Proc. Natl. Acad. Sci. USA
Vol. 76, No. 5, pp. 2165–2169, May 1979
Biochemistry

Structural studies on oncornavirus-related sequences in chicken genomic DNA: Two-step analyses of *Eco*RI and *Bgl* I restriction digests and tentative mapping of a ubiquitous endogenous provirus

(avian sarcoma virus/avian leukosis virus/*sarc* DNA sequences)

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Communicated by B. L. Horecker, February 9, 1979

ABSTRACT **DNA from a variety of uninfected chicken cell types has been analyzed by using restriction endonuclease digestion and RPC-5 ion-exchange chromatography followed by agarose gel electrophoresis. Endogenous retrovirus sequences were detected by using a ^{32}P-labeled avian leukosis viral RNA probe. One simple pattern was identified in an individual containing unexpressed endogenous proviral genes (gs$^-$ chf$^-$ phenotype for group-specific antigens and chicken helper factor) that was common to all individuals studied. A tentative restriction map has been derived for this and one other gs$^-$ chf$^-$ endogenous provirus. Other gs$^-$ chf$^-$ individuals and individuals with other phenotypes (e.g., gs$^+$ chf$^+$ and gs$_L$ chf$_{hE}$) showed more complicated patterns that often included additional bands and thus probably additional proviruses. RNA from an avian sarcoma virus was used to detect cellular sequences (*sarc*) homologous to the viral transforming gene (*src*). Results have revealed that a single restriction endonuclease *Eco*RI fragment of 13 × 10^6 daltons contains the majority of these sequences and confirm that they are not adjacent to the endogenous provirus.**

Uninfected chicken cells possess DNA sequences homologous to at least part of the RNA genomes of the avian retroviruses (1–5). Nucleic acid hybridization has shown these endogenous proviral sequences to be present in a few copies per haploid genome; however, a precise determination of the copy number is not possible by this technique (1, 4). Cells from certain chicken lines spontaneously release an endogenous virus, Rous-associated virus type 0 (RAV-0), (6, 7). These lines and other normally nonproducing lines that can be chemically induced to release virus (8, 9) must contain a complete viral genome that is capable of expression. In other lines, partial expression of the endogenous provirus results in production of viral core proteins [group-specific antigens (gs)] and envelope proteins [chicken helper factors (chf)] (10, 11). In addition to the gs$^-$ chf$^-$ phenotype, which does not produce either viral product, two other phenotypes have been described: gs$^+$ chf$^+$, in which both products are made (12, 13), and gs$_L$ chf$_{hE}$ in which gs antigens are produced at low levels and helper is produced at high levels (14, 15). By nucleic acid hybridization, the amount of viral specific DNA appears to be the same in all three phenotypes (1, 2, 16). However, Hayward and Hanafusa (17, 18) have shown quantitative and qualitative differences in the virus-specific RNAs. Thus, differences in expression may be regulated at the transcriptional level.

In addition to the endogenous provirus, all chicken cells contain DNA homologous to the transformation-specific (*src*) gene of avian sarcoma viruses (19). These cellular sequences (called "*sarc*") are not linked to the provirus (20) and are independently transcribed into poly(A)-RNA that is found in polysomes (21, 22). The function of *sarc* in normal cells is unknown.

To investigate the organization of the provirus in normal cellular DNA and its possible relationship to viral expression, we have analyzed restriction endonuclease-digested chicken DNA by the method of Southern (23). To increase resolution and sensitivity, DNA was fractionated by RPC-5 chromatography, then by agarose gel electrophoresis. Digestion with *Eco*RI and *Bgl* I have allowed identification and partial purification of a cellular *sarc* sequence and construction of a partial restriction map of two endogenous proviruses, one of which appears to be present in all DNAs analyzed. A preliminary report was presented at the 1978 Cold Spring Harbor RNA Tumor Virus Meeting.

MATERIALS AND METHODS

Cells and Viruses. The preparations of Rous-associated virus type 2 (RAV-2) and Schmidt–Ruppin B Rous sarcoma virus (SR-RSV-B) have been described (24). Chicken embryo fibroblasts were prepared from 11-day-old gs$^-$ chf$^-$ embryos (Spafas, Norwich, CT) and grown in M 199 medium (GIBCO), supplemented with 8% fetal calf serum, 2% heat-inactivated chicken serum, 100 units of penicillin per ml, 100 mg of streptomycin per ml, 2 mM glutamine, and 10% tryptose–phosphate broth (Difco). Fibroblasts were infected by using standard techniques (25, 26). Four days after infection, serum supplementation was reduced to 2% and virus-containing medium was collected every 8 or 12 hr. Supernatants were cleared by low-speed centrifugation and the virus was pelleted at 36,000 × *g* for 2–5 hr. RAV-0 was prepared from virus-producing embryo fibroblasts from a line 7 × 15 cross obtained from L. Crittenden (27).

Viral RNA Probes and Filter Hybridization. 70S RNA was isolated from pelleted virus by oligo(dT)-cellulose chromatography (Collaborative Research, Waltham, MA) (28), heat denatured, and recycled over oligo(dT)-cellulose. The 35S RNA was purified on a 15–30% neutral sucrose gradient. RNA was labeled *in vitro* by the phage T4 polynucleotide kinase reaction (29). [γ-^{32}P]ATP (specific activity ≈1250 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) was prepared by a modification of published procedures (30). Specific activities of probes were 5 to 50 × 10^6 Cerenkov cpm/μg. Blotting and filter hybridizations have been described (23, 31, 32).

Phenotypes. Expression of gs antigen and chf in chicken embryo fibroblasts was assayed by published procedures (13,

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Abbreviations: gs, group-specific antigens; chf, chicken helper factor; RSV, Rous sarcoma virus; SR-RSV, Schmidt–Ruppin RSV; RAV, Rous-associated virus; ASV, avian sarcoma virus.

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17). In adults, gs antigen was assayed in muscle obtained by biopsy. The chf phenotype was determined by fusing erythrocytes with RSV(−)-transformed cells and assaying for infectious virus. Erythrocytes (3×10^7) and 1.5×10^6 Bryan RSV(−)-transformed gs$^-$ chf$^-$ chicken cells or Japanese quail cells were suspended in 0.5 ml of medium containing about 1000 hemagglutinin units of UV-inactivated Sendai virus, incubated at 0°C for 20 min then at 37°C for 40 min, then seeded. After 2 days, culture fluid was assayed for infectious RSV(chf). Quantitative results of this assay correlated well with the conventional feather follicle assay.

RESULTS

Detection of *Eco*RI Fragments of Endogenous Provirus with RAV-2 RNA. DNA from pooled erythrocytes from five mature gs$^-$ chf$^-$ chickens was digested with *Eco*RI, fractionated by RPC-5 chromatography followed by agarose gel electrophoresis (33, 34), then assayed by blotting and hybridization with ^{32}P-labeled RAV-2 RNA. The results in Fig. 1*A* show hybridization to specific bands in three sets of fractions. In set I, a band at 5×10^6 and (in short exposures) a discrete band at 16×10^6 daltons have been shown to be rDNA (31, 32). These bands were detected because of contamination of the viral RNA probe with rRNA; however, they served as a useful internal monitor of endonuclease digestion, which appeared complete in this preparation (32). In set II a 2.4×10^6 dalton fragment and in set III three fragments at 5.2, 6.2, and 10×10^6 daltons were hybridized with probe. Thus four *Eco*RI fragments containing sequences homologous to RAV-2 are identified.

*Eco*RI digests of DNA from individual hens who contributed to the erythrocyte pool were analyzed. Autoradiograms of the DNA from two individuals are shown in Fig. 1*B*. DNA from individual 22 has the same pattern as that in the RPC-5 analysis. In DNA from individual 88, the 6.2×10^6 dalton fragment is missing [the additional bands at 5.0 and 16.5×10^6 daltons in both DNAs are rDNA, verified by competition hybridization (not shown)]. Thus, two different *Eco*RI restriction patterns occur in individuals from the same line and flock, both classified as gs$^-$ chf$^-$ for the expression of viral products.

The RPC-5 fractions described in Fig. 1*A* were also screened for proviral sequences with endogenous virus RAV-0 probe. The fragments containing proviral sequences were the same as those detected with RAV-2, a more readily obtained virus.

Detection of *sarc* and Endogenous Proviral Sequences in *Eco*RI Fragments with RSV RNA. To detect cellular *sarc* sequences homologous to the *src* gene(s) of avian sarcoma viruses, the same RPC-5 fractions were analyzed, using ^{32}P-labeled RNA from SR-RSV-B. Results with selected fractions are shown in Fig. 2. Hybridization to the band at 2.4×10^6 daltons in set II fractions and to three bands at 5.2, 6.2, and 10×10^6 daltons in set III fractions is the same as seen with either RAV-2 or RAV-0 probe. In addition, we detected strong hybridization to a 13×10^6 dalton band in a set of fractions (IV) that eluted from RPC-5 between the two provirus-containing sets. Blots of the DNAs from sets II, III, and IV were probed with either RAV-2 [^{32}P]RNA (Fig. 2*B*) or RSV [^{32}P]RNA plus unlabeled RAV-2 RNA competitor that does not contain *src* sequences (Fig. 2*C*). No hybridization to a 13×10^6 dalton fragment in region IV was detected with the RAV-2 probe; hybridization to II and III was the same as in Fig. 1*A*. Fig. 2*C* shows that

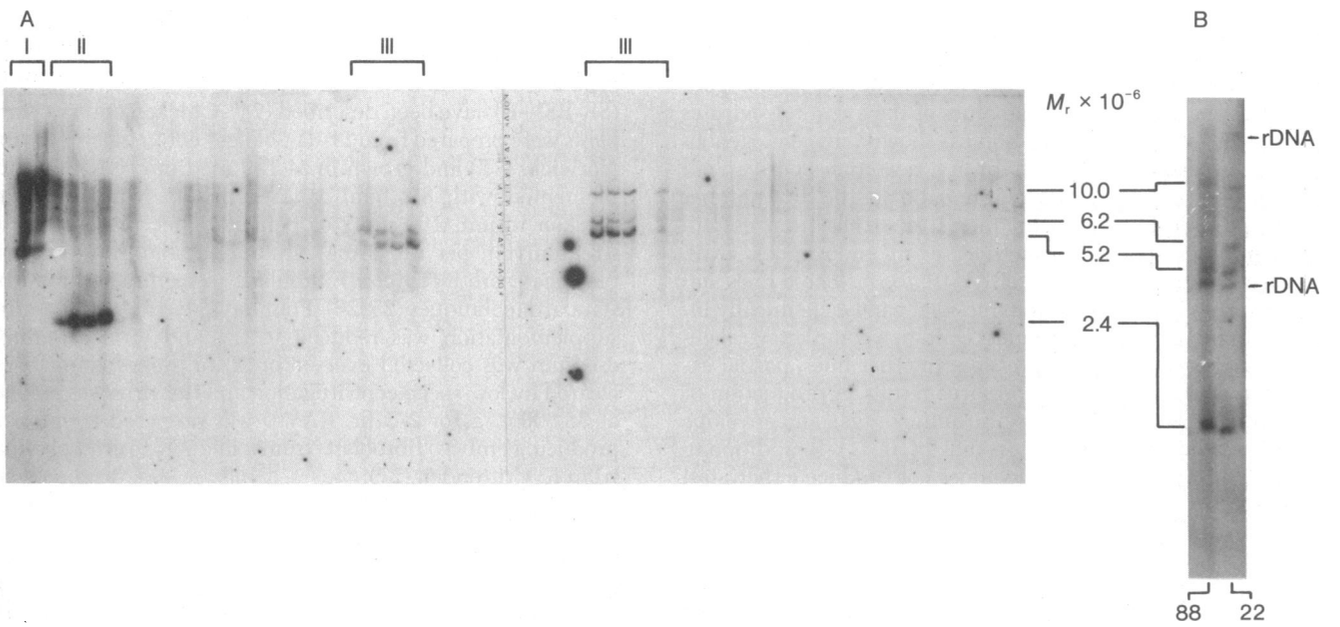

FIG. 1. Hybridization of RAV-2 [^{32}P]RNA to endogenous proviral DNA. (*A*) DNA was prepared from erythrocytes of gs$^-$ chf$^-$ chickens by a method similar to that of Chambon and coworkers (35). Approximately 60 mg of DNA was obtained from 20–30 ml of packed erythrocytes, which were diluted to 10^7–10^8 cells per ml for extraction. The DNA was extensively dialyzed against 10 mM Tris·HCl (pH 7.4)/10 mM NaCl/1 mM EDTA and then against 10 mM Tris·HCl (pH 7.4)/10 mM NaCl/0.1 mM EDTA. DNA was digested with restriction endonuclease *Eco*RI, extracted with phenol, and precipitated with ethanol at −20°C. Digested DNA (32 mg) was subjected to chromatography on a 150-ml RPC-5 column (33). Approximately 400 fractions, 7 ml each, were collected. Pairs of adjacent DNA-containing fractions were pooled and the DNA was concentrated by ethanol precipitation. Small samples (containing about 0.6% of the original column fractions) of each of 55 pools were subjected to electrophoresis in 0.7% agarose. Molecular weights were established by comparison with *Eco*RI-digested phage λ DNA run in an adjacent slot of the gel (not shown). The leftmost tracks contain DNA that eluted from RPC-5 first. DNA was transferred to cellulose nitrate membranes by the method of Southern (23). Hybridization was for 65 hr with a mixture containing approximately 30 ng of RAV-2 [^{32}P]RNA (specific activity 8×10^7 cpm/μg) per ml (32). The gel was transferred in two sections; the break is in the middle of fraction set III, therefore all set III fractions are actually contiguous. (*B*) DNA extracted from erythrocytes obtained from gs$^-$ chf$^-$ individuals numbers 22 and 88 was digested with *Eco*RI. Of each digest, 10 μg was fractionated by electrophoresis on 0.7% agarose gels blotted onto cellulose nitrate and hybridized with RAV-2 [^{32}P]RNA for 60 hr.

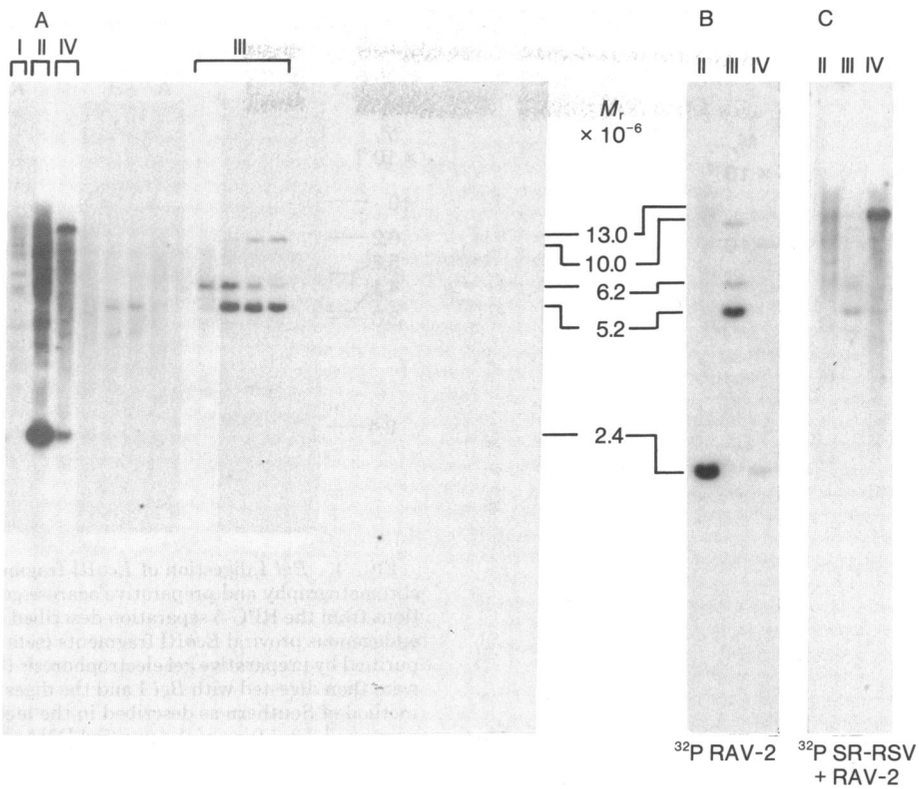

FIG. 2. Hybridization of SR-RSV [^{32}P]RNA to endogenous proviral and *sarc* sequences. (*A*) The pooled fractions described in the legend to Fig. 1 were further combined so that each new pool contained DNA from four contiguous RPC-5 column fractions. These pools were fractionated by 0.7% agarose gel electrophoresis and transferred onto cellulose nitrate membranes. Hybridization was for 65 hr with a reaction mixture containing ≈30 ng of SR-RSV [^{32}P]RNA (specific activity 9 × 10^6 cpm/μg) per ml. (*B* and *C*) Fractions from regions II, III, and IV were pooled, as in *A*, subjected to electrophoresis, and transferred to cellulose nitrate filters. Molecular weights were determined as in Fig. 1. Filter *B* was hybridized with RAV-2 [^{32}P]RNA (≈30 ng/ml, specific activity 3 × 10^7 cpm/μg) for 66 hr. Filter *C* was hybridized with SR-RSV [^{32}P]RNA (30 ng/ml, 9 × 10^6 cpm/μg) in the presence of unlabeled RAV-2 RNA (5 μg/ml) for 66 hr.

hybridization of RSV RNA to the endogenous proviral sequences in II and III was almost completely blocked by RAV-2 RNA, whereas hybridization to the 13 × 10^6 dalton fragment in IV was not. Therefore, this fragment contains sequences homologous to those present in the sarcoma virus but missing from the leukosis virus genome. The small amount of hybridization detected in bands between sets II and III was also blocked by RAV-2 (not shown). Thus, these bands may represent additional virus-related sequences or cellular sequences whose RNA contaminates both viral preparations.

Detection of *Bgl* I Fragments of Endogenous Provirus. Digestion of chicken genomic DNA with several restriction enzymes showed that *Bgl* I gave a simple pattern for endogenous provirus. Fig. 3 shows the results of *Bgl* I digests for DNAs from individuals 88 and 22. In 88, RAV-2 RNA hybridized to a single band with a molecular mass of 10 × 10^6 daltons. In addition to the 10 × 10^6 dalton fragment, at least one other band of 12–14 × 10^6 daltons hybridized with the probe in DNA from individual 22. Because a restriction enzyme that generates a single fragment bearing a complete provirus is useful, *Bgl* I-digested DNA from individual 88 was further characterized by RPC-5 chromatography (not shown). In the presence of rRNA competitor, RAV-2 RNA probe hybridized to a single 10 × 10^6 dalton band.

In another series of experiments the four *Eco*RI endogenous provirus-containing fragments were further purified by preparative gel electrophoresis of the sets II and III RPC-5 fractions shown in Fig. 1, digested with *Bgl* I, and analyzed again by gel electrophoresis and hybridization. The results (Fig. 4) showed no change in size for the 2.4 × 10^6 dalton *Eco*RI fragment; however, the 5.2 × 10^6 became 4.1 × 10^6 daltons; the 6.2 × 10^6 became 5.9 × 10^6; and the 10 × 10^6 became 3.8 × 10^6. The faint disperse band of hybridization around 3 × 10^6 daltons in the purified 5.2 × 10^6 dalton preparation is probably the result of degradation during preparative electrophoresis because it is not present in the starting RPC-5 fractions (see track III, Fig. 2*B*).

From the data in Figs. 3 and 4 we conclude that the 10 × 10^6 dalton *Bgl* I fragment in DNA from individual 88 probably includes all of its endogenous provirus sequences and the 2.4 × 10^6 dalton *Eco*RI fragment lies between the 5.2 × 10^6 and the 10 × 10^6 dalton *Eco*RI fragments. Preliminary data obtained by using 3′-end-specific RAV-2 probes indicate that the 10 × 10^6 dalton *Eco*RI fragment contains the 3′ end of the endogenous provirus.

Detection of Endogenous Proviral Sequences in Other Phenotypes. DNAs from 14 individual birds, including gs^- chf^-, gs^+ chf^+, gs_L chf_{HE}, and producers of RAV-0, were digested with *Eco*RI, fractionated by agarose gel electrophoresis, and analyzed as described for Fig. 1*B* (unpublished observations). In every case, the 2.4, 5.2, and 10 × 10^6 dalton fragments characteristic of DNA from individual 88 were detected, and they were the only fragments consistently present. Similarity in patterns of additional bands detected in most DNAs appeared to be specific for related individuals, but we could deduce no simple correlation between *Eco*RI restriction pattern and phenotype.

Bgl I digests of these same DNAs were analyzed in a similar way. In each case, proviral sequences were detected in a band at 10 × 10^6 daltons; however, in every DNA except that of in-

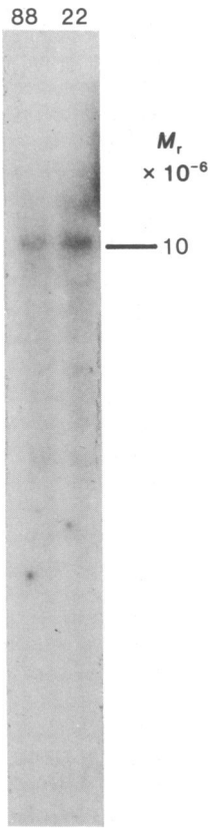

FIG. 3. Detection of endogenous provirus after *Bgl* I digestion. DNA extracted from erythrocytes as described in the legend to Fig. 1 was incubated at 37°C for 1 hr with 2 units per μg of DNA of restriction endonuclease *Bgl* I (New England BioLabs) in 10 mM Tris·HCl, pH 7.4/66 mM KCl/10 mM $MgCl_2$/1 mM dithiothreitol/100 μg of bovine serum albumin per ml. Samples (10 μg) of *Bgl* I-digested DNA from chickens 88 and 22 were fractionated on agarose gels and transferred to cellulose nitrate filters. Filters were hybridized at 37°C for 64 hr with RAV-2 [^{32}P]RNA (30 ng/ml, 12 × 10^7 cpm/μg) and unlabeled 18 and 28S rRNA (5 μg/ml).

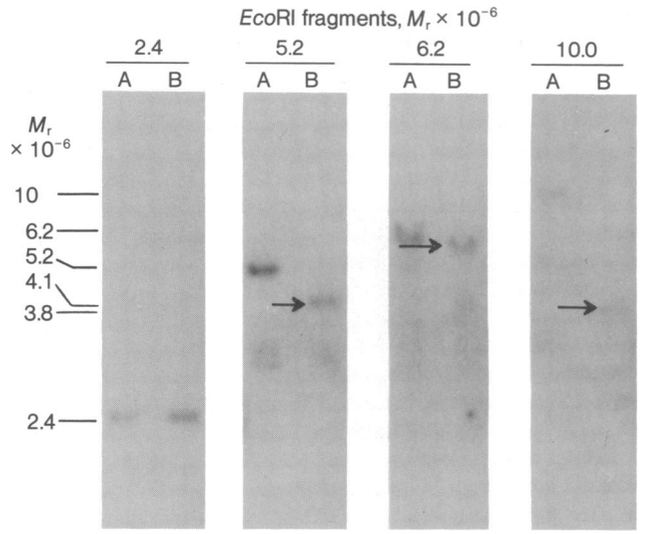

FIG. 4. *Bgl* I digestion of *Eco*RI fragments purified by RPC-5 chromatography and preparative agarose gel electrophoresis. Fractions from the RPC-5 separation described in Fig. 1 that contained endogenous proviral *Eco*RI fragments (sets II and III) were further purified by preparative gel electrophoresis (36). The purified DNAs were then digested with *Bgl* I and the digests were analyzed by the method of Southern as described in the legend to Fig. 1. Each well contained 0.3–1.0 μg of the purified DNAs before (*A*) and after (*B*) *Bgl* I digestion.

dividual 88, additional bands, larger or smaller, were also seen. As was the case with *Eco*RI fragments, the *Bgl* I fragment characteristic of individual 88 was the only one consistently present.

DISCUSSION

The two-step fractionation system of RPC-5 chromatography and gel electrophoresis has proven valuable in screening for unique sequences in restriction digests of complex genomes (34). It provides both increased resolution and sensitivity because the equivalent of 100–200 μg of unfractionated genomic DNA may be analyzed on a single analytical gel. The ability to identify and separate restriction fragments containing virus-related sequences from those bearing rDNA has been particularly useful in our studies. Cellular RNA including rRNA is encapsidated in avian tumor viruses (37), the source of our RNA probe. Because rDNA genes are present in chicken in about 200 copies (38), even a 1% contamination of the probe with the rRNA will result in detectable hybridization to rDNA, which may obscure the proviral hybridization pattern even in the presence of unlabeled rRNA competitor. The two-step fractionation circumvents this possible source of confusion and has simplified the interpretation of our data.

Of all chicken DNAs examined by *Eco*RI and *Bgl* I restriction digestion, the DNA from individual 88 yields the simplest pattern. Its proviral sequences are of particular interest because they are the only ones common to all DNAs examined and may represent some basic proviral unit. The single 10 × 10^6 dalton *Bgl* I fragment is large enough to contain the complete provirus [M_r ≈5–6 × 10^6 (39)] in a continuous segment. Two *Eco*RI sites within the provirus could generate a 2.4 × 10^6 dalton internal fragment and 5.2 and 10 × 10^6 dalton fragments that contain the remaining proviral sequences and flanking cellular sequences. *Eco*RI/*Bgl* I double digests support this interpretation. *Eco*RI digestion of the related RAV-2 provirus yields a 2.4 × 10^6 dalton internal fragment (L. Boone, personal communication), and restriction mapping of avian sarcoma virus (ASV) integrated into mammalian DNA (40) and provirus synthesized *in vitro* (41) show an internal fragment of approximately the same size. Absence of other internal fragments indicates that the endogenous proviral genome lacks the *Eco*RI site located close to the 3′ end of ASV (ref 41; L. Boone, personal communication) and duplicated at both ends in integrated and unintegrated exogenous proviral DNA *in vivo* (refs. 40, 41; L. Boone, personal communication). This difference is consistent with the observation that endogenous and exogenous avian tumor viruses show significant sequence divergence at their 3′ ends (42).

Variation in cellular sequences flanking the endogenous provirus could result in the loss or gain of *Eco*RI restriction sites, thereby altering the restriction pattern. With several copies of provirus per cell, more than three fragments containing provirus sequences could be generated from only two internal sites if the proviruses were inserted into more than one type of cellular sequence. This could explain the four-band *Eco*RI pattern and the two-band *Bgl* I pattern of DNA from individual 22 and the even more complex patterns found in other individuals. Fig. 5 shows a tentative restriction map for two integrated gs^- chf^- endogenous proviruses based on our results and those published for ASV (40–42). The diagram shows the provirus as a continuous segment but interruption by cellular sequences cannot be excluded.

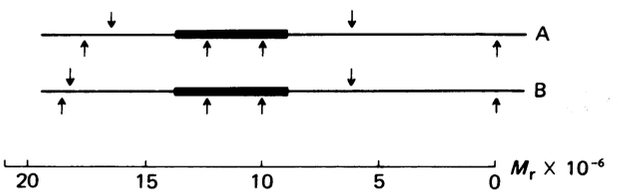

FIG. 5. Tentative restriction map of two integrated gs⁻ chf⁻ proviruses. Heavy bars indicate the approximate locations of proviral sequences, based on our studies and results of others (40, 41). ↑, *Eco*RI sites; ↓, *Bgl* I sites. A indicates the integrated provirus present in DNA from chicken 88 and all other individuals analyzed. B indicates the additional provirus detected in chicken 22 DNA.

The conservation of the pattern of the gs⁻ chf⁻ individual 88 among all chickens tested suggests that changes in the internal or flanking sequences do not occur frequently. Thus, the additional fragments seen in other lines and phenotypes may not result from single-site mutations, but could reflect proviral gene translocations or could even result from exogenous virus infections followed by proviral insertion at various locations, some of which effect phenotype. Results from our limited survey did not allow us to deduce any simple correlation between a given pattern and phenotype.

After the manuscript of this paper was completed, we learned of results from a related study by Astrin (43) that included restriction analysis of DNA from more than 150 individuals of different phenotypes. As with our observations, a complicated pattern of fragments was found, suggesting that endogenous provirus may be integrated at many sites. One site identified in a gs⁻ chf⁻ bird and designated *ev* 1 by Astrin was common to all DNAs studied. *Eco*RI restriction data suggest that *ev* 1 and our chicken 88 provirus site are identical. Other gs⁻ chf⁻ sites were identified that were present in addition to *ev* 1; this is confirmed by our results with chicken 22. Finally, *Sst* I restriction analysis and results from controlled mating experiments revealed four loci that were always accompanied by specific phenotypes (a gs⁻ chf⁺ locus, a gs⁻ chf⁺ locus, and two V⁺ loci). It will be of interest to compare the DNA of these and provirus integrated at the chicken 88 (*ev* 1) site.

Further analysis of the cellular *sarc* sequences should also be of interest. From our results, these sequences occur mainly in a 13×10^6 dalton *Eco*RI fragment. However, a small amount of hybridization to specific bands in the early RPC-5 fractions was also detected, and it is possible that other fragments may contain additional *src*- or virus-related sequences. Molecular cloning of fragments containing these and other virus-related sequences will make it possible to study their structure and function in detail and should simplify comparisons of various fragment patterns. DNA fractionated by the procedures described here should provide convenient starting material for these efforts.

We thank P. DeBona for unceasing efforts in supplying RAV-2 RNA and L. Baldassari for preparing SR-RSV-B and RAV-0 RNAs. We appreciated the gift of restriction enzymes from Dr. R. Roberts during the early stages of this work. Dr. P. Leder generously supplied advice, materials, and facilities for the large-scale RPC-5 fractionation that was performed by one of us (S.T.) while working in his laboratory. Dr. Leder and Drs. L. Enquist and G. Vande Woude at the National Institutes of Health were kind enough to run large-scale preparative electrophoreses for us on their "gene machines." The work was supported in part by U.S. Public Health Service Grant CA14935 from the National Cancer Institute.

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