken (spontaneously infected with ILV-E7 produced from the ev-2 locus); 49 clones of line 100(+) cells were isolated. Cellular DNA was prepared from each clone and analyzed by using the XbaI-specific endonuclease. XbaI cleaves ILV-E7 once at 3.4 kbp (Fig. 1) and so should produce two viral DNA fragments for every complete copy of exogenously derived integrated ILV-E7 DNA sequences. XbaI digestion of line 100(+) cellular DNA (containing only ev-1 and ev-2) produces three virus-specific DNA fragments of 5 x 10^6, 8 x 10^6, and 13 x 10^6 daltons. DNA from all the clones analyzed contained the same three XbaI-specific fragments. Cellular DNA containing ev-1 yields only 5.0 x 10^6 and 8.0 x 10^6-dalton DNA fragments (data not shown). XbaI analysis identifies only the 13 x 10^6-dalton fragment as derived from ev-2. It appears, however, that the 5.0 x 10^6-dalton fragment derived from line 100(-) DNA is a doublet, consistent with the ev-2 locus being cleaved once (data not shown). XbaI cleavage of the ev-2 locus once is consistent with XbaI analysis of the ILV-E7 linear DNA molecule derived from that locus (Fig. 1).

The number of exogenously derived ILV-E7 DNA proviruses present in the line 100(+) clones was determined by number of additional XbaI-specific DNA fragments identified after endonuclease analysis of the DNA prepared from each of the clones. Results typical of the analyses of the 49 clones are shown in Fig. 5. Eight clones contained one copy of exogenously introduced ILV-E7, 18 clones contained two copies, and 11 clones contained three copies, whereas 13 clones contained four or more copies. Approximately one clone in six contained an uneven number of additional potential junction fragments (clones no. 9 and 10, Fig. 5). We attribute this to the greatly reduced ability to detect fragments greater than 13 x 10^6 to 15 x 10^6 daltons (Fig. 5). In all clones containing a single exogenous copy of ILV-E7, the two additional XbaI-specific fragments had a combined molecular mass of greater than 6 x 10^6 daltons, consistent with the fragments being cell-virus junction fragments. The DNA from several clones (containing one, two, or three copies of exogenous ILV-E7) were analyzed with HindIII. This analysis revealed that these DNA preparations yielded an even number of potential junction fragments specific for HindIII and consistent with the results of the XbaI analysis (data not shown). The results of this analysis demonstrated that each clone possessed a unique pattern of virus-specific DNA fragments, further suggested that each insertion event was located at a different site within the cellular DNA.

Analysis of the level of virus production from clonal populations of line 15(+) and line 100(+) fibroblasts. The results presented above indicate that two different endogenous avian retroviruses insert viral DNA sequences into multiple locations within the host DNA after exogenous viral infection. We wanted to determine whether different clones of line 15(+) and line 100(+) cells produced virus at equal rates. We therefore examined the amount of virus produced by different clones by measuring reverse transcriptase activity in the culture fluids for 55 clones of line 15(+) and line 100(+) fibroblasts containing between one and six cop-
ies of exogenously introduced ILV-E sequences. Analysis of virus production from clones containing a single copy of exogenous ILV-E revealed that production varied more than 100-fold among the nine clones studied (Table 2). Table 3 lists the results of similar analyses of 46 additional clones containing two, three, four, or more exogenous copies of an ILV-E provirus. These clones showed essentially the same variation in virus expression. They have been placed into three categories based on the amount of virus each produced. The first category consists of a single clone containing two proviruses which did not produce detectable virus. The second category contains four clones which produced 5- to 10-fold less virus than that produced from an equivalent number of uncloned cells from the same embryo. The remaining 41 clones produced levels of virus within two- or threefold (above or below) that produced by similar uncloned cultures. These results indicate that independent clones, containing one or more ILV-E-proviruses integrated at distinct sites within the cellular genome, produce virus at characteristically different rates. The probability that a clone would produce virus at a rate approximately equal to that of an uncloned population increased with the number of copies of ILV-E present. Virus produced from a clone representative of each of the three categories presented in Table 3 replicated with equal efficiency when used to infect fresh uncloned line 15(−) fibroblasts, making it unlikely that the rate of virus replication in the original clone was the result of a stable viral specific property (data not shown). These results suggest that the rate of virus production expressed by a clone is controlled in a cis-acting fashion specific for individual proviruses and not by cellular controls that exhibit clonal variation.

Three restriction endonucleases were used to analyze the integrated structure of the ILV-E proviruses present in 10 of the clones described above. One line 15(+) clone and eight line 100(+) clones, all characterized as containing a single

![Image](http://jvi.asm.org/content/63/5/1570/fig5)

**FIG. 5.** XbaI restriction endonuclease analysis of clonal populations of line 100(+) fibroblasts. Line 100(+) fibroblasts were cloned and used to prepare DNA for analysis with XbaI endonuclease. The digested DNA was analyzed on 0.7% agarose gels as described in the legend to Fig. 2. Molecular masses indicated are given in megadaltons.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>RNA-directed DNA polymerase activity&lt;sup&gt;a&lt;/sup&gt; (cpm/4 × 10&lt;sup&gt;6&lt;/sup&gt; cells/16 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line 100</td>
<td>200</td>
</tr>
<tr>
<td>Line 100</td>
<td>800</td>
</tr>
<tr>
<td>Line 100</td>
<td>1,000</td>
</tr>
<tr>
<td>Line 100</td>
<td>3,000</td>
</tr>
<tr>
<td>Line 100</td>
<td>11,000</td>
</tr>
<tr>
<td>Line 15</td>
<td>27,000</td>
</tr>
<tr>
<td>Line 100</td>
<td>30,000</td>
</tr>
<tr>
<td>Line 100</td>
<td>32,000</td>
</tr>
<tr>
<td>Line 100</td>
<td>55,000</td>
</tr>
<tr>
<td>Line 100(−)</td>
<td>200</td>
</tr>
<tr>
<td>Line 100(+)</td>
<td>58,000</td>
</tr>
</tbody>
</table>

<sup>a</sup> Culture fluid from clonal populations of line 15 or line 100 fibroblasts containing a single copy of exogenous ILV-E sequences, as determined by restriction enzyme analysis, was assayed for the presence of reverse transcriptase activity.

<sup>b</sup> Reaction conditions using polyadenylic acid-polydeoxymyridic acid were as described in the text. A background value of 200 cpm was obtained in this experiment for a reaction that contained no added enzyme. All values greater than 1,000 cpm have been rounded off to the nearest thousand.

<sup>c</sup> Cells for these samples were from an uncloned population. Analysis of four clones of line 100(−) fibroblasts showed no more than a twofold difference in polymerase activity when compared with the activity produced by the uncloned population.
Table 3. Virus production from clonal populations of line 15(+) and line 100(+) fibroblasts containing more than one ILV-E provirus

<table>
<thead>
<tr>
<th>No. of copies/cell</th>
<th>No. of clones</th>
<th>RNA-directed DNA polymerase activity (cpm/4 x 10^6 cells/16 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>&lt;500 (200)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>500-10,000 (6,000)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>&gt;10,000 (42,000)</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>&lt;500</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>500-10,000 (8,700)</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>&gt;10,000 (73,000)</td>
</tr>
<tr>
<td>4 or more</td>
<td>0</td>
<td>&lt;500</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>500-10,000 (8,700)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>&gt;10,000 (105,000)</td>
</tr>
</tbody>
</table>

* The number of copies was determined by analysis with either SacI or XbaI.
* Individual clones have been placed in different categories according to the amount of virus detected.
* The value in parentheses represents the average enzyme activity for all the clones in that category.

proivirus (Table 2), and one line 100(+) clone containing two apparently inactive proviruses (Table 3), were analyzed by using HincII, HhaI, and BamHI endonucleases. The analyses of all these ILV-E proviruses showed that, despite the fact that they produced significantly different amounts of virus, no significant differences in their structures were observed. HincII analysis demonstrated that the DNA from all clones contained the HincII-specific 2.2 x 10^6-dalton fragment located between 3.0 and 6.15 kbp (Fig. 6, Fig. 1, and data not shown). The presence of this fragment indicates that DNA sequences corresponding to a 3.15-kbp region of the linear DNA were present within each of the integrated ILV-E proviruses found in these clones. Similarly, the absence of a 2.5 x 10^6-dalton DNA fragment after this HincII analysis of the line 100(+) clones (Fig. 6) indicated that integration did not involve the region between 2.85 and 6.4 kbp on the linear map. HhaI analysis demonstrated that the HhaI specific 0.38 x 10^5, 0.43 x 10^5, and 0.9 x 10^5-dalton fragments located within the internal portions of the ILV-E map (Fig. 1) were obtained after the analysis of DNA from all 10 of these clones (Fig. 7 and data not shown). It is likely, therefore, that all of the proviruses, even those apparently not expressed, contained a 4-kbp segment of DNA (approximately 3 to 7 kbp) as it is arranged in the linear DNA intermediate. Additional information concerning the arrangement of the integrated viral DNA was obtained after analysis of these clones by BamHI. BamHI analysis of DNA from all the clones demonstrated the absence of a 3.0 x 10^6-dalton DNA fragment as would have been expected had integration occurred between 0.6 and 2.85 kbp (data not shown, Table 1, and Fig. 1). This information, together with that obtained from HindIII and HhaI analyses, suggests that integration did not involve the region between 0.6 and 7.0 kbp on the linear map. It would appear that a majority of the ILV-E integration events lead to complete proviruses colinear with viral genomic RNA inserted at multiple sites within the cell genome. Production of virus from these ILV-E proviruses, however, varied more than 100-fold.

**DISCUSSION**

We have presented experiments that have examined the integration of ILV-E DNA sequences in CEF derived from two inbred lines of chickens. Our results support several conclusions. The ILV-E provirus is inserted into multiple sites within both types of chicken cells. The

![Fig. 6. HincII restriction endonuclease analysis of clonal populations of CEF. DNA prepared from clones was digested to completion with HincII and analyzed on 1.0% agarose gels as described in the legend to Fig. 2. Clone 1 was prepared from line 15(+) fibroblasts. Clones 2 through 6 were prepared from line 100(+) fibroblasts. Clones 1 through 5 contain a single ILV-E provirus, and clone 6 contains two copies. Molecular masses are given in megadaltons.](http://jvi.asm.org/)
and that is flanked by a direct terminal repeat. We have not been able to examine the structure of the integrated retroviral provirus in the same detail that has been possible in some of these studies. We have been hindered both by the presence of endogenous sequences that are apparently identical to those acquired by exogenous infection and by the limited amounts of DNA with which we have had to work. Our data, however, do indicate that provirus integration does not utilize the region from 0.6 to 7.0 kbp on the linear map. This finding is consistent with more detailed DNA sequence analysis of cloned retroviral proviruses (9, 13, 18, 29). Examination of 67 integration events provided no evidence for insertion of less than a complete virus. Further, analysis of over 80 clones of ILV-E-infected fibroblasts has provided numerous examples of viral sequence insertion into multiple sites after infection of either clonal or nonclonal CEF.

The primary focus of our investigation has been to examine the expression of ILV-E sequences within clonal populations of CEF that are permissive for ILV-E replication. Our studies have provided information indicating not only that significant variation exists in the rate of virus production from clones containing a single provirus, but also that some exogenously acquired proviruses are apparently expressed as inefficiently as the endogenous locus ev-2. Use of reverse transcriptase activity to measure virus production appears to cause underestimation of the differences in expression between exogenous and endogenous sequences (6, 16, 30). Since two of the clones we have examined produced no more virus than uncloned populations of line 100(−) cells, cells containing only the endogenous sequences ev-1 and ev-2, the variation in virus production we have observed may in fact be significantly greater than 100-fold.

In demonstrating variation in virus production, we have been careful to examine the expression of nine clones containing a single provirus. Our restriction enzyme analyses indicate that all of these clones contain complete proviruses that are colinear with the viral genomic RNA. Since 8 of the clones are producing detectable virus (Table 2), the biological characterization of these clones supports our structural analysis. It is possible, however, that the single clone from which we detect no virus production contains a provirus characterized by a minor deletion that we have not observed. Further, a recent study has identified a viral promoter located in the 3′ region of the long terminal repeat (35). Should this promoter be required for virus production, it is possible that integration in the negative clone resulted in the absence of a functional promoter located at the 3′ end of the provirus.

![Fig. 7. HhaI restriction endonuclease analysis of clonal populations of CEF. DNA prepared from clones was digested to completion with HhaI and analyzed on 1.4% gels. DNA transfer was completed with 20x SSC. Otherwise, the analysis was described in the legend to Fig. 2. The clones are described in the legend to Fig. 6. Molecular masses are given in megadaltons.](http://jvi.asm.org/)
As listed in Table 3, we isolated and characterized a clone containing two apparently inactive proviruses. Although interpretation of our data is limited because two proviruses were present, our restriction endonuclease analysis provided no indication that either viral structure was incomplete or oriented in a noncolinear fashion.

Two recent investigations have examined the expression of integrated avian retroviral sequences. In one study, individual proviruses were isolated by recombinant DNA cloning after spleen necrosis virus infection of chicken cells (22). The levels of expression observed differed among the viral DNA structures examined by nearly 10,000-fold as measured by DNA transfection. The variation observed in our study was only 100- to 200-fold. It is possible that the 10,000-fold difference observed in the spleen necrosis virus study reflects, in part, differences introduced by molecular cloning that were not characteristic of the expression of the proviruses as they existed in the cell. Since individual insertion events could not be characterized before molecular cloning, this explanation remains unresolved. In a second study, clonal populations of ILV-E-infected cells, some of which may have contained a single provirus, were prepared and analyzed for virus production (17). The rate of virus production observed differed by 30-fold. This value may, however, represent an overestimate since the clones that differed most were prepared in a nonpermissive cell with a nonadapted virus (20, 23). Consequently, some of the variation in expression may result from heterogeneity in either proviral sequences or proviral formation.

Several recent studies have suggested that methylation of DNA may play a role in regulation of expression of both viral and cellular genes (5, 12, 21, 34). Although it has been suggested that methylated DNA is associated with reduced transcriptional activity, it is clear that such a general correlation is an oversimplification (4, 34). Our results have shown that for the nine proviruses we have examined, several sites apparently modified in the endogenous form of the viral sequences (16) are equally accessible in all of the clones regardless of the level of viral expression. It is clear, however, that our results are a very crude measure of potential modification and that a more defined analysis of DNA modification may reveal a role for modification in regulating exogenous ILV-E sequence expression. It is certainly possible the expression in one or more of the clones is affected by methylation in specific regions of the provirus.

We have observed significant variation in virus production from clonal populations of permissive cells containing a single complete copy of an exogenous ILV-E provirus. Our analysis suggests that the variation in expression may have resulted from differences in the adjacent cellular DNA sequences present as a consequence of integration into multiple sites. Our results indicate that, as the number of integrated copies of ILV-E sequences present in a clonal population of cells increases, the proportion of clones producing large amounts of virus increases. This observation suggests that, if in fact cellular sequences play a role in regulating expression of viral sequences, they do so in cis-acting fashion.

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