Avian Sarcoma Virus-Transformed Quail Clones Defective in the Production of Focus-Forming Virus

WILLIAM S. MASON,1* TERESA W. HSU,1 CAROL YEATER,1 JAN L. SABRAN,2 GEORGE E. MARK,1 AKIRA KAJI,2 AND JOHN M. TAYLOR1

The Institute for Cancer Research, Fox Chase Center, Philadelphia, Pennsylvania 19111,1 and Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania 191742

Received for publication 20 December 1978

Quail embryo fibroblasts were infected at low multiplicity with avian sarcoma virus, and transformed cells were selected by their ability to form colonies in agar. Five clones that failed to produce focus-forming virus were examined for (i) intactness of the integrated proviral DNA, (ii) intracellular viral RNA production, (iii) intracellular viral antigen production, (iv) production of virus particles, and (v) rescue of a functional src gene and of parental host range determinants by superinfection with Rous-associated virus-60, an avian leukosis virus of subgroup E. Deletions in the integrated viral DNA were apparent in three of the five nonproducer clones. In one clone producing focus-forming virus, analysis of the integrated viral DNA revealed an insertion in the region of the genome that codes for src.

In a previous publication we described the state of the integrated proviral DNA in transformed clones of chicken, duck, and quail cells isolated after low-multiplicity infection with avian sarcoma virus (27). Within one group of transformed quail clones derived from the cells of a single embryo, we found that 7 out of 18 transformed clones failed to produce focus-forming virus. The purpose of this manuscript is to present a preliminary characterization of five of these defective clones and of one interesting nondefective clone.

MATERIALS AND METHODS

Cells and viruses. The sources of embryonated eggs and the procedures for the preparation of chicken and Japanese quail embryo fibroblast cultures were as described (36). The chicken embryo fibroblasts were either C/E or C/BE, i.e., resistant to infection by avian tumor viruses of subgroups E or B plus E, respectively. The quail embryo fibroblasts were Q/BD, resistant to viruses of subgroups B and D, but susceptible to infection by viruses of subgroups A, C, and E.

For isolation of clones of transformed quail cells we used both the Prague strain of Rous sarcoma virus (RSV) of subgroup A (wtPR-A) and the Bratislava 77 strain of avian sarcoma virus of subgroup C (wtB77). The procedures and media for the isolation and growth of clones of transformed quail cells were previously described (27). For rescue of transforming virus from defective clones we used Rous-associated virus-60 (RAV-60), an avian leukosis virus of subgroup E (8) that is infectious for Japanese quail embryo fibroblasts but not for C/E or C/BE chicken embryo fibroblasts. Cell culture was at 37–38°C. wtB77 was provided by E. Hunter (University of Alabama, Birmingham); RAV-60 was obtained from H. Hanafusa (Rockefeller University, New York, N.Y.). A Bryan strain RSV-infected quail cell line, Q-BH-RSV(−) cl. 3 (7), was provided by R. Friis, Justus Liebig Universitat, Gießen, West Germany.

Virus assays. Focus assays on chicken or quail embryo fibroblasts were done using standard procedures (36), and concentrations of sarcoma viruses are expressed as focus-forming units per milliliter. Transformation-defective sarcoma viruses and avian leukosis viruses are not detected by the focus assay.

To test for the presence of transformation-defective viruses or avian leukosis viruses, gs(−) chf(−) chicken embryo fibroblast cultures (38) were infected with the sample in question and passaged three times. The cells were grown in Ham F10 medium supplemented with 10% (vol/vol) tryptose phosphate broth, 10% (vol/vol) calf serum, and 2% (vol/vol) heat-inactivated (1 h, 56°C) chick serum, except that infection and transfer were done in Ham F10 supplemented with 10% (vol/vol) tryptose phosphate broth, 5% (vol/vol) calf serum, and 0.0002% (wt/vol) polybrene (GM + PB). After the third passage, the cells on a 100-mm-diameter petri dish were washed three times with 10 ml of ice-cold phosphate-buffered saline (0.15 M NaCl, 0.0028 M KH2PO4, 0.0072 M Na2HPO4, pH 7.1), scraped into 0.5 ml of phosphate-buffered saline, and stored at −80°C for subsequent assay. The samples were sonically disrupted prior to assay for the group-specific (gs) antigen of avian tumor viruses by the COFAL assay (2, 28, 32). For this assay, antiserum was obtained from a pigeon bearing an RSV-induced tumor.

Radioactive labeling of viruses and cells. Labeling was done on 100-mm-diameter petri dishes with monolayers that were ca. 70 to 90% confluent. The monolayers were first washed three times with 37°C Earle basal salt solution containing 0.066% (wt/vol)
NaHCO₃ and 1% (vol/vol) dimethyl sulfoxide. For labeling of virus, the cells were incubated for 16 h in 5 ml of Ham F10 medium containing 2% the normal concentration of methionine, 5% (vol/vol) calf serum, 1% (vol/vol) dimethyl sulfoxide, and 100 μCi of [³⁵S]methionine (200 to 300 Ci/mmol; New England Nu-
clear Corp., Boston, Mass.). The virus was then har-
vester from the tissue culture fluids as described be-
low. To label the cells for immune precipitation, 1.5
ml of Earle salt solution containing 0.056% (wt/vol)
NaHCO₃, 1% (vol/vol) dimethyl sulfoxide and 100 μCi
of [³⁵S]methionine was added for 20 min at 37°C. The
label was removed, and the cells were washed two
-times with 5 ml of prewarmed GM + 1% (vol/vol)
dimethyl sulfoxide and then incubated for an addi-
tional 2.5 h. At the end of this incubation, the cells
were washed once with 10 ml of ice-cold phosphat-
buffered saline containing 1% (vol/vol) dimethyl sul-
oxide and stored at −80°C, prior to lysis for immune
precipitation.

Purification of viruses. Radioactive virus was par-
tially purified from tissue culture fluids, essentially
as previously described (5). Briefly, the media were
clarified, 0.1 mg of purified wtPR-A virus was added
as carrier, the media were layered over 3 ml of 35%
(wt/vol) sucrose in 0.1 M NaCl-0.01 M Tris-chloride-
0.001 M EDTA (pH 7.4), and the viruses were sedi-
mented through the sucrose by centrifugation for 2 h,
38,000 rpm, 4°C, in a Spincgo SW40 rotor. The pellets
were then suspended for sodium dodecyl sulfate
(SDS)-polyacrylamide gel electrophoresis (15).

Immunoprecipitation. Cells were lysed and clar-
ified prior to direct immunoprecipitation, as described
(5). To half of the cell lysate (900 μl) were added 5 μg
of wtPR-A virus and 0.1 ml of rabbit anti-gs serum.
As a control, to an equal volume of cell lysate was added
5 μg of human serum albumin and 0.1 ml of rabbit
anti-human serum albumin (Behring Diagnostics, So-
merville, N.J.). The immunoprecipitates were collected
after an overnight incubation at 4°C and resus-
pended for SDS-polyacrylamide gel electrophoresis.

The anti-gag serum was prepared by three subcu-
taneous injections of a female New Zealand white
rabbit at 2- to 3-week intervals with 0.5 mg of Nonidet-
P40-disrupted avian myeloblastosis virus in Freund
adjuvant. The first was in complete adjuvant, the next
two in incomplete adjuvant. Eight weeks after the
third injection, avian myeloblastosis virus was injected
intraperitoneally without adjuvant. The serum was
collected 10 days later. Avian myeloblastosis virus for
these injections was gradient purified (23) from
chicken plasma obtained from D. Bolognesi (Duke
University, Durham, N.C.).

Gelelectrophoresis. Discontinuous SDS-poly-
acylamide gel electrophoresis was carried out as de-
scribed by Laemmli (15), except that the spacer and
separation gels contained twice the recommended con-
centrations of Tris-chloride and the ratio of acrylamide
to bisacrylamide in the separation gel was adjusted
according to the formula of Blattler et al. (1). Gels
were prepared and fluorographed using preexposed
film, as described (3, 16).

Determination of the average number of mol-
eules of viral RNA per transformed cell. Total
nucleic acid was obtained from 4 × 10⁶ cells using
an SDS-Pronase treatment followed by two phenol
extractions (27). The total nucleic acid was annealed
with [³²P]-labeled DNA probes to the viral RNA (34). A
representative probe labeled with [³²P]dATP (350 Ci/
mml; New England Nuclear) was prepared using
avian myeloblastosis virus reverse transcriptase,
wtPR-C RSV 70S RNA, and calf thymus DNA
primers, all as previously described (35). A probe for
those sequences located near the 3′ terminus, exclud-
ing polyadenylic acid [poly(A)], of the viral RNA was
prepared using a similar reaction except that we used
as template poly(A)-containing viral RNA, selected by
two passages through a column of oligodeoxynu-
dylic acid-cellulose (21). As primer we used 5′-p(DT)₂-
rG-3′ obtained from Collaborative Research (Wal-
tham, Mass.), which was subsequently removed by
treatment with alkali.

To determine the average number of viral RNA
molecules per cell, the kinetics of annealing data were
entered into a Digital 11/70 computer programmed
for this analysis (22).

Analysis of integrated viral DNA. The proce-
dures for analysis of integrated viral sequences were
described in a previous report (27). Briefly, DNA
extracted from infected cells was subjected to restric-
tion endonuclease digestion followed by electropho-
resis on slab gels of 0.7% (wt/vol) agarose. The DNA
was then transferred to nitrocellulose filters by the
method of Southern (30), and viral sequences were
detected by hybridization with a [³²P]-labeled comple-
mentary DNA (cDNA) probe (11). For detection of
ribosomal sequences, we prepared a cDNA probe to a
mixture of purified 28S and 18S ribosomal RNA using
calf thymus DNA primers (35). Unintegrated viral
DNA (11), used as a marker, was a gift from R.
Guntaka, Columbia University, New York, N.Y. It is
important to note that these unintegrated viral DNA
intermediates contain the large terminal repeat
(LTR), of which at least part is also maintained in
integrated viral DNA (11, 27).

RESULTS

"Nonproducer" quail clones. As noted
above, 7 out of 18 clones of avian sarcoma virus-
transformed quail embryo fibroblasts failed to
produce focus-forming virus. These isolates are
designated as "nonproducer" clones. Two of the
clones did not grow well, and were discarded.
The remaining clones have been subjected to a
detailed analysis of the integrated viral DNA
and to a preliminary analysis of virus gene
expression, with the aim of establishing the
block(s) to virus replication. On the assumption
that the failure to produce infectious progeny is
due to a virus mutation, we have designated the
viruses carried by the clones as replication de-
fective (rd) (37). Accordingly, the viruses infect-
ing the five clones are designated rdPH9PR-A,
rdPH18PR-A, rdPH2B77-C, rdPH10B77-C,
and rdPH11B77-C. The corresponding quail cell
clones are called Q-Fra-A-8, Q-Fra-A-18, Q-B77-2,
Q-B77-10, and Q-B77-11, respectively.
TABLE 1. Synthesis of viral RNA and avian tumor virus gs antigen

<table>
<thead>
<tr>
<th>Quail clonea</th>
<th>Viral RNAa (molecules per cell)</th>
<th>gs antigen concn</th>
<th>Relative particle productionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q-PrA-9</td>
<td>1,500</td>
<td>5.7-11.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Q-PrA-18</td>
<td>1,300</td>
<td>7.7-15.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Q-B77-1</td>
<td>1,600 (2,200)</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>Q-B77-2</td>
<td>60 (370)</td>
<td>&lt;0.5</td>
<td>0</td>
</tr>
<tr>
<td>Q-B77-10</td>
<td>500</td>
<td>7.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Q-B77-11</td>
<td>1,000</td>
<td>8.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Q-PrA-4</td>
<td>2,200</td>
<td>16.5</td>
<td>=1</td>
</tr>
<tr>
<td>Q-B77-8</td>
<td>300</td>
<td>14.4</td>
<td>0.7</td>
</tr>
</tbody>
</table>

a The nonproducer quail clones are described in the text. Q-PrA-4, Q-B77-1, and Q-B77-8 are producer clones isolated from infected cells of the same quail embryo as the nonproducer clones.

b The amount of viral RNA per cell was determined as described in the text, assuming 25 × 10⁻¹⁴ g of total RNA per cell and using a representative cDNA probe. The numbers in parentheses were determined using a probe specific for sequences near the 3' terminus of the viral RNA (Fig. 1).

c The COFAL reactivity was expressed as the reciprocal of the highest sample dilution producing a positive response, determined on twofold serial dilutions of cell lysates. The results have been normalized by dividing by the protein concentration (milligrams per milliliter) in each sample (19).

d To quantify the production of virus particles, virions were labeled with [³⁵S]methionine, purified, disrupted, and subjected to electrophoresis on 18% (wt/vol) polyacrylamide-SDS slab gels, as described in the text. After fluorography, films were scanned at 620 nm with a densitometer, and the areas under the peaks were determined for the major virion proteins. The numbers presented are the relative yields of virus particles determined from the quantitation of the major virion protein, P27. Results obtained by normalizing to P19 or P12/P15 differed by a factor of two or less from those presented. Q-B77-2 did not produce detectable yields of virus particles. The yield of particles produced by Q-PrA-4 was taken as unity.

* ND, Not done.

To characterize the nature of the defect in the five nonproducer clones, we first measured four parameters of virus infection. These were synthesis of viral RNA, synthesis of avian tumor virus gs antigens, release of virus particles, and rescue of the src gene by superinfection with RAV-60 avian leukosis virus.

In the following section, we show that the inability to produce infectious progeny virus can be attributed neither to a failure to transcribe the provirus into RNA nor to an inability to translate that RNA into the typical group-specific (gs) antigens of the avian tumor viruses. Nonproducer clones synthesize viral RNA and avian tumor virus gs antigens. The results of the analysis for viral RNA and gs-antigen synthesis are presented in Table 1 and in Fig. 1. Intracellular viral RNA was quantitated by hybridization, in RNA excess, with a cDNA probe representative of the entire viral genome. Avian tumor virus gs antigen was measured in the COFAL assay using serum from an RSV-tumor-bearing pigeon.

The results of a typical hybridization with a cDNA probe representative of the viral genome and nucleic acid isolated from a producer clone, Q-B77-1, are shown in Fig. 1b. Qualitatively similar results were obtained with four of the nonproducer clones, Q-PrA-9, Q-PrA-18, Q-B77-10, and Q-B77-11, as well as with the three other producer clones, Q-PrA-4, Q-B77-1, and Q-B77-8. The amount of viral RNA per cell (Table 1) varied from 500 to 2,200 copies per cell, with the exceptions of the producer clone, Q-B77-8 (300 copies per cell), and the nonproducer clone, Q-B77-2 (ca. 60 copies per cell).

![Fig. 1. Annealing kinetics of [³²P]cDNA probes, representing total and 3'-specific avian sarcoma virus sequences, to nucleic acid extracted from transformed quail clones. [³²P]cDNA probes (6,000 cpm per reaction), prepared by calf thymus-primed transcription (●—●), total or 5'-p[³²P]-rG-3'-primed transcription (X—X), of wtPR-C RNA, were annealed to the total nucleic acid extracted from a producer quail clone (Q-B77-1) and a nonproducer quail clone (Q-B77-2). At the indicated values of Ct, samples were removed, and the formation of hybrids was assayed by resistance to nuclease S1. Each annealing curve has been corrected to 100% annealing of the [³²P]cDNA to an excess of purified 70S RNA of wtPR-C. The actual extent of annealing of each probe with virion RNA was approximately 85%.](http://jvi.asm.org/Downloadedfromhttp://jvi.asm.org)
In contrast to the hybridization of Q-B77-1 presented in Fig. 1b, only 40% of a representative cDNA probe was annealed, at a log_{10}(C/t) of 3, to nucleic acid extracted from clone Q-B77-2 (Fig. 1a). A greater extent of annealing was achieved with a cDNA probe transcribed from poly(A)-selected viral 36S RNA. This result suggests that it is principally those sequences specific to the 3′ region of the viral genome that are transcribed in clone Q-B77-2.

The results of molecular hybridization indicated that each nonproducer clone, except possibly Q-B77-2, synthesized RNA coding for the gs-antigenic determinants associated with the gag gene products. Consistent with this, each quail clone but Q-B77-2 synthesized gs antigen detectable in the COFAL assay (Table 1). The maximum variation between COFAL-positive clones was ca. twofold, but the cells of the COFAL-negative clone, Q-B77-2, accumulated less than 1/10 the amount of gs antigen that the cells of the COFAL-positive clones did.

The positive results of the viral RNA and COFAL assays (Table 1) allowed that some of the nonproducer clones might release noninfectious particles. To test this possibility, the cells were grown for 16 h in the presence of [3H]methionine, and any virus particles released were sedimented from the supernatant fluids by ultracentrifugation and analyzed by electrophoresis on SDS-polyacrylamide gels. By this analysis, each of the clones, except Q-B77-2, produced approximately equivalent amounts of typical virus particles containing P27, P19, and P15 + P12 as did the wild-type virus-infected clone, Q-PrA-4 (Table 1). In addition, we have demonstrated that these virus particles, labeled by growth in the presence of [3H]uridine, banded at a density of ca. 1.15 g/ml after isopycnic centrifugation in sucrose density gradients, and that these particles contained 70S RNA (G. Seal, C. Yeater, and W. Mason, unpublished data). The particles released by the clones were then assayed for reverse transcriptase activity, using calf thymus DNA as an exogenous template. DNA polymerase activity was detected in the particles released by Q-PrA-18, Q-B77-10, and Q-B77-11, but was not detected in the particles released by Q-PrA-9. Unfortunately, we were unable to determine whether [35S]-labeled Q-PrA-9 virus contained the α and β subunits of reverse transcriptase, since a high background of cell-specific proteins in the purified virus preparations reduced the resolution in the region of the polyacrylamide gel containing these subunits.

The presence of typical C-type particles suggested that some of the clones might produce transformation-defective virus. Despite the production of virus particles by four out of five nonproducer clones, none of the nonproducer clones produced infectious but transformation-defective virus. The supernatant fluids from the nonproducer clones did not transfer transformation-defective, replication-competent avian tumor viruses to cultures of susceptible chicken embryo fibroblasts.

Rescue of transforming viruses by superinfection with RAV-60. To further characterize virus expression in the transformed quail clones, we have attempted to rescue viral transforming and host-range determinants by superinfection with RAV-60 avian leukemia virus. The rescue of viral RNA sequences specifying transformation (src) was detected in the focus assay. Simultaneous rescue of subgroup A or C host range determinants was recognized by the ability of the rescued transforming viruses to form foci on C/BE cells resistant to the subgroup E host range of RAV-60. We were able to rescue focus-forming virus from all of the nonproducer clones (Table 2). The absolute efficiency of rescue of focus-forming virus from Q-B77-2 was approximately 100-fold lower than from any other clone, suggesting a defect either in the packaging of the transforming gene into virions or in the utilization of these sequences during initiation of infection in the focus assay. Moreover, transforming virus rescued from Q-B77-2 did not form foci on C/BE, indicating that subgroup C host range determinants were absent or defective in this clone. The relatively low efficiency of rescue of subgroup C virus from Q-B77-10 suggests that this clone, also, might carry a defective env gene. For the remaining nonproducer clones, the efficiency of rescue of transforming virus of the parental host range more closely paralleled that seen with the producer clones. We have not determined whether rescue of the parental host range occurred by complementation or as a consequence of intragenic recombination in env (39).

These results suggest that viral RNA synthesized by all the nonproducer clones, except possibly Q-B77-2, was efficiently packaged into virions released after superinfection with RAV-60. Thus, these mutants differ from the packaging mutant, rd5E21PR-E (M. Linial, E. Medeiros, and W. S. Hayward, Cell, in press). In addition, all the mutants except possibly Q-B77-2 coded for the determinants of the host range of the parental viruses.

Analysis of integrated viral DNA. Probably the most informative property of the defective quail clones that we have examined is the status of the integrated viral DNA. For this we have used digestion of cell DNA by one of sev-
<table>
<thead>
<tr>
<th>Quail clone</th>
<th>Virus production (FFU/ml) assayed on:</th>
<th>(Q/BD)/(Q/BE)£</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q-PrA-4 + RAV-60</td>
<td>3.4 x 10^6</td>
<td>9.3 x 10^3</td>
</tr>
<tr>
<td>Q-PrA-9 + RAV-60</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Q-PrA-18 + RAV-60</td>
<td>7.1 x 10^4</td>
<td>2.6 x 10^4</td>
</tr>
<tr>
<td>Q-B77-1 + RAV-60</td>
<td>1 x 10^4</td>
<td>9.4 x 10^2</td>
</tr>
<tr>
<td>Q-B77-2 + RAV-60</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Q-B77-8 + RAV-60</td>
<td>6 x 10^3</td>
<td>3.3 x 10^2</td>
</tr>
<tr>
<td>Q-B77-10 + RAV-60</td>
<td>1.1 x 10^3</td>
<td>5.8 x 10^4</td>
</tr>
<tr>
<td>Q-B77-11 + RAV-60</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Q-BH-RSV (−) cl. 3</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Controls</td>
<td>wtPR-A: 1.7 x 10^6</td>
<td>6.4 x 10^3</td>
</tr>
<tr>
<td></td>
<td>wtB77: 8 x 10^5</td>
<td>1.1 x 10^3</td>
</tr>
</tbody>
</table>

* A total of 10^6 cells per 60-mm-diameter petri dish were infected with 0.2 ml of RAV-60 and passed twice at 1:2 dilution at 6- to 11-day intervals. Supernatant fluids harvested 3 to 5 days after the second passage were assayed for focus-forming units (FFU) per milliliter on C/BE chicken embryo fibroblasts or on quail embryo fibroblasts.

† Data are presented for cells that received RAV-60 as well as for cells that were not superinfected. Q-PrA-4, Q-B77-1, and Q-B77-8 are producer clones. Q-BH-RSV (−) clone 3 has been described (7). Relative plating efficiencies of subgroups A, C, and E avian viruses on C/BE and quail embryo fibroblasts. wtPR-A and wtB77 were grown on chicken embryo fibroblasts.

£ <10; Focus-forming units were not detected in 0.3 ml of supernatant fluids.

...eral restriction endonucleases, followed by agarose gel electrophoresis of the DNA fragments. As in a previous study (27), unintegrated viral DNA was not observed in the transformed quail clones (data not shown).

In a previous study of the integrated viral DNA of wtPR-A or wtB77 productively transformed clones of chicken, quail, or duck embryo fibroblasts, we observed a large terminal repeat (LTR) of at least 300 base pairs at each end of the integrated viral DNA (27). The restriction endonuclease PvuI makes one cut in the LTR and releases from integrated viral DNA a fragment of unit length (5.85 x 10^6 daltons [5.85 Mdal]); that is, a fragment the size of a transcript of the viral RNA, minus the poly(A) and one copy of the short terminal repeat. Digestion of the integrated DNA of the nonproducer clones also yielded a single fragment, equal to, or in some cases larger or smaller than, unit size (data not shown). This result suggested that the integrated viral DNA of the nonproducers also contained the flanking copies of the LTR but that in some clones intervening sequences had been lost. Moreover, as with the producer quail clones, analysis of fragments produced by digestion with the restriction endonuclease KpnI suggested that each clone contained a single integrated provirus.

KpnI cleaves viral DNA once, approximately in the middle of the genome (33; P. Shank, S. Hughes, H. J. Kung, R. V. Guntaka, H. E. Varmus, and J. M. Bishop, personal communication). Thus, if viral DNA within a cloned population is integrated at a unique site, two cell-virus junction fragments should be released by KpnI, the size of the fragments depending upon the location of adjacent cellular KpnI sites relative to the site of integration. In Table 3 we show that for all the clones but Q-B77-2 two cell-virus junction fragments were obtained. The fragment sizes are different between clones, suggesting that the integration site on the host DNA is different in each. Q-B77-2 yielded only a single fragment of 9.5 Mdal. Results presented below suggest that Q-B77-2 has lost the viral KpnI site, implying that the 9.5-Mdal fragment contained the entire provirus. The results with KpnI indicate that each clone contains one provirus integrated at a single site, but does not rule out the possibility that the clones also contain some viral sequences integrated at random within each cloned population.

The restriction endonuclease EcoRI cuts the integrated viral DNA at a site in the LTR, as well as at two internal sites on the viral DNA, yielding three restriction fragments of 2.35 Mdal (A), 1.96 Mdal (B), and 1.53 Mdal (C), respectively.

<table>
<thead>
<tr>
<th>Quail clone</th>
<th>KpnI junction band (Mdal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q-PrA-9</td>
<td>12.2, 6.4</td>
</tr>
<tr>
<td>Q-PrA-18</td>
<td>8.1, 7.1</td>
</tr>
<tr>
<td>Q-B77-1</td>
<td>13.7, 11.5</td>
</tr>
<tr>
<td>Q-B77-2</td>
<td>9.5</td>
</tr>
<tr>
<td>Q-B77-10</td>
<td>6.9, 3.3</td>
</tr>
<tr>
<td>Q-B77-11</td>
<td>13.0, 6.6</td>
</tr>
<tr>
<td>Q-PrA-4</td>
<td>9.3, 3.1</td>
</tr>
<tr>
<td>Q-B77-8</td>
<td>4.4, 3.8</td>
</tr>
</tbody>
</table>

* See text for details.
tively, representing the entire viral genome (11) (see Fig. 3). Relative to the 3' terminus of the viral RNA genome, these fragments appear in the order B, A, C (33).

The results of an EcoRI analysis of the integrated viral DNAs of the defective quail clones are shown in Fig. 2. Lane 1 shows an EcoRI digestion of unintegrated linear viral DNA intermediates which yielded fragments A, B, and C. Lane 2 shows the EcoRI digest of the producer clone, Q-B77-1, with the surprising result that fragment B is replaced by a new fragment (B') which is 0.17 Mdal larger than B. The EcoRI analysis of the nonproducer clone, Q-B77-10 (lane 3), suggests that a deletion of 0.09 Mdal has occurred in fragment B, yielding fragment B". In Q-PrA-9 cells (lane 4), fragments A and C are missing, while a new fragment, A + C, appears. A + C is 0.6 Mdal smaller than the summed molecular masses of fragments A and C, suggesting that a deletion in either A, C, or both has occurred. Clone Q-B77-2 has lost fragments A and C, indicating deletion of substantial portion(s) of both these fragments (lane 5). In lane 6 we show that certain common bands observed in all the clones also hybridized with a ribosomal cDNA probe, indicating that our viral cDNA probe had some contamination with ribosomal sequences. As discussed in a previous report (27), even 1% contamination of the probe with rRNA-specific sequences due to packaging of a small amount of rRNA into virions would lead to detection of ribosomal DNA because of the reiteration of these sequences within the cellular genome. The EcoRI fragments of clones Q-PrA-18 and Q-B77-11 were of normal size, at the level of resolution afforded by this analysis.

To more precisely locate the deletions and the insertion/duplication detected by our EcoRI analyses of the defective quail clones, we examined the products of digestion of each clone with the restriction endonucleases PvuI, XhoI, and BglIII, as appropriate. The cleavage sites for these enzymes on integrated viral DNA are shown in Fig. 3. The results of our analysis of each defective clone are also shown in Fig. 3, which presents a schematic representation of the size and location of each deletion and insertion or duplication. As shown, Q-B77-1 has an insertion of 0.17 Mdal, in src, Q-B77-10 has a deletion of 0.09 Mdal in env, Q-PrA-9 has a deletion of 0.41 Mdal in pol and/or env, and Q-B77-2 has a deletion of 3.97 Mdal encompassing gag, pol, and env. Although the Q-B77-10 deletion maps near the env-src junction, we have placed this deletion in env because this clone does not produce infectious virus; however, some overlap into src cannot yet be ruled out. Q-PrA-

![Fig. 2. Agarose gel electrophoresis of EcoRI restriction fragments of integrated proviral sequences in transformed quail clones. From 4 to 8 μg of cell DNA was digested with 4 units of EcoRI per μg, followed by slab gel electrophoresis, transfer to nitrocellulose filters, and hybridization with [32P]cDNA. Lanes 1-5, hybridization with a cDNA probe to viral 70S RNA; lane 6, hybridization with a cDNA probe to purified rRNA. Lane 7, HindIII digest of phage λ DNA labeled with [32P]dCTP, in vitro, as described (33). Lane 1, unintegrated intermediates (11); lane 2, Q-B77-1; lane 3, B77-Q-10; lane 4, Q-PrA-9; lane 5, Q-B77-2; lane 6, Q-B77-2. The numbers on the right are the masses in megadaltons of the HindIII fragments of phage λ DNA, used as molecular weight markers. The notations on the left are explained in the text. The two fragments at the top of the gels, not detected with a ribosomal probe, are also seen in uninfected quail cells, as are the ribosomal fragments.

9 may also contain a mutation near the junction of gag and pol, since the EcoRI cleavage site location in this region is missing in Q-PrA-9. Although Fig. 3 shows the deletions and insertion(s) in the mutants as single changes, it is important to note that the changes in size of the DNA between adjacent restriction endonuclease sites may actually result from a number of noncontiguous deletions or insertions.
In the following section, we present a preliminary analysis of the intracellular viral proteins synthesized by one of the nonproducer clones, Q-PrA-9.

Clone Q-PrA-9 synthesizes a defective form of Pr180. The results of our analysis of integrated viral DNA indicated that Q-PrA-9 has a deletion mapping near the junction of \textit{env} and \textit{pol}. If the DNA sequences detected by our analysis were transcribed into viral mRNA specific for \textit{pol}, the reverse transcriptase precursor, Pr180 (9, 20, 26), would not be synthesized. Cells were labeled with \([^{35}\text{S}]\)methionine, and cell-associated viral proteins were immunoprecipitated with an antiserum to whole virus. As expected, Pr180 was not synthesized in Q-PrA-9 cells (Fig. 4). Instead, we detected a novel protein (designated X) with a molecular weight of ca. 130,000 to 140,000. Pr76 appears to be of normal size; however, processing of Pr76 to the gag structural

Fig. 3. The sizes and locations of the insertions or deletions on the integrated proviral DNA in clones Q-B77-1, Q-B77-10, Q-PrA-9, and Q-B77-2, defining mutations in PH1B77-C, rdPH10B77-C, rdPH9PR-A, and rdPH2B77-C. Cleavage sites of restriction endonucleases PstI, XhoI, EcoRI, BglII (P. Shank et al., personal communication), and KpnI are also shown on the proviral DNA genome. The circle on the right corresponds to the 5' terminus of the DNA transcript complementary to the viral RNA (PH1B77-C). The additional sequences were located between the BglII site at 1.09 Mdal and the XhoI site at 1.45 Mdal. Both of these sites are located on src (P. Shank et al., personal communication). The size of the insertion or duplication, 0.17 ± 0.04 Mdal, was derived from comparison of EcoRI and BglII fragments of Q-B77-1 with those of unintegrated DNA intermediates. (rdPH10B77-C) The deletion was located between the XhoI site at 1.45 Mdal and the EcoRI site at 2.06 Mdal. The size of this deletion, 0.09 ± 0.025 Mdal, was determined from analysis of XhoI and EcoRI digests. (rdPH9PR-A) The deletion was located to the left of the KpnI site at 2.71 Mdal. A preliminary experiment indicated that the deletion was located to the right of an HpaI site at 4.22 Mdal (not shown). The BglII site at 3.26 Mdal is missing, suggesting that the mutation encompasses this site, as shown. The size of the deletion, at least 0.41 ± 0.04 Mdal, was derived from the fragments produced by XhoI and a double digestion with PstI and KpnI, although the EcoRI data indicated a slightly larger deletion, 0.6 Mdal. Although the major deletion is indicated as mapping to the right of the HpaI site at 4.22 Mdal, an alteration has also occurred to the left of this site, since the EcoRI site at 4.4 Mdal was not detected (Fig. 2). (rdPH2B77-C) Digestion with PstI yielded a fragment of size 1.76 Mdal, whereas digestion with EcoRI yielded a single fragment of 1.9 Mdal. The presence of src was indicated by the rescue of this gene by superinfection with RAV-6 (Table 2). We believe, therefore, that the 1.9-Mdal EcoRI fragment is released by cleavages at the EcoRI sites at 0.095 Mdal and either 2.06 or 5.95 Mdal from the 5' terminus. In the figure, the open brackets define the locations of the deletions and insertion/duplication, and the hatched or filled brackets indicate the size. It should be noted that the mutations could be at multiple sites within the indicated locations.

Fig. 4. Immune precipitation of viral proteins from lysates of Q-PrA-4, Q-PrA-9, and Q-B77-2. The procedure is described in the text. Electrophoresis was on a 12.5% (wt/vol) polyacrylamide gel. Lanes a, h: WtPR-A, grown in chicken embryo fibroblasts and purified as described in the text. Lane b: Q-PrA-4, anti-gs. Lane c: Q-PrA-4, anti-human serum albumin. Lane d: Q-PrA-9, anti-gs. Lane e: Q-PrA-9, anti-human serum albumin. Lane f: Q-B77-2, anti-gs. Lane g: Q-B77-2, anti-human serum albumin.
proteins P27, P19, P12, and P15 may be partially defective. By radioactive labeling, Q-PrA-9 cells contained less processed gag proteins and produced slightly less virus than Q-PrA-4 cells (Table 1). Since the apparent reduction in the size of Pr180 (ca. 40,000 to 50,000 daltons) is greater than predicted from the size of the deletion, which is sufficient to account for ca. 21,000 daltons, at least part of the deletion probably maps at an internal location in pol, rather than at the 3' end of pol or across the pol-env junction.

As predicted from the results of the DNA mapping and COFAL experiments, Q-B77-2 did not synthesize any gp proteins recognized by an antiserum raised against total virion proteins (Fig. 4).

**DISCUSSION**

We have described a preliminary characterization of five transformed quail clones with nonconditional defects in virus replication. In three instances, restriction enzyme analysis has revealed that the defect is a consequence of a deletion in the viral genome. The mechanism of formation of these deletions is unknown. A common feature of the defective clones we have studied is the maintenance of an LTR. This maintenance is consistent with the proposed importance of the LTR in the initiation and termination of transcription of integrated viral DNA into RNA, as discussed in a previous report (27).

Although 7 nonconditional mutants were obtained out of 18 clones examined, in a subsequent experiment with cells of a different quail embryo and using a new focus clone of utPR-A, only 1 transformed clone in 35 failed to produce progeny virus. A similar low level of defects was reported previously in RSV-transformed quail cells, and mutants with nonconditional defects in pol (18) and in the packaging of viral RNA (Linial et al., Cell, in press) were described. Modified viral genomes in RSV-transformed mammalian cells have been found by P. Shank and co-workers (P. Shank, S. Hughes, H. J. Kung, J. E. Majors, N. Quintrell, R. V. Gunataka, J. M. Bishop, and H. E. Varmus, personal communication) and by K. Steimer and D. Boettiger (personal communication; 31). In addition, S. Martin and co-workers (personal communication) have obtained a number of deletion mutants similar to rdPH2B77-C, using UV-inactivated RSV.

Irrespective of the mechanisms by which deletions and insertions were formed, several potentially useful mutants are described in this report. Q-B77-1 is transformed and produces phenotypically wild-type virus (PH1B77-C), yet has an insertion in src sufficient to code for an additional 80 amino acids. It is difficult to envision the retention of a functional src product following such a large alteration, and it will be interesting to see whether or not these cells synthesize a larger form of the src gene product, P60 (4, 12). Alternatively, the additional sequences may be removed by RNA splicing or protein processing.

The provirus of rdPH9PR-A has a deletion in pol and a phenotype reminiscent of the Schmidt-Ruppin RSV mutant, rdNY78a (13). Unlike this mutant, however, rdPH9PR-A has a functional env gene, and may be similar to a48T, isolated as a recombinant between the Bryan strain of RSV and RAV-49 avian leukemia virus (25). In contrast to a48T, rdPH9PR-A has a nondefective parent and should be useful for precise mapping of pol on the wild-type genome.

Finally, rdPH10B77-C has a deletion near the 3' end of env and may, therefore, be a gp37 mutant (14, 29). This mutant could prove useful in analyses of the processing of the glycoprotein precursor Pr95 (6, 9, 14, 24, 29) to gp85 and gp37, and in the assembly of the gp85-gp37 dimer, VGP (17).

**ACKNOWLEDGMENTS**

We are grateful to H. Hanafusa (Rockefeller University, New York, N.Y.), E. Hunter (University of Alabama, Birmingham, Ala.), and R. Friis (Justus Liebig Universität, Giesen, West Germany) for providing viruses and to R. Gunataka (Columbia University, New York, N.Y.) for providing the unintegrated viral DNA intermediates.

This work was supported by grants VC-155C and NP-241 from the American Cancer Society, by Public Health Service grants RR-05539, CA-08297, CA-16641, and CA-18497 from the National Institutes of Health, by grant PCM76-61525 from the National Science Foundation, and by an appropriation from the Commonwealth of Pennsylvania.

**LITERATURE CITED**


