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Integration of Rous Sarcoma Virus DNA into Chicken Embryo Fibroblasts: No Preferred Proviral Acceptor Site in the DNA of Clones of Singly Infected Transformed Chicken Cells

TEENA L. LERNER,[1]* ANN M. SKALKA,[2] AND HIDESABURO HANAFUSA[1]

The Rockefeller University, New York, New York 10021,[1] and Roche Institute of Molecular Biology, Nutley, New Jersey 07110[2]

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We analyzed retroviral integration into a host genome by using avian sarcoma virus infection of natural target cells under conditions where secondary integration via virus spread was inhibited. This was accomplished by using the noninfectious *pol*$^-$ *env*$^-$ α variant of the Bryan high-titer strain of Rous sarcoma virus. A total of 12 independent Bryan high-titer Rous sarcoma virus-transformed chicken embryo fibroblast clones were obtained and mapped by using restriction endonucleases. Provirus-cell junction fragments were identified with appropriate hybridization probes. We found that expression of the viral genes could occur after proviral integration at many sites on the chicken genome and that there was no apparent preference for specific integration sites.

Retrovirus replication involves reverse transcription of an RNA genome and subsequent integration of a DNA copy into the host genomic DNA. The integrated proviral DNA is a linear nonpermuted copy of the viral RNA with long terminal repeats (LTR) at both ends (9, 14, 25, 39). Thus, the integration event seems to be highly specific with respect to the viral DNA. We sought to determine whether there was any specificity for proviral integration on host DNA.

Recently, there have been many reports dealing with this question. No preferential integration has been found in mouse cells infected with murine leukemia virus (3, 44) or mouse mammary tumor virus (9, 15). In the avian system, workers have found that Rous-associated virus-0 (RAV-0) (28), avian myeloblastosis-associated virus-2 (4), and reticuloendotheliosis virus (31, 42) are integrated nonspecifically into chicken embryo fibroblasts. With avian sarcoma virus, similar results were obtained in infected rat (10, 25), duck (14), and quail cells (39). However, there was no report on infected cells of chickens (the natural host) until Hughes et al. (27) recently presented evidence that avian sarcoma virus integration is random in chicken cells.

The results presented here confirm and extend the findings of Hughes et al. Under conditions which selected for initial integration events, Rous sarcoma virus (RSV) integrated into chicken DNA at no preferred sites. In addition, expression of the proviral genes was compatible with proviral integration at multiple sites.

Although the techniques used in our study of proviral integration were similar to the methods used in the other studies mentioned above, we attempted to overcome the following two difficulties which were encountered in previous analyses of avian sarcoma virus-infected chicken cell clones (27, 39): (i) the ambiguity in identification of fragments corresponding to exogenous proviruses and (ii) the lack of clonality of the exogenous proviruses found in chicken cell clones grown by conventional methods.

We overcame the problem of distinguishing between endogenous and exogenous proviral bands by including in our DNA analyses the uninfected chicken embryo fibroblasts from which the infected clones were derived. The lack of clonality of exogenous proviruses within a colony of avian sarcoma virus-infected chicken cells is probably due to virus spread. Even within a clone, new integration is occurring constantly through reinfection of cells by newly formed virus. To prevent further integration events, we used conditions which prevented secondary infection. The α variant of the Bryan high-titer strain of RSV (BH-RSVα) is defective in the functioning of both the *pol* gene and the *env* gene (17, 19, 37, 47) and therefore is noninfectious. A cell infected with highly diluted BH-RSVα should divide and grow into a clone in which each cell contains a provirus only at the original integration site(s). An analysis of such

BH-RSVα-infected chicken cell clones showed that, in fact, clonality of proviral integration sites was maintained.

MATERIALS AND METHODS

Cells and viruses. A clone of 3Y-1 rat cells infected with BH-RSV was obtained from S. Kawai and was grown in minimal essential medium as described previously (30). A stock of an RAV-1 pseudotype of BH-RSVα [RSVα(RAV-1)] was generously provided by T. Hanafusa. All preparations of RSVα(RAV-1) contained a small percentage of *pol*$^+$ RSV(RAV-1), which is thought to be a consequence of recombination events which occurred after superinfection with helper virus (18). BH-RSVα-transformed chicken cells were grown in Scherer or Hams F-10 medium as previously described (16). Chicken embryo fibroblasts were from *gs*$^-$*chf*$^-$ SPAFAS embryos.

Cloning of BH-RSV-infected chicken cells. Clonal populations of chicken cells harboring BH-RSV proviruses were obtained by infecting chicken embryo fibroblasts with RSVα(RAV-1) at high virus dilutions (multiplicity of infection, 10^{-5} to 10^{-6} focus-forming units per cell). Within 24 h after infection, the cells were overlaid with agar medium containing an antibody that neutralized RAV-1. Individual foci picked on day 8 were cultured separately and were monitored continuously for morphology, infectivity, and virion polymerase activity. Clones of cells that were morphologically transformed but did not produce infectious virus (as assayed by focus formation) were monitored further for the production of virus particles by sucrose gradient banding of [^{35}S]methionine-labeled particles from labeled cultures. Transformed clones that produced noninfectious virions were designated BH-RSV clones. Those BH-RSV clones which, in addition, released polymerase-negative particles were designated BH-RSVα clones, and the clones in which sedimentable polymerase activity was detected were designated BH-RSV(−) clones. [BH-RSV(−) clones have been referred to in previous studies as BH-RSVβ.] The viral proteins of the BH-RSV clones were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Intracellularly produced viral proteins were detected by imunoprecipitation of [^{35}S]methionine-labeled cells with anti-virion antiserum. To analyze virion-associated proteins, cultures were labeled with [^{35}S]methionine, and virions were purified by sucrose gradient sedimentation (38).

DNA extraction procedure. DNA was extracted from pellets containing 10^8 cells by the procedure of R. Junghans, Roche Institute of Molecular Biology (personal communication). The cell pack was lysed in extraction buffer containing 70% guanidine thiocyanate, 10% β-mercaptoethanol, 0.02 M Tris (pH 8), and 0.001 M EDTA. CsCl crystals were added to a final concentration of 1.4 g/ml, and the final volume was adjusted to 2 ml with extraction buffer. The lysate was layered on top of a preformed CsCl step gradient containing 1.8- and 1.6-g/ml CsCl solutions in TEN buffer (0.01 M Tris, pH 7.4, 0.001 M EDTA, 0.01 M NaCl). DNA was banded by centrifugation for 72 h at 34,000 rpm in a fixed-angle Beckman T40 or Ti50 rotor, and the DNA-containing fractions were identified by viscosity or agarose gel electrophoresis with ethidium bromide staining or both and were dialyzed against TEN buffer for a minimum of five buffer changes (at least 8 h/buffer change). Dialyzed DNA was then used directly in restriction enzyme reactions.

Enzymes and enzyme reactions. We used the restriction endonuclease reaction conditions and enzyme buffers recommended by the manufacturer (New England Biolabs), except that we used an excess of at least twofold over the recommended amounts of enzyme to digest chromosomal DNA.

All digestion reactions were monitored by incubating a sample (usually 5%) of the chromosomal reaction mixture with 1 μg of λ or adenovirus type 2 DNA. This marker DNA was subjected to electrophoresis on an agarose gel, stained with ethidium bromide, and examined under UV light for completeness of digestion. Chromosomal samples were precipitated in ethanol and electrophoresed on agarose gels only after completeness of digestion was demonstrated. Samples were heated to 65°C for 5 min before electrophoresis.

Nick translation was performed as described by Maniatis et al. (34). The labeled nucleotide used was 10 mCi/ml of [α-^{32}P]dCTP (specific activity, >2,000 Ci/mmol; Amersham Corp.) in aqueous solution, and DNA polymerase I was obtained from Boehringer Mannheim; specific activities of 10^8 cpm/μg of DNA were obtained routinely.

Gel electrophoresis and filter hybridization. DNA samples were electrophoresed on submerged horizontal agarose gels (20 by 25 by 0.5 cm) in electrophoresis buffer (0.04 M Tris-acetate, pH 7.8, 0.005 M sodium acetate, 0.001 M EDTA). The agarose concentration was 0.7% unless otherwise indicated, and the size markers which we used were ^{32}P-labeled λ *Hin*dIII fragments. DNA was visualized by ethidium bromide (0.5 μg/ml) staining and UV light excitation. Southern transfer (43) to nitrocellulose filters (Schleicher & Schuell Co.), hybridization to radioactive probes, and washing of filters were performed as previously described (5), except that unlabeled salmon sperm carrier DNA was added to the hybridization solution at a concentration of 250 μg/ml. Filters which were to be rehybridized to a second radioactive probe were soaked in a pre-hybridization solution (hybridization solution with water substituted for formamide) at 80°C for 10 min to remove the first probe.

Radioactive probes. (i) RAV-2$_{rep}$ probe. RAV-2 DNA purified from a λRAV-2 clone (29) was cut into two fragments with *Hin*dIII and *Sal*I. Such cleavage generated one fragment 4.25 kilobases (kb) long, which contained a portion of the *env* gene, the "c" region, the LTR, and the entire *gag* gene, and another fragment 3.3 kb long, which contained the entire *pol* gene and part of the *env* gene. These two fragments have been subcloned separately into the plasmid pBR322 (T. Takeya, unpublished data). ^{32}P-labeled RAV-2$_{rep}$ probe was prepared by nick translation of a 1:1 mixture of these two pBR322 subclones.

(ii) *pol* probe. ^{32}P-labeled *pol* probe was prepared by nick translation of the pBR322 subclone containing the 3.3-kb *Hin*dIII-*Sal*I RAV-2 fragment described above.

(iii) cDNA$_{5'}$. ^{32}P-labeled cDNA$_{5'}$, which consisted of the first 101 nucleotides from the 5′ end of RAV-2

(formerly known as strong stop DNA), was generously provided by B. Neel, The Rockefeller University. This probe was made by previously described procedures (20, 21).

(iv) LTR probe. ^{32}P-labeled LTR DNA, which contained all of the 5′ and 3′ sequences present in one copy of the LTR of the Schmidt-Ruppin A strain of RSV, was obtained by *Eco*RI digestion of a Schmidt-Ruppin A RSV clone which contained two copies of the LTR (T. Takeya, H. Hanafusa, R. Junghans, G. Ju, and A. M. Skalka, Mol. Cell. Biol., in press), followed by nick translation of the purified 350-nucleotide fragment.

(v) *src* probe. ^{32}P-labeled *src* probe was made either by nick translation of a pBR322 subclone (Takeya, unpublished data) containing the 2.0-megadalton *Eco*RI *src*-containing restriction fragment from the Schmidt-Ruppin A RSV clone mentioned above or by nick translation of a purified 600-base pair *Hae*III fragment cut from the 5′ region of the *src* gene of the subclone.

RESULTS

Restriction map of BH-RSV. To determine which restriction enzymes would be suitable in our study of BH-RSVα-infected chicken cells, we constructed a restriction map of the provirus by using a clonal line of 3Y-1 rat cells which contained integrated provirus from the parent mutant, BH-RSV(−) (*gag*$^+$ *pol*$^+$ *env*$^-$ *src*$^+$). *Eco*RI cuts within the LTRs of avian sarcoma and leukosis viruses (12, 14, 22, 24, 29, 40, 45); therefore, this enzyme is very useful for mapping studies since it generates the same internal proviral fragments regardless of the cellular site of integration.

Identification of *Eco*RI fragments. BH-RSV contains a deletion of approximately 1,500 nucleotides in the *env* region (13). This deletion includes an *Eco*RI recognition site which has been identified in the *env* gene of every avian sarcoma and leukemia virus mapped to date (12, 14, 22, 23, 24, 29, 40, 41, 45). Digestion of BH-RSV-infected 3Y-1 rat cell DNA with *Eco*RI yielded two fragments (2.45 and 5.4 kb) which hybridized to viral sequences (Fig. 1, lane 1). Since the 2.45-kb fragment comigrated with a 2.45-kb *Eco*RI fragment from cloned Schmidt-Ruppin A RSV DNA, hybridized to cDNA$_{5'}$, and did not hybridize to the *pol* probe (data not shown), this fragment appeared to be identical to the 2.45-kb (ca. 1.5-megadalton) *gag*-containing *Eco*RI fragment that has been identified in all avian leukemia and sarcoma viruses. The other fragment (5.4 kb) appeared only in *Eco*RI digests of BH-RSV DNA, whereas the two fragments that were 3.8 and 3.3 kb long (ca. 2.5 and 2.0 megadaltons) and normally were present in *Eco*RI digests of avian sarcoma virus DNA did not appear in this virus strain. The 5.4-kb fragment hybridized to *pol* and *src* probes and did not hybridize to cDNA$_{5'}$ (data not shown); the size and genetic content of this new fragment is consistent with its being a fusion product of the 3.3- and 3.8-kb fragments, minus approximately 1.5 kb of *env* sequences.

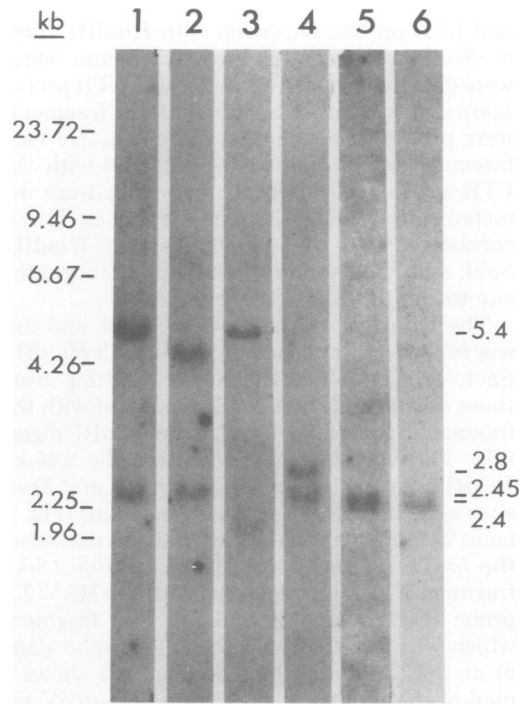


FIG. 1. *BH-RSV-infected rat cellular DNA digested with restriction enzymes and hybridized to viral probes. Samples containing 10 μg of total cellular DNA were digested with the restriction endonucleases specified below, subjected to electrophoresis on agarose gels, and transferred to nitrocellulose paper as described in the text. The blots were then hybridized to the viral probes specified below. The positions of λ molecular weight markers run in a parallel lane are indicated on the left. Sizes are expressed in kilobases. Lane 1, EcoRI digestion, hybridization to RAV-2$_{rep}$ probe; lane 2, EcoRI-HindIII double digestion, hybridization to RAV-2$_{rep}$ probe; lane 3, EcoRI-SacI double digestion, hybridization to RAV-2$_{rep}$ probe; lane 4, EcoRI-KpnI double digestion, hybridization to RAV-2$_{rep}$ probe; lane 5, EcoRI-KpnI double digestion, hybridization to LTR probe; lane 6, EcoRI-KpnI double digestion, hybridization to src probe. Lanes 5 and 6 were rehybridizations of the sample in lane 4.*

Mapping other enzyme sites in relation to *Eco*RI sites. For integration studies, restriction enzymes which cleave at only a limited number of sites within a provirus are useful. DNA extracted from the BH-RSV-infected 3Y-1 rat cells was digested with *Hin*dIII, *Sac*I, *Kpn*I, and *Pvu*I and then hybridized to RAV-2$_{rep}$, *src*,

and LTR probes. Digestion with *Hin*dIII, *Sac*I, or *Kpn*I produced two large fragments which were detected by both RAV-2_{rep} and LTR probes (data not shown). Therefore, these fragments were provirus-cell junction fragments. No viral fragments other than those detected with the LTR probe (i.e., internal fragments) were detected with the RAV-2_{rep} and *src* probes. This is consistent with the hypothesis that *Hin*dIII, *Sac*I, and *Kpn*I each have only one recognition site within the BH-RSV provirus.

The DNA was digested with *Eco*RI, and this was followed by a second digestion with *Hin*dIII, *Sac*I, or *Kpn*I. The fragments resulting from these double digestions were compared with the fragments generated by a single *Eco*RI digestion. The *Sac*I site mapped within the 2.45-kb *Eco*RI fragment, whereas the *Hin*dIII and *Kpn*I sites mapped within the 5.4-kb fragment (Fig. 1, lanes 2 through 4). In the *Eco*RI-*Kpn*I digestion, the 5.4-kb fragment was cleaved into a 2.8-kb fragment which was detected with the RAV-2_{rep} probe (Fig. 1, lane 4) and a 2.4-kb fragment which was detected with the LTR probe (lane 5) or the *src* probe (lane 6). Figure 2 shows a map of these enzyme sites on the BH-RSV genome. For the three enzymes other than *Eco*RI, the single recognition site in BH-RSV corresponded to a site mapped previously at the same position on the avian sarcoma virus genome. However, for *Hin*dIII and *Sac*I, a second site assigned to *env* in many avian sarcoma virus strains appeared to be included in the BH-RSV deletion (12, 14, 22, 29, 40, 45).

*Pvu*I cuts most avian sarcoma virus DNAs in the 3′ sequences of the LTR, generating a unit-length provirus band upon hybridization to a viral probe (12, 14, 24, 29, 40, 45, 46). Although the LTR of BH-RSV contained the *Eco*RI recognition sites, the *Pvu*I sites were absent (data not shown).

Analysis of BH-RSVα-infected chicken cells: study of integration sites in clonal cells. We analyzed the DNAs from 12 independent clones of BH-RSV-infected chicken embryo fibroblasts. The DNAs from the two uninfected embryos from which these 12 clones were derived were analyzed in parallel. Of the 12 clones, 11 were BH-RSVα, and 1 was BH-RSV(−); all 12 produced virion particles, as well as all of the intracellular viral precursor and structural proteins, but none produced the envelope proteins (data not shown).

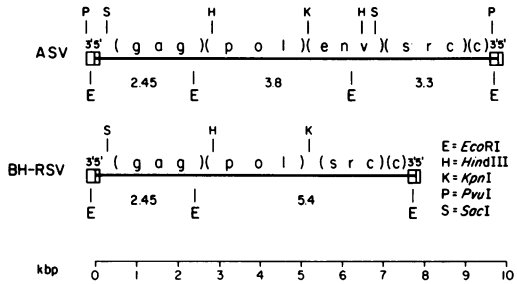
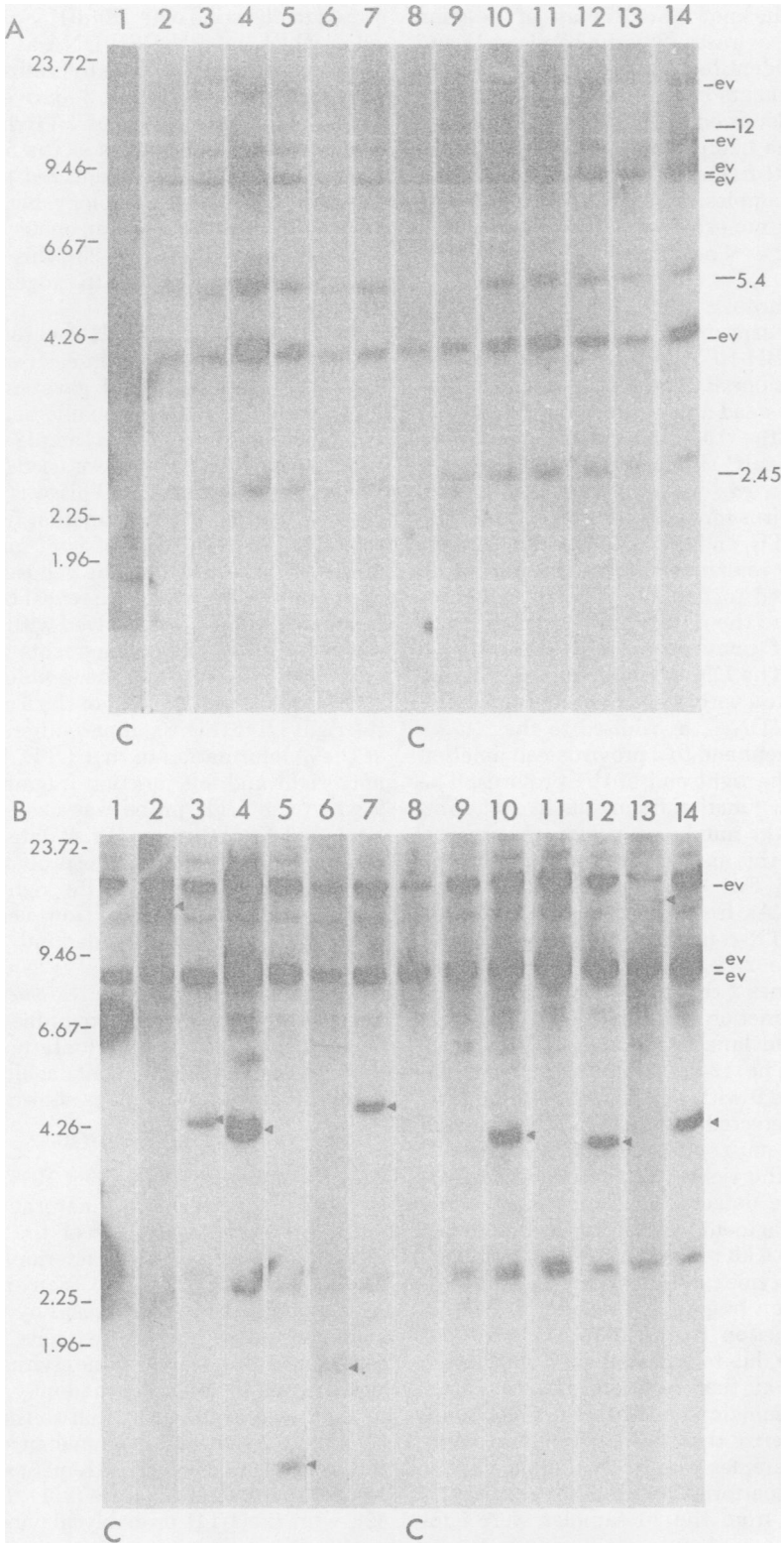


FIG. 2. *Restriction map of integrated proviral BH-RSV DNA compared with avian sarcoma virus (ASV) DNA. The restriction enzyme cleavage sites on the BH-RSV genome were determined by single and double digestions, followed by hybridizations to specific viral probes as described in the text. Restriction enzyme sites on the avian sarcoma virus genome are shown for comparison. The avian sarcoma virus data were compiled from previous reports (12, 22). The boundaries of the viral genes are approximate. The sizes (in kilobases) of the EcoRI fragments of BH-RSV and avian sarcoma virus are indicated; these were determined by agarose gel electrophoresis. The boxes at the ends of the genomes represent the 3′ and 5′ LTRs of BH-RSV and avian sarcoma virus. kbp, kilobase pair.*

Integrity of the BH-RSV proviruses. The DNAs from the 12 BH-RSV clones and the 2 uninfected chicken embryo fibroblast clones were digested with *Eco*RI. The structural integrity of each provirus was ascertained by the presence of the 2.45- and 5.4-kb internal BH-RSV *Eco*RI fragments.

Figure 3A shows an analysis of the cellular DNAs cut with *Eco*RI and hybridized to the RAV-2_{rep} probe. The RAV-2_{rep} probe was used to avoid detecting the cellular *src* bands. The same five bands (15, 11, 8.6, 8.4, and 4.2 kb; the 8.6- and 8.4-kb bands ran as a doublet) were detected in the two uninfected cell DNAs (Fig. 3A, lanes 1 and 8). Based upon hybridization with specific probes (data not shown) and com-

FIG. 3. *Clonal chicken cellular DNA digested with EcoRI and hybridized to viral probes. Samples (8 μg) of total cellular DNA were digested with EcoRI, subjected to agarose gel electrophoresis, and transferred to nitrocellulose paper as described in the text. (In lane 1 only 6 μg of DNA was used.) Lanes 1 and 8, cellular DNAs from control uninfected chicken embryo fibroblasts; lanes 2 through 7 and 9 through 13, independent BH-RSVα clones; lane 14, independent BH-RSV(−) clone. Clones 2 through 7 were derived from the uninfected cells in lane 1 and clones 9 through 14 were derived from the uninfected cells in lane 8. The positions of the λ molecular weight markers run in a parallel lane are shown on the left. Endogenous proviral bands are indicated by ev on the right. All sizes are in kilobases. (A) Blot hybridized to RAV-2_{rep} probe. The internal BH-RSV fragments are indicated by their sizes on the right. Lane 1 is from a longer exposure. (B) Same blot as in (A) rehybridized to cDNA$_{5'}$. The BH-RSV proviral-cellular junction fragments are indicated by arrowheads.*


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
23.72-
9.46-
6.67-
4.26-
2.25-
1.96-
-ev
-12
-ev
=ev
=ev
-5.4
-ev
-2.45
C
C
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
23.72-
9.46-
6.67-
4.26-
2.25-
1.96-
-ev
=ev
=ev
C
C

parison with the known *Eco*RI maps of the avian endogenous proviruses (26), the bands at 15 and 8.4 kb were identified as the proviral-cellular junction fragments of ev-1, the 11- and 8.6-kb bands were identified as the junction fragments of ev-4, and the 4.2-kb band was identified as the internal *Eco*RI fragment of both ev-1 and ev-4. Thus, all 14 samples used in these experiments contained the nonexpressed endogenous proviruses ev-1 and ev-4 as defined by S. Astrin (1, 2, 21).

Clones 2 through 7 and 10 through 14 all contained the expected 2.45- and 5.4-kb internal fragments of BH-RSV. We detected a new pattern only in clone 9. The 5.4-kb fragment was absent, and instead a new band was detected at 12 kb. Since the 2.45-kb band in clone 9 was normal, the *Eco*RI site in the left LTR and the internal site in *gag* were both present. Therefore, this provirus must have lost the *Eco*RI site in the right LTR, and the 12-kb band must have contained the remainder of the proviral 5.4-kb fragment linked to flanking cellular sequences downstream (to the right) of the provirus.

Number of proviruses and clonality of proviruses. The DNAs from clones 1 through 14 were digested with *Eco*RI and hybridized to $cDNA_{5'}$. The $cDNA_{5'}$ hybridized to the 2.45-kb internal fragment and to a provirus-cell junction fragment at the right end of the provirus. The number of new junction fragments in each sample indicated the number of proviruses present. The results of this analysis are shown in Fig. 3B. The bands in Fig. 3B, lanes 1 and 8, which contained DNAs from uninfected cells, represented the LTR-containing fragments of ev-1 and ev-4. The 2.45-kb internal fragment was detected in lanes 2 through 7 and 9 through 14. One unique junction fragment was detected in each sample in lanes 2 through 7, 10, and 12 through 14. The 12-kb junction fragment detected in clone 9 with the $RAV\text{-}2_{rep}$ probe (Fig. 3A) was not detected with $cDNA_{5'}$; the defect in this provirus must also have affected the 5′ sequences of the right LTR. No junction fragment could be detected in clone 11, probably because the fragment was too small. Fragments smaller than 0.6 kb ran off this gel. Experiments with other enzymes did demonstrate the presence of junction fragments in clone 11 (see below). The junction fragment of clone 13 was difficult to see due to a partial dead spot in the nitrocellulose at that location; after a longer exposure this junction band became much more evident. To verify that the junction fragments in all of the samples were truly unique, similar analyses were performed with other enzymes.

The DNAs from the 14 samples were subjected to digestion with *Hin*dIII, *Sac*I, and *Kpn*I, all of which cut BH-RSV DNA at only one site (Fig. 2). These DNAs were hybridized to a probe specific for the LTR. The 3′-derived sequences of the avian sarcoma virus LTR have at least twice as many nucleotides as the 5′-derived sequences, and our probe reflected this 2:1 composition. Since the homology between the 3′ regions of the LTRs of exogenous and endogenous viruses is limited (8, 23), this probe shows preferential hybridization to exogenous proviral sequences.

As Fig. 4 shows, *Hin*dIII generated a unique set of junction fragments in each sample. Analyses with *Sac*I and *Kpn*I gave similar results (data not shown). Occasionally, only one of the two junction fragments in a sample was detected when a particular enzyme was used (for example, Fig. 4, lanes 11 and 12). This was expected in cases where the second junction fragment was too large (or in the case of *Sac*I fragments, too small) to be resolved on the gel used. However, each sample which failed to reveal both junction fragments when it was cleaved with one enzyme did reveal two junction fragments with the two other enzymes tested. Clone 9, which lacked the *Eco*RI site as well as most of the 5′ sequences of the right LTR (Fig. 3), apparently retained most of the 3′ information in that LTR. We detected both right and left junction fragments in clone 9 when the LTR probe was used on *Hin*dIII, *Sac*I, and *Kpn*I digests (Fig. 4; data not shown). If clone 9 had a deletion, it is probable that only the last 150 base pairs of the right LTR were missing from the provirus. However, it is possible that the deletion extended into the flanking cellular sequences.

The blots from the *Hin*dIII, *Sac*I, and *Kpn*I analyses were rehybridized to probes for selected parts of the RSV genome (data not shown) so that the restriction fragments could be ordered for construction of the maps shown in Fig. 5.

DISCUSSION

In this study, we examined RSV integration into cells of chickens, the natural host of this virus. Since the discovery of the site-specific integration mechanism of bacteriophage lambda into *Escherichia coli* (7, 33), many investigators have examined the mechanism by which other elements integrate into host cells. It has been established that DNA tumor viruses, such as simian virus 40, integrate randomly with respect to their own genomes as well as the host DNA (6, 32). In avian and mammalian retroviruses, the integration event is extremely specific with respect to the viral genomes (9, 11, 14, 25, 27, 39, 42), with the LTR probably playing an impor-

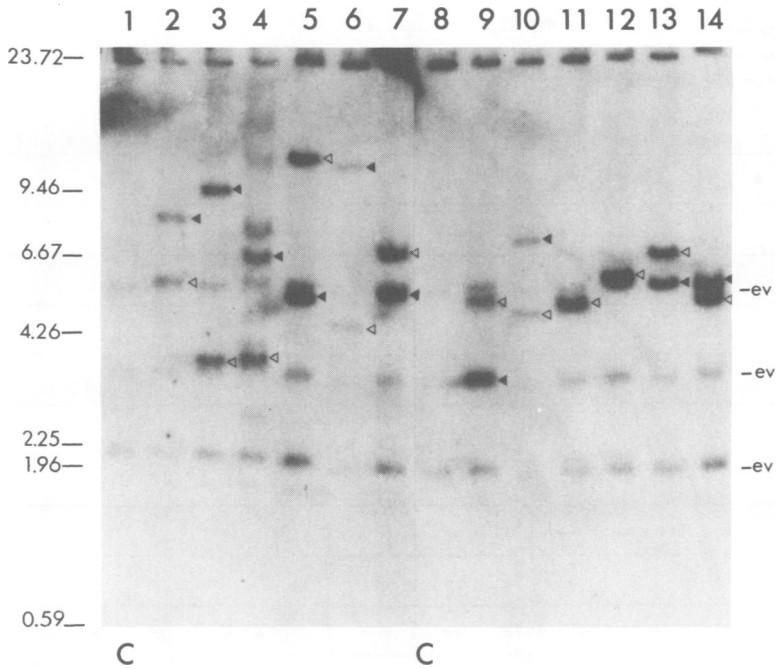


FIG. 4. *Clonal chicken cellular DNA digested with HindIII and hybridized to the LTR probe. The contents of the lanes, the markings on the right, left, and bottom, and the experimental details were all as described in the legend to Fig. 3, except that the restriction enzyme was HindIII and the probe was the LTR. The junction fragments which mapped to the right end of the BH-RSV provirus are indicated by solid arrowheads; the junction fragments which mapped to the left end of the BH-RSV provirus are indicated by open arrowheads. The order of the fragments was determined by rehybridization of this blot to other viral probes (data not shown). The extra fragments in lane 4 were products of partial digestion.*

tant role in this specificity. However, for a number of avian and mammalian systems, retrovirus integration takes place at many locations on the host genome (see above).

In this work we studied the question of specificity by using an avian retrovirus and its natural host cells under conditions which selected for initial integration events. The virus which we used (BH-RSVα) contains genetic defects in the *pol* and *env* genes. Initial infection was possible because both of the essential proteins encoded by these genes were obtained from a complementing virus. Once infection was established under conditions where cells were infected by a single BH-RSVα(RAV-1) particle without a helper virus, the only viral genome present in the cells was the genome of the replication-defective virus BH-RSVα. Thus, multiple infection and virus spread were prevented effectively.

Our results (Fig 3B and 4) verified that we obtained clones of infected chicken cells which harbored only a single provirus. This is in contrast to the results of other workers, who were unable to obtain clonal populations of avian sarcoma virus-infected chicken cells by conventional methods (27, 39). Although the conditions which we used selected for infection by a single virus particle, the number of integration events which could be effected by a single particle was not under selective pressure. Therefore, our results confirm the hypothesis that single particles introduce single integration events. The appearance of multiple proviruses in a clone of infected cells, as has been reported by many investigators (4, 14, 27, 39, 44), can be attributed primarily to viral reinfection.

The clones described here were selected initially on the basis of transformed phenotype (*src*) and subsequently were found to be positive for production of virus particles (*gag*). Thus, in each clone examined, the provirus was capable of supporting expression of the entire viral genome, regardless of the site of integration. Furthermore, with the cloning procedure used, we did not detect any grossly aberrant proviruses. We found that clone 9 had a defect in its right LTR. The transformed phenotype of clone 9 was indistinguishable from the transformed phenotypes of the other 11 clones, as were the levels of intracellular and virion-associated viral struc-

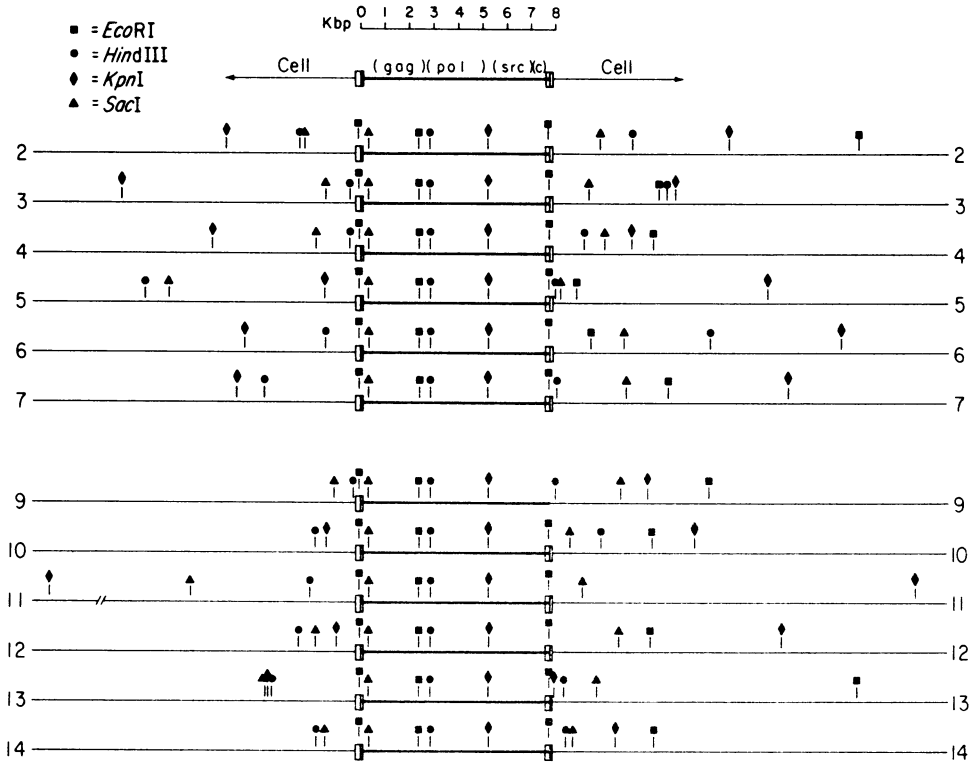


FIG. 5. *Restriction maps of the cellular integration sites of 12 independent BH-RSV-infected chicken cell clones. Cleavage sites for the restriction enzymes were determined by digestion of total cellular DNA and hybridization to specific viral probes, as described in the text. The scale and genetic map are shown at the top. The LTRs are depicted by boxes, as described in the legend to Fig. 2. The numbers at the ends of each line are the clone numbers, as described in the legend to Fig. 3. The break in the cellular DNA of clone 11 represents 6 kb. A right LTR is not depicted for the provirus in clone 9 since we determined that the provirus was missing viral sequences in that region (see text). There may be a deletion which spans the last 150 base pairs of proviral DNA; this deletion may also include an undetermined length of cellular DNA in the region immediately flanking the provirus of clone 9. Kbp, kilobase pairs.*

tural proteins (data not shown). In addition, transforming virus was rescued successfully from a plate of clone 9 cells superinfected with helper virus. It seems that the defect in this provirus did not affect any viral function. Even if the signals for termination and polyadenylation of viral RNA transcripts were affected, there is evidence that viral RNA could be synthesized by using cellular signals (48).

Using four restriction enzymes, we found no similarities among the cellular integration sites of the 12 independent clones studied (Fig. 5). In addition, there was no evidence that more than one provirus integrated into the same site in opposite orientations. We found enzymes which cleaved at or very near the integration site in some but not all clones (Fig. 5). Thus, we concluded that the base sequences 100 to 500 nucleotides around the integration sites differed among the clones. However, as in other similar studies, in this study we could not eliminate the possibilities that (i) very short sequence similarities existed at or near the various possible integration sites and (ii) the provirus recognized specific cellular sequences but integrated distal to such sites. Since the clones that we studied all survived the integration event, it was impossible to determine the number, if any, of "forbidden" sites for integration (i.e., into vital cellular genes). Within the limits mentioned above, we detected no apparent preferred or specific acceptor site(s) for avian sarcoma virus. This finding supports the conclusions of other workers (10, 14, 25, 27, 39).

Keshet and Temin proposed that integration can occur at multiple sites in a genome, but that biological expression of the proviral genes can only occur via integration at a "limited" number of sites (31, 36). We found 12 expressed proviruses integrated at 12 different sites. Thus, if this limited number exists, it must be significantly larger than 12.

Recently, Neel et al. presented a promoter-insertion model for oncogenesis by avian leukosis virus (35). Although avian leukosis virus can express all of its replicative functions via random integration, Neel et al. found that in avian leukosis virus-induced tumors, the proviruses were all integrated at a specific site. In light of these findings, it seems likely that viral integration at specific sites should be found only in cases where there is selection for expression of a function which is not encoded on the viral genome (such as tumor formation by avian leukosis virus). Transformation by avian sarcoma virus would not be in this category.

This study also revealed some interesting results concerning the nature of the BH-RSV(−) and BH-RSVα mutations. BH-RSV(−) DNA contained the single *Kpn*I site (Fig. 2) that is found in other avian leukosis and sarcoma viruses. Therefore, the *env* deletion in this mutant must originate at a location downstream from the *Kpn*I site and then extend through the *env* *Eco*RI site and possibly through the *env* *Hin*dIII and *Sac*I sites (Fig. 2). (*Hin*dIII sites or *Sac*I sites or both have been reported in the *env* gene of many but not all avian sarcoma and leukemia virus strains.) BH-RSVα DNA also contained the *Kpn*I site (data not shown), which indicated that the additional defect in this isolate was not due to an extension of the BH-RSV *env* deletion into the *pol* region. In addition, the sizes of the internal restriction fragments of BH-RSVα were indistinguishable from the sizes of the internal restriction fragments of BH-RSV(−) (Fig. 3A, compare lanes 2 through 7, and 10 through 13, to 14). As has been speculated previously (L.-H. Wang, S. Kawai, P. Duesberg, and H. Hanafusa, unpublished data), it does not seem that the *pol* defect in BH-RSVα is due to a major deletion.

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