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Genome of Reticuloendotheliosis Virus: Characterization by Use of Cloned Proviral DNA

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Reticuloendotheliosis virus is an avian type C retrovirus that is capable of transforming fibroblasts and hematopoietic cells both in vivo and in vitro. This virus is highly related to the three other members of the reticuloendotheliosis virus group, including spleen necrosis virus, but it is apparently unrelated to the avian leukosis-sarcoma virus family. Previous studies have shown that it consists of a replication-competent helper virus (designated REV-A) and a defective component (designated REV) that is responsible for transformation. In this study we used restriction endonuclease mapping and heteroduplex analysis to characterize the proviral DNAs of REV-A and REV. Both producer and nonproducer transformed chicken spleen cells were used as sources of REV proviral DNA; this genome was mapped in detail, and fragments of it were cloned in λgtWES·λB. The infected canine thymus line Cf2Th(REV-A) was used as a source of REV-A proviral DNA. The restriction maps and heteroduplexes of the REV and REV-A genomes showed that (proceeding from 5′ to 3′) (i) REV contains a large fraction of the REV-A *gag* gene (assuming a gene order of *gag-pol-env* and gene sizes similar to those of other type C viruses), for the two genomes are very similar over a distance of 2.1 kilobases beginning at their 5′ termini; (ii) most or all of REV-A *pol* is deleted in REV; (iii) REV contains a 1.1 kilobase segment derived from the 3′ end of REV-A *pol* or the 5′ end of *env* or both; (iv) this *env* region in REV is followed by a 1.9-kilobase segment which is unrelated to REV-A; and (v) the helper-unrelated segment of REV extends essentially all of the way to the beginning of the 3′ long terminal repeat. Therefore, like avian myeloblastosis virus but unlike the other avian acute leukemia viruses and most mammalian and avian sarcoma viruses, REV appears to be an *env* gene recombinant. We also found that the REV-specific segment is derived from avian DNA, for a cloned REV fragment was able to hybridize with the DNA from an uninfected chicken. Therefore, like the other acute transforming viruses, REV appears to be the product of recombination between a replication-competent virus and host DNA. Two other defective genomes in virus-producing chicken cells were also cloned and characterized. One was very similar to REV in its presumptive *gag* and *env* segments, but instead of a host-derived insertion it contained additional *env* sequences. The second was similar (but not identical) to the first in its *gag* and *env* regions and appeared to contain an additional 1-kilobase inversion of REV-A sequences.

The reticuloendotheliosis viruses are a closely related group of avian type C retroviruses which exhibit no homology with the avian leukosis-sarcoma virus family (20, 25). The members of this group are reticuloendotheliosis virus, spleen necrosis virus (SNV), chick syncytial virus, and duck infectious anemia virus. Reticuloendotheliosis virus is the only oncogenic member of the group and is able to transform fibroblasts and hematopoietic cells both in vivo and in vitro (11, 16, 17).

Like the other avian acute leukemia viruses and the mammalian sarcoma viruses, reticuloendotheliosis virus has been shown to be a mixture of a replication-competent helper virus (designated REV-A) and a defective genome (designated REV) which is responsible for transformation (16). In our initial report we showed that REV(REV-A) cDNA synthesized in the endogenous reverse transcriptase reaction contained some molecules that were able to hybridize with REV(REV-A) RNA but not with REV-A RNA [we refer to the oncogenic virus stock which contains both the REV genome and the REV-A genome as REV(REV-A)]; these molecules presumably represented sequences unique to the

transforming genome (38). The finding that REV(REV-A) RNA contained several oligonucleotide fragments not found in REV-A RNA (5) led to the same conclusion. We then showed that the helper-unrelated sequences were found in a 5.7-kilobase (kb) RNA isolated from REV(REV-A) (13). As with the other known acute transforming viruses, this RNA also contained helper-related sequences. These results were confirmed and extended by Hu et al. (19), who heteroduplexed full-length REV-A cDNA with REV(REV-A) RNA. Finally, we reported that the REV(REV-A)-specific sequences are able to hybridize with DNAs from uninfected chickens, turkeys, and pheasants, but that REV-A sequences are not. The cellular sequences were present in low copy numbers (less than five copies per haploid genome) and were very similar but not identical to the viral sequences (the melting temperature depression of the hybrid was about 5°C) (38). This result was confirmed recently by Wong and Lai (43). Thus, like the other known acute transforming viruses, the REV transforming genome appeared to be a recombinant between the helper virus genome and host DNA.

Although highly suggestive, none of these studies established that the recombinant RNA was in fact the transforming genome. Since REV(REV-A) RNA contains several species in addition to the helper and the 5.7-kb molecule (13, 19), this has not been a trivial issue. Therefore, we used restriction mapping to characterize the REV genome found in nonproducer transformed cells and compared it with the map of the REV-A genome. Fragments of REV-A and REV were also cloned out of a producer cell, and the relatedness of these fragments was examined by heteroduplex analysis. In this way, we were able to establish unequivocally that the REV genome present in nonproducer transformed cells contains both REV-A-related sequences and host-related sequences. Thus, this genome appears to be the product of recombination between the helper virus REV-A genome and avian host DNA.

MATERIALS AND METHODS

Virus and cells. The growth and purification of REV(REV-A) from a transformed chicken bone marrow cell line (11) and of REV-A from the canine thymus line Cf2Th have been described previously (38). The transformed chicken spleen cell lines S3D6, S0C6, and AS-1, all of which produce REV(REV-A), and the two nonproducer chicken spleen cell lines np3 and np4 were established by Jacalyn Hoelzer and Ann Soria (AS-1), as previously described (17).

Preparation of REV-A proviral DNA. Uninfected Cf2Th cells were treated with 2 μg of polybrene per ml for 1 h and then exposed for 1 h to clarified medium taken from cultures of Cf2Th(REV-A) cells. Cells were lysed 48 h later, and low-molecular-weight DNA was prepared as described by Hirt (15) and Yang et al. (45). The Hirt supernatant fraction was extracted with phenol-chloroform, ethanol precipitated, and after RNase A treatment, subjected to centrifugation (type 40 rotor, 34,000 rpm, 20°C, 48 h) in a CsCl density gradient containing 200 μg of ethidium bromide per ml. Fractions from the gradient were assayed for viral DNA content by electrophoresis, blotting, and hybridizing with REV-A [^{32}P]cDNA (see below). For the experiments described below, fractions containing linear REV-A viral DNA were used.

Preparation of DNA for restriction endonuclease analysis. Cell pellets or tissues were suspended with a Bellco tissue homogenizer in 0.01 M Tris (pH 8)–0.01 M sodium chloride–0.001 M EDTA (TNE) supplemented with 100 μg of self-digested pronase per ml. Sodium dodecyl sulfate was added to a final concentration of 1%, and the mixture was incubated at 37°C for 3 h. After phenol-chloroform extraction, the DNA was spooled out of the aqueous phase after 2 volumes of ethanol was added. The DNA was dissolved in TNE, treated with 25 μg of RNase A per ml, extracted with phenol-chloroform, dialyzed, ethanol precipitated, and resuspended in TNE at a concentration of 2 to 4 mg/ml.

Synthesis of [^{32}P]DNA. The preparation of REV-A 70S RNA and REV(REV-A) 50 to 70S RNA by sucrose gradient centrifugation and the synthesis of [^{32}P]cDNAs from these RNAs have been described previously (13). Nick-translation of cloned DNAs was performed by the method of Rigby et al. (34). Typically the specific activity of the reaction product was 10^8 cpm/μg.

Restriction endonuclease analysis of DNAs. DNAs were digested with restriction endonucleases under the conditions suggested by the manufacturers (New England Biolabs and Bethesda Research Laboratories). The amount of DNA per sample varied from 10 ng (when cloned DNA was used) to about 10 μg (when tissue DNA was used). The molecular weight markers used were a *Hin*dIII digest of λ DNA and a *Hae*III digest of φX174 DNA. Digested DNAs were subjected to electrophoresis through 1% agarose (Seakem) gels, blotted onto nitrocellulose (39), and hybridized with [^{32}P]DNA. When [^{32}P]cDNAs were used, hybridization was carried out at 67°C for 1 or 2 days in a solution containing 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate), 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin (Pentex), 50 μg of herring sperm DNA per ml, 10 μg of rRNA per ml, 0.1% sodium dodecyl sulfate, and 1×10^6 to 4×10^6 cpm of [^{32}P]cDNA per ml. Several washes were then used, the most stringent of which was 1× SSC at 67°C. When nick-translated [^{32}P]DNA was used, the filters were prehybridized for 3 h at 45°C in the above-described mixture. Then hybridization was carried out at 45°C for 1 to 2 days in a solution containing 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, 0.02% bovine serum albumin 5× SSC, 0.02 M sodium phosphate, 0.1% sodium dodecyl sulfate, 50 μg of herring sperm DNA per ml, 50% recrystalized formamide (Eastman), 10% dextran sulfate (Pharmacia Fine Chemicals), and 10^6 cpm of [^{32}P]DNA per ml. The most stringent of several washes was 0.1× SSC at 55°C. After washing, the filters were dried and exposed to Kodak XAR film at −70°C by using a Du Pont Lightning Plus intensifying screen if necessary.

Preparation of bacteriophage DNA. Bacteriophage λgtWES·λB (23) was propagated in *Escherichia coli* strain LE392 in T broth (1% tryptone [Difco Laboratories], 0.5% sodium chloride, 0.01 M magnesium sulfate) and purified by polyethylene glycol adsorption (44) and banding in CsCl gradients. The banded phage were dialyzed against 0.01 M Tris (pH 7.5)–0.01 M magnesium chloride and incubated with a solution containing 50 μg of pancreatic RNase (Calbiochem) per ml and 1 μg of DNase I (Sigma Chemical Co.) per ml for 60 min at 37°C. Then the solution was made 0.08 M EDTA and 0.1% sodium dodecyl sulfate and incubated with 200 μg of proteinase K (EM Biochemicals) per ml for 30 min at 55°C. The DNA was extracted sequentially with equal volumes of Tris-saturated phenol (pH 8.0), phenol-chloroform (1:1), and chloroform-octanol (24:1). The aqueous phase was dialyzed against TNE.

λgtWES·λB cohesive ends were annealed by incubation in 0.1 M Tris (pH 8.0)–0.01 M magnesium chloride for 60 min at 42°C. *Sac*I arms were prepared by digestion to completion with *Sac*I (Bethesda Research Laboratories) and sedimentation in a 20 to 50% sucrose gradient containing 1 M sodium chloride and 0.02 M Tris (pH 8.0) for 20 h at 30,000 rpm and 20°C in a Beckman SW41 rotor (26). The fractions were analyzed by agarose gel electrophoresis, and the fractions containing the annealed *Sac*I arms were pooled and dialyzed against TNE.

Isolation and size selection of cellular DNA. DNA from the producer cell line S3D6 was digested to completion with *Sac*I and electrophoresed on a preparative gel containing 1% low-melting-point agarose. The digestion products of the desired size were cut out, extracted by using 1-butanol and hexadecyltrimethylammonium bromide (22), further purified from residual agarose by benzoylated naphtholated DEAE-cellulose chromatography (35), ethanol precipitated, and suspended in TNE.

Ligations. Purified *Sac*I arms of λgtWES·λB and size-selected *Sac*I digestion products of cellular DNA were ligated at a molar ratio of vector to insert of 2:1 and a final DNA concentration of 100 μg/ml in a reaction mixture containing 0.066 M Tris (pH 7.6), 0.066 M magnesium chloride, 0.01 M dithiothreitol, 66 μM ATP, and 50 U of T4 ligase (Bethesda Research Laboratories) per ml. Ligations were carried out at 9°C for 24 h.

Packaging. In vitro packaging extracts were prepared from lysogenic strains NS428 and NS433, and packaging reactions were carried out as described by Sternberg and Enquist (41). The usual packaging efficiencies ranged from 5×10^5 to 5×10^6 PFU/μg of ligated DNA.

Recombinant phage were propagated in strain LE392 in 15-cm petri dishes (5×10^4 PFU/dish) and were screened for REV-related inserts by adsorption of phage DNA to nitrocellulose filters in situ (4) and hybridization with REV-A or REV(REV-A) [^{32}P]cDNA. Positive plaques were picked, replated, and rescreened, and finally phage DNA was prepared as described above for λgtWES·λB.

Subcloning. A 650-base pair fragment of the cloned REV genome extending from the *Hin*dIII site at map position 2.90 to the *Eco*RI site at map position 3.55 was excised from the λgtWES·λB vector by digestion with *Hin*dIII and *Eco*RI, and this fragment was purified by agarose gel electrophoresis. Then the fragment was electroeluted from the gel and further purified from contaminating agarose by benzoylated naphtholated DEAE-cellulose chromatography (35). The purified fragment was ligated into the *Hin*dIII-*Eco*RI sites of pBR322, and the hybrid plasmid was transfected into *E. coli* strain HB101 (8). Clones containing the hybrid plasmid were selected on the basis of ampicillin resistance and tetracycline sensitivity. To isolate plasmid DNA, 1-liter cultures were grown to an absorbance at 560 nm of 0.8. Then 100 μg of chloramphenicol per ml was added, and the cultures were held at 37°C for 12 h. The plasmids were extracted from pelleted bacteria, and supercoiled DNA was purified on cesium chloride-ethidium bromide gradients (42).

Heteroduplexes. The conditions used for alkali denaturation of cloned DNAs, renaturation, and spreading of the heteroduplexes from a hyperphase containing 0.1 M Tris (pH 8.5), 0.01 M EDTA, and 50% deionized formamide onto a hypophase containing 0.01 M Tris, 0.001 M EDTA, and 20% formamide have been described previously (46).

RESULTS

Restriction endonuclease map of REV-A DNA. Both chromosomal DNA from chronically infected canine cells and unintegrated DNA isolated from canine cells 24 to 48 h after infection with REV-A were used as sources of REV-A viral DNA. We observed no differences in the cleavage patterns between these two DNAs. Figure 1 shows the data which established the cleavage sites for eight enzymes which cut the DNA once, twice, or three times. For other enzymes we simply state below the sizes of the resulting fragments and explain how the positions of the cleavage sites were obtained.

The unintegrated linear REV-A DNA was about 8.6 kb long (Fig. 1A). *Sac*I cut this linear DNA into three apparent fragments of 7.3, 0.75, and 0.55 kb, whereas *Sal*I digestion resulted in two fragments of 7.7 and 0.9 kb. Double digestion with both *Sal*I and *Sac*I revealed that *Sal*I cut about 0.1 kb from the 7.3-kb *Sac*I fragment. The three cleavage sites implied by these data are shown in Fig. 1E. Since a 0.75-kb *Sac*I fragment was also found in integrated DNA from infected canine cells, there must be a *Sac*I site at the extreme left end of the genome. Since the same constellation of *Sac*I and *Sal*I sites has been reported for SNV DNA and since the orientation of the genome was established in SNV experiments (21), we assigned the left end in Fig. 1E to the 5′ terminus of the genome. More precisely, a *Sac*I site occupies bases 10 through 15 of the 569-base pair SNV long terminal repeat (LTR) (36). The many similarities between the REV-A and SNV restriction maps (see below) justify this reliance on the SNV data.

In addition to the eight enzymes used in the experiments shown in Fig. 1, six others were

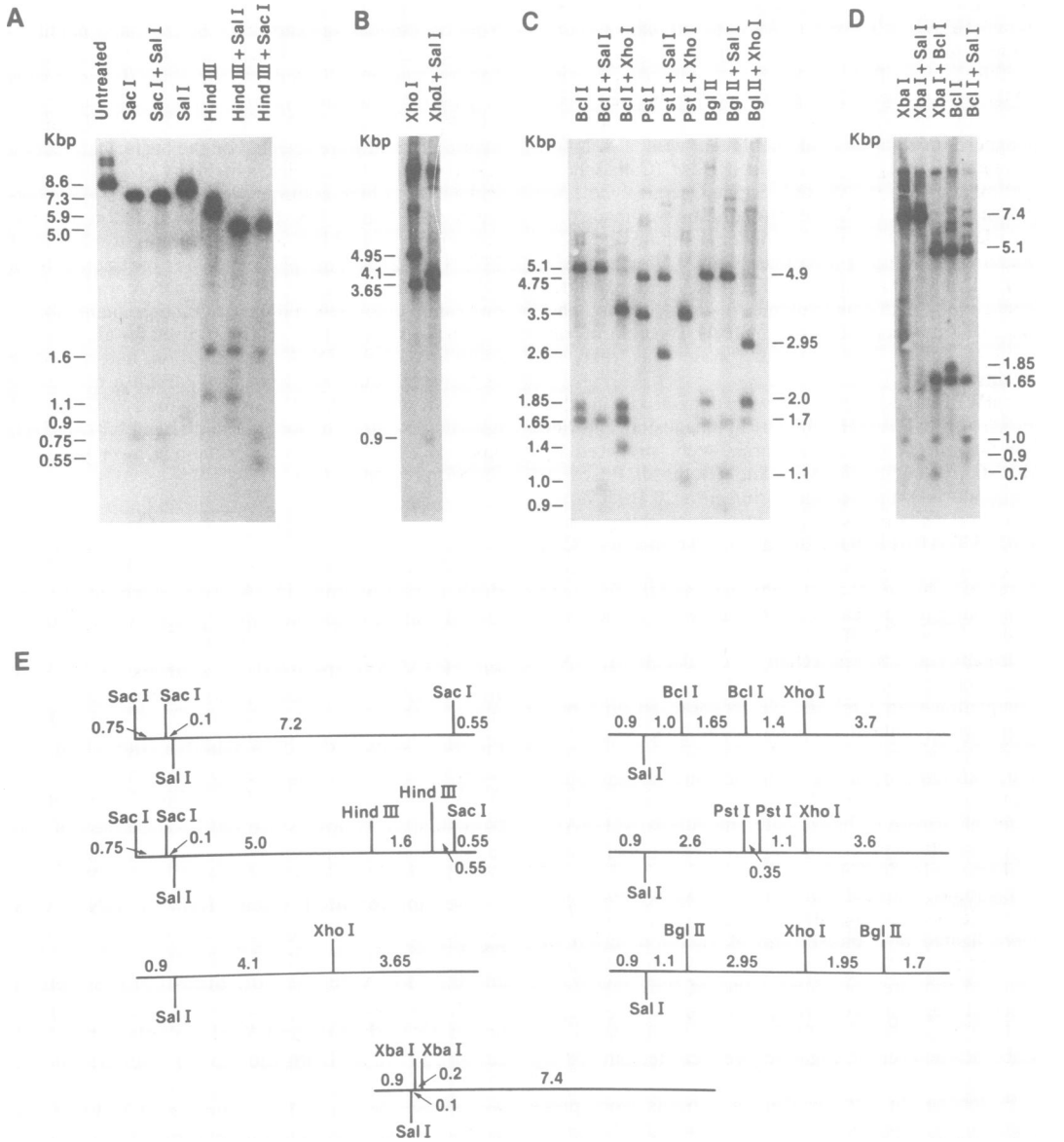

FIG. 1. Restriction endonuclease digests of REV-A DNA. Unintegrated linear REV-A DNA was digested as described in the text, subjected to electrophoresis through 1% agarose gels, blotted onto nitrocellulose (39), and hybridized with REV-A [^{32}P]cDNA. (A through D) Autoradiographs of washed filters. In the untreated sample, the two faint apparent high-molecular-weight bands are probably nicked circular REV-A DNA which copurified with the linear molecules in a cesium chloride-ethidium bromide gradient. They appear to differ in size by the length of one LTR. Since the intensity of these bands is low, it was always possible to distinguish their digestion products from those of the linear DNA. (E) Maps of the cleavage sites for the eight endonucleases, drawn by using the fragment sizes found in (A) through (D). The arrangement of *Pst*I sites was confirmed by double digestion with *Bcl*I and *Pst*I. We found that *Pst*I reduced the size of the 1.65-kb *Bcl*I fragment by about 50 nucleotide pairs and the size of the 5.1-kb *Bcl*I fragment by about 0.3 kb. The *Xba*I site at map position 1.2 was also established through double digestion with *Bcl*I. Whereas *Sal*I cleaved the 1.85-kb *Bcl*I fragment into 1.0- and 0.85-kb pieces, *Xba*I digestion of the 1.85-kb *Bcl*I fragment resulted in 1.0- and 0.7-kb pieces, leaving 0.15 kb unaccounted for (D). In a second experiment, the missing fragment was 0.19 kb. Thus, there must have been a second *Xba*I site within about 0.2 kb of the first. Kbp, kilobase pairs.

tested, yielding the fragment sizes given in Table 1. Of the five *Bam*HI fragments found in unintegrated REV-A DNA, only the 2.25-, 1.9-, and 0.8-kb fragments were also found after digestion of chromosomal DNA from chronically infected canine cells; therefore, these are the only internal fragments. Digestion with *Bam*HI plus *Pst*I, *Xho*I, or *Hin*dIII unambiguously established the order of these fragments. The end fragments were verified by double digestion with *Bam*HI plus *Sac*I or *Sal*I. Of the five *Sma*I fragments shown in Table 1, the 1.9-, 1.55-, and 0.9-kb fragments were also found in digests of chromosomal DNA. Double digestion with *Sma*I and *Sal*I established that the first *Sma*I site is located at map position 3.0, and digestion with *Sma*I plus *Xho*I, *Pst*I, or *Bgl*II enabled ordering of the internal fragments. Digestion of unintegrated REV-A DNA with *Kpn*I yielded four fragments. These were ordered and the cleavage sites were mapped by double digestion with *Sal*I, *Xba*I, *Xho*I, *Bgl*II, or *Hin*dIII. The order of the five *Bst*EII fragments was determined by double digestions with *Sal*I, *Xho*I, *Sma*I, or *Bgl*II.

Not all of the cleavage sites for the two remaining enzymes (*Bgl*I and *Ava*I) were determined, since a number of the fragments are quite small. Double digestion with *Bgl*I and *Sal*I, *Sac*I, or *Bgl*II established that there are *Bgl*I sites at map positions 4.0 and 7.35, but there are also three additional sites between these two points. One of these sites is at either map position 5.65 or map position 5.85, but the other two have not been mapped with certainty. Several of the numerous *Ava*I sites have been mapped. As in SNV DNA, *Ava*I cuts REV-A DNA in the LTR, for *Sal*I cuts the 1.7-kb *Ava*I fragment into 1.2- and 0.5-kb pieces. The 1.1-kb *Ava*I fragment is located at the 3′ end of the genome, for both *Sac*I and *Hin*dIII cleave it, whereas the 1.4-kb *Ava*I fragment extends from map position 5.9 to map position 7.3, based on results of digestion with *Bgl*II. The region from map position 2.1 to map position 5.9 has not been mapped with *Ava*I. This region evidently contains many sites, for digestion produces a number of small fragments. A 14-enzyme restriction map of REV-A DNA is shown in Fig. 2, together with the map of REV DNA. Construction of the latter is described below.

Identification of REV DNA in transformed cells. Knowing the restriction map of REV-A DNA, we then asked which fragments, if any, are also present in REV-transformed chicken cells. As expected, all REV(REV-A)-producing cells tested contained the 7.3-kb *Sac*I fragment already identified in REV-A DNA. DNA of this approximate size was purified from a *Sac*I digest

TABLE 1. Restriction endonuclease fragments of unintegrated REV-A and REV DNAs[a]

Enzyme	Fragment sizes (kilobase pairs)	
	REV-A	REV
None	8.6	5.8
*Eco*RI	8.6	3.55, 1.05, 1.2
*Hpa*I	8.6	NT
*Pvu*I	8.6	5.8
*Pvu*II	8.6	3.35, 0.9, 0.45, 1.1
*Sal*I	0.9, 7.7	0.9, 4.9
*Xho*I	4.95, 3.65	5.8
*Sac*I	0.75, 7.3, 0.55	0.75, 3.05, 0.6, 0.8, 0.55
*Hin*dIII	5.9, 1.6, 1.1	2.9, 0.8, 2.1
*Bcl*I	1.85, 1.65, 5.1	1.85, 3.95
*Pst*I	3.5, 0.35, 4.75	3.5, 1.2, 1.1
*Bgl*II	2.0, 4.9, 1.7	2.0, 3.8
*Xba*I	1.0, 0.2, 7.4	1.0, 0.2, 0.8, 1.2, 2.6
*Bam*HI	1.9, 1.9, 2.25, 0.8, 1.7	0.55, 1.4, 2.35, 1.45
*Sma*I	3.0, 0.9, 1.9, 1.55, 1.25	2.85, 2.95
*Kpn*I	0.7, 0.85, 2.1, 4.9	0.7, 0.75, 4.35
*Bst*EII	4.9, 0.6, 0.5, 0.3, 2.3	2.6, 3.2
*Bgl*I	4.0, (1.3, 1.1, 0.6, 0.4), 1.25	2.65, 0.85, 2.3
*Ava*I	0.4, 1.7 (0.9, 0.6, 0.5, and others), 1.4, 1.1, 0.2	0.4, 1.7, 0.4, 0.3, 0.2, 2.6, 0.2

[a] Restriction maps were constructed as described in the text and in the legends to Fig. 1 and 4. Since all mapping experiments with REV were performed either on integrated DNA in nonproducer cell clones or on molecularly cloned REV DNA segments, end fragments were not measured directly. The dimensions of the fragments were calculated based on (i) the 5.8-kb size of unintegrated linear REV DNA found in chicken embryo fibroblasts 48 h after infection with REV(REV-A) and (ii) the assignment of REV segments from positions 0 to 0.6 and 5.2 to 5.8 as LTRs based on the similarity of their restriction enzyme sites to those of REV-A and SNV. Fragments are listed in order from 5′ to 3′. The order was not determined for the fragments in parentheses. NT, Not tested.

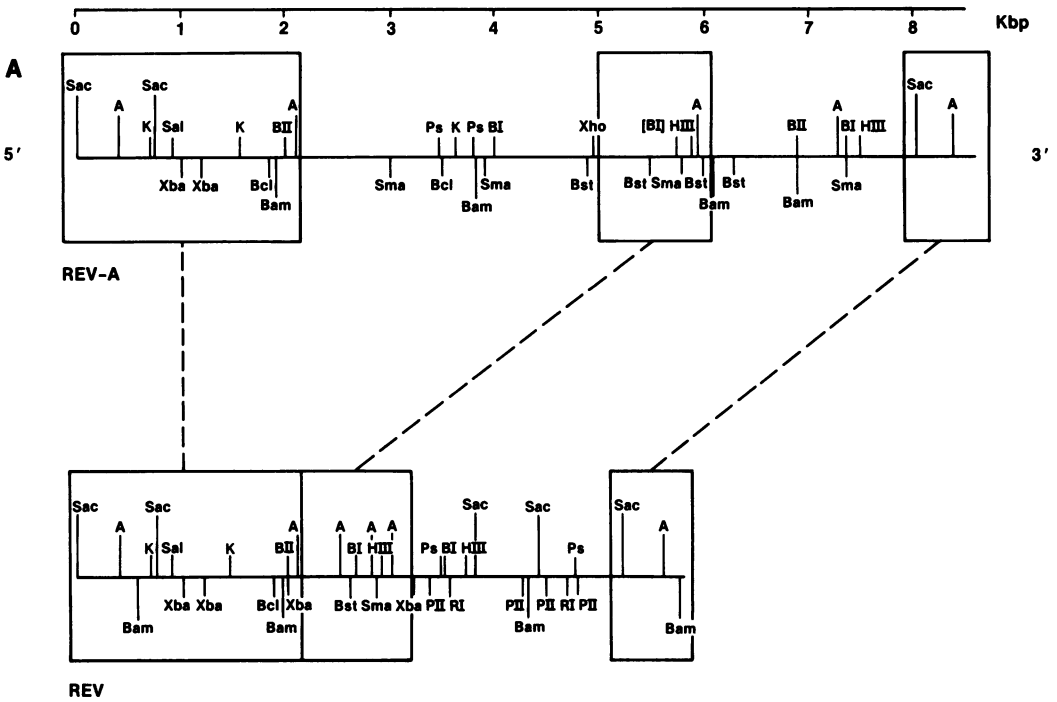

FIG. 2. Restriction endonuclease maps of REV-A and REV. The maps were constructed as described in the text and in the legends to Fig. 1 and 4. The regions of similarity between the two genomes are enclosed in boxes. The LTR of SNV, which is highly related to REV-A, is 569 base pairs long; a *Sac*I site occupies bases 10 through 15 (36). (A) *Eco*RI, *Hpa*I, *Pvu*I, and *Pvu*II do not cut the REV-A genome. There are three *Bgl*I sites between map positions 4.0 and 7.35; one of these is at map position 5.65 or 5.85 and is shown in brackets. There are several *Ava*I sites between map positions 2.1 and 5.9 which have not been mapped. (B) *Xho*I and *Pvu*I do not cut the REV genome. A, *Ava*I; Bam, *Bam*HI; Bcl, *Bcl*I; Bst, *Bst*EII; BI, *Bgl*I; BII, *Bgl*II; HIII, *Hin*dIII; K, *Kpn*I; Ps, *Pst*I; PII, *Pvu*II; RI, *Eco*RI; Sac, *Sac*I; Sal, *Sal*I; Sma, *Sma*I; Xba, *Xba*I; Xho, *Xho*I. Kbp, kilobase pairs.

of the producer cell line S3D6 and was cloned into λgtWES·λB; the identity of the resulting clones was verified by mapping. This cloned DNA, which contains most of the REV-A genome, was then used as a probe to detect related sequences in nonproducer transformed chicken cells. As expected, the 7.3-kb cloned DNA hybridized with the 7.3-kb *Sac*I fragments in four chicken cell lines producing REV(REV-A) and in the canine cell line producing REV-A; no such fragment was detected in *Sac*I digests of two independent nonproducer chicken cell lines (Fig. 3). However, DNAs from the nonproducers were able to hybridize with the probe, for *Sac*I fragments of 3.05 and 0.8 kb were observed. Hybridizing DNAs in this size range were also apparent in all four producer lines, but not in uninfected chicken DNA. Therefore, we concluded that these two fragments were derived from the genome responsible for transformation.

In addition to containing sequences able to hybridize with REV-A, the 3.05- and 0.8-kb *Sac*I fragments also appeared to contain other sequences not found in the helper. This conclusion was first reached through the use of an REV-(REV-A) cDNA from which most sequences able to hybridize with REV-A 70S RNA had been removed by hydroxyapatite chromatography. We found that this preabsorbed cDNA still hybridized intensely to both fragments, as well as to an additional fragment of 0.6 kb (data not shown). Neither the 3.05-, 0.8-, nor 0.6-kb fragment was found in uninfected chicken DNA. Thus, just as previously found for the 5.7-kb RNA in REV(REV-A) preparations (13), the REV-A-related genome found in nonproducer transformed cells also contains sequences unique to the transforming genome. Restriction endonuclease mapping of REV confirmed this conclusion.

Restriction endonuclease map of REV DNA. A detailed restriction map of REV was constructed initially by using chromosomal DNA from nonproducer transformed chicken cells. Subsequently, *Sac*I fragments of the genome from producer line S3D6 were cloned into λgtWES·λB. Since mapping data are much easi-

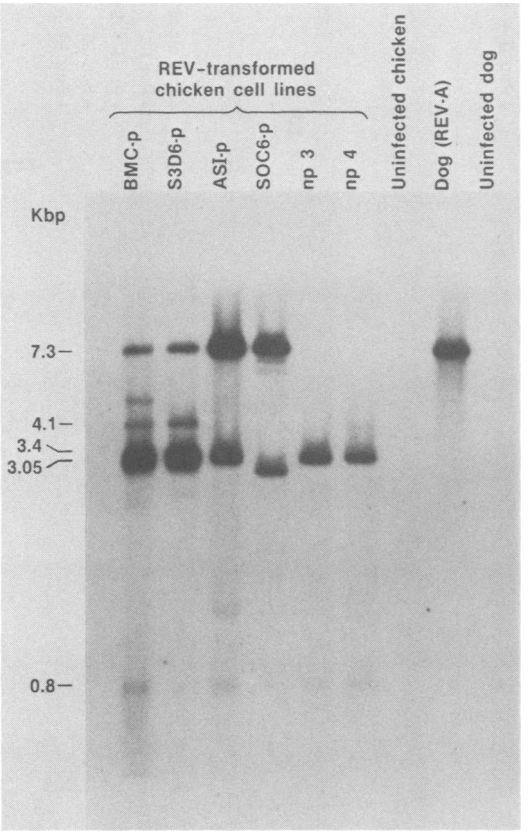

FIG. 3. Detection of REV-A and REV in infected cells. DNAs from the cell lines indicated were digested with *Sac*I, electrophoresed, blotted onto nitrocellulose, and hybridized with a nick-translated 7.3-kb *Sac*I fragment cloned out of producer line S3D6 into λgtWES·λB. This fragment contains most of the REV-A genome (see Fig. 1 and 2). The amount of DNA per lane varied from 3 μg for S3D6 and dog (REV-A) DNAs (which contain multiple copies of the viral genome) to 17 μg for the nonproducer and uninfected chicken DNAs. The two nonproducer cell clones contained only a single copy of REV per cell since digestion with each of several enzymes which cleave the REV genome once (*Bcl*I, *Bgl*I, *Sma*I, *Bst*EII) resulted in only two hybridizing fragments. The 3.05-kb fragment in SOC6-p DNA is actually 2.85 kb long, and the position of its deletion(s) has not been mapped. The 4.1- and 3.4-kb fragments in BMC-p and S3D6-p originated from deletion mutants of REV-A. An analysis of these genomes is given in the text and in Fig. 8 and 9. p, Producer; np, nonproducer; Kbp, kilobase pairs.

er to follow with cloned DNA than with cellular DNA, we present the former here. In every respect tested, the restriction sites in the cloned DNA were identical to those established for cellular DNA.

The results of digesting the 3.05-kb REV *Sac*I fragment with 10 enzymes are shown in Fig. 4A and B. With the exception of *Hin*dIII (which is discussed below), these enzymes have single sites within the DNA. Since *Sal*I cuts very close to one end of the 3.05-kb fragment, removing about 0.15 kb, it was convenient to map sites for the other enzymes simply by determining which of the two resulting fragments was cut by *Sal*I. This led to the map shown in Fig. 4C.

The cleavage sites for *Kpn*I, *Bgl*I, *Xba*I, and *Ava*I were also determined. *Kpn*I cut the 3.05-kb *Sac*I fragment once, and double digestion with *Sal*I showed that its site is located at map position 0.7 (Fig. 4D). *Bgl*I digestion of the 3.05-kb REV *Sac*I fragment resulted in three pieces (1.85, 0.9, and 0.3 kb), whose locations were mapped by double digestion with *Sal*I, *Pvu*II, or *Eco*RI. The 3.05-kb fragment was cut by *Xba*I into five pieces (1.25, 0.75, 0.6, 0.3, and 0.2 kb), even the smallest of which was detectable in the autoradiograph of the hybridized Southern blot. These fragments were ordered by double digestion with *Sal*I, *Kpn*I, *Bam*HI, *Sma*I, or *Pvu*II to give the arrangement shown in Fig. 4D. The *Hin*dIII site at map position 2.95 was deduced because *Hin*dIII cut not only the 1.25-kb *Xba*I fragment (map positions 1.25 to 2.5), but also the 0.6-kb *Xba*I fragment (map positions 2.45 to 3.05). Finally, *Ava*I digestion of the 3.05-kb *Sac*I fragment yielded five pieces (1.35, 0.8, 0.4, 0.25, and 0.2 kb), whose order was determined by double digestion with *Sal*I, *Bam*HI, *Bst*EII, *Sma*I, *Pvu*II, or *Bgl*I.

The map of this 3.05-kb *Sac*I fragment, which is shown as part of the complete REV map in Fig. 2, can now be compared with that of REV-A. The two genomes are very similar, having eight cleavage sites in common, for a distance of at least 1.35 kb beginning at the *Sac*I site at map position 0.75. Thus, assuming a gene order of *gag-pol-env*, much of the REV-A *gag* gene is also present in REV. Thereafter colinearity ends, for the grouping of sites in REV-A between map positions 3.0 and 5.0 (which presumably includes much of the *pol* gene) has no counterpart in the REV fragment. However, the next cluster of sites in REV (including *Bst*EII, *Bgl*I, *Sma*I, *Hin*dIII, and *Ava*I) does bear a striking resemblance to a cluster located in REV-A between map positions 5.5 and 6.0 (which presumably includes the 3′ end of *pol* or the 5′ end of *env* or both). Beyond the similar *Bst*EII-*Bgl*I cluster, the succeeding sites in the REV *Sac*I fragment (*Xba*I-*Pvu*II-*Pst*I-*Bgl*I-*Eco*RI-*Hin*dIII-*Sac*I) have no counterparts in REV-A; these sites are evidently unique to the transforming genome. If this is true, then fragments wholly within this unique region should not be able to hybridize with REV-A cDNA, whereas REV(REV-A) cDNA should allow their

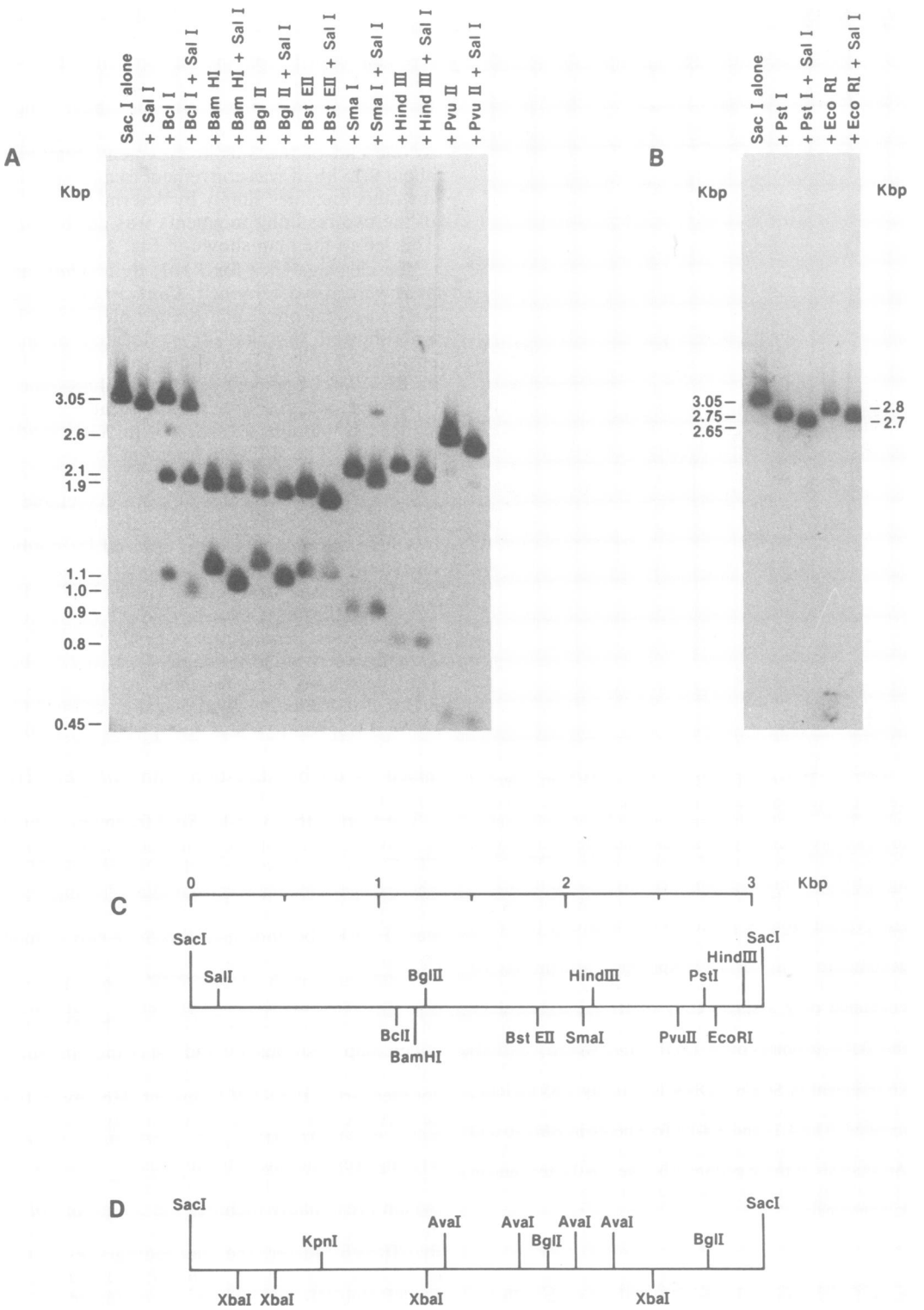

FIG. 4. Restriction endonuclease digest of a cloned 3.05-kb *Sac*I fragment of REV DNA. λgtWES·λB DNA containing the 3.05-kb *Sac*I fragment of REV was digested with endonucleases, subjected to electrophoresis, blotted onto nitrocellulose, and hybridized with REV(REV-A) cDNA. (A and B) Autoradiographs of the washed filters. (C) Map. (D) Cleavage sites for *Xba*I, *Kpn*I, *Ava*I, and *Bgl*I determined as described in the text. The *Bcl*I digests (A) were incomplete presumably because of the high frequency of methylation of the *Bcl*I site in *E. coli* K-12. Digestion of chromosomal DNA always proceeded to completion. Kbp, kilobase pairs.

detection. Figure 5 shows that this indeed is the case. Whereas the 0.9-kb *Bgl*I fragment (map positions 2.65 to 3.55) and the 0.75-kb *Ava*I-*Sac*I fragment (positions 3.0 to 3.8) are able to hybridize with both cDNAs, the *Xba*I-*Sac*I, *Pvu*II-*Sac*I, *Pst*I-*Sac*I, and *Bgl*I-*Sac*I fragments hybridize only with REV(REV-A) cDNA.

Beyond the 3.05-kb *Sac*I fragment, the remainder of the REV genome was mapped by using DNA from nonproducer transformed cells. Of the 17 enzymes used with REV, only *Bam*HI, *Pvu*II, *Eco*RI, *Pst*I, *Ava*I, *Kpn*I, and *Sac*I had cleavage sites outside the 3.05-kb *Sac*I fragment. These sites were located through double digestions, and several of them were confirmed by testing a λgtWES·λB clone of the 0.8-kb *Sac*I fragment located toward the 3′ end of the genome (map positions 4.4 to 5.2).

As Fig. 2 shows, the region unique to REV extends well beyond the 3′ terminus of the 3.05-kb *Sac*I fragment. In fact, no similarity to REV-A occurs until the *Sac*I site at map position 5.2, which marks the beginning of the 3′ LTR. As mentioned above, REV(REV-A) cDNA but not REV-A cDNA is able to hybridize to fragments wholly within this region. The fragments tested include the 1.1-kb *Xba*I-*Bam*HI fragment (map positions 3.2 to 4.3), the 0.9-kb *Pvu*II fragment (map positions 3.35 to 4.25), the 0.6-kb *Sac*I fragment (map positions 3.8 to 4.4), and the 1.1-kb *Eco*RI fragment (map positions 3.55 to 4.65) (data not shown). Thus, the region unique to REV begins at map positions 3.0 to 3.2 and extends through positions 4.7 to 5.2, giving a total size of 1.5 to 2.2 kb. This region is linked at its 5′ end to a segment apparently derived from the REV-A *env* gene and at its 3′ end to as much as a few hundred base pairs of REV-A *env*, followed by the REV-A 3′ LTR.

Heteroduplexes between REV-A and REV. The above conclusions about the structure of REV were confirmed by the results of heteroduplexing with REV-A DNA. When the cloned 7.3-kb *Sac*I fragment from REV-A was hybridized with the cloned 3.05-kb fragment from REV, the structure shown in Fig. 6 resulted. Starting from the left (the presumptive 5′ end), the two molecules were paired over a distance of 1.45 ± 0.09 kb. Since these fragments begin at the *Sac*I site at map position 0.75, this pairing extends to map position 2.2 (1.45 plus 0.75). A deletion loop of 2.94 ± 0.18 kb was followed by pairing over a distance of 1.12 ± 0.07 kb. As expected, there was no homology at the 3′ ends of these molecules, for the 3.05-kb fragment ends within the region unique to REV. Heteroduplexing the REV-A 7.3-kb *Sac*I fragment (which lacks all of the 5′ LTR and almost all of the 3′ LTR) with REV RNA showed that the REV-specific region must extend almost all the way to the beginning

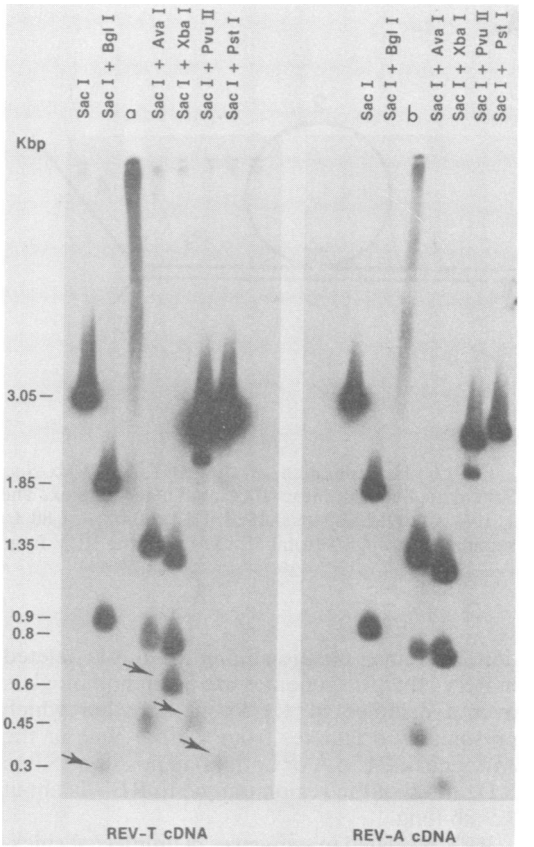

FIG. 5. REV contains sequences not found in REV-A cDNA. The 3.05-kb *Sac*I fragment of REV in λgtWES·λB was digested, electrophoresed, blotted onto nitrocellulose, and hybridized with [^{32}P]cDNA from either REV(REV-A) or REV-A. The arrows indicate the fragments detected only by REV(REV-A) cDNA. The 0.3-kb *Sac*I-*Xba*I fragment detected with REV-A cDNA also appeared after a longer exposure of the filter hybridized with REV(REV-A) cDNA. Lanes a and b contained incomplete digests. The minor band at about 2 kb in the *Sac*I-*Pvu*II digests was not reproducible.

of the 3′ LTR, for in only two cases did the molecules appear to be paired at their 3′ ends; this pairing extended over a distance of ≤100 bases (data not shown). These RNA-DNA heteroduplexes also provided an independent means of establishing the 5′-3′ orientation of the molecules, for the RNAs had circular simian virus 40 DNA molecules linked via polybromodeoxyuridine to the polyadenylic acid tracts at their 3′ ends.

Taken together, the mapping and heteroduplex data show that REV and REV-A share common sequences for a distance of 2.1 kb from their 5′ termini, that the 2.9 kb from map posi-

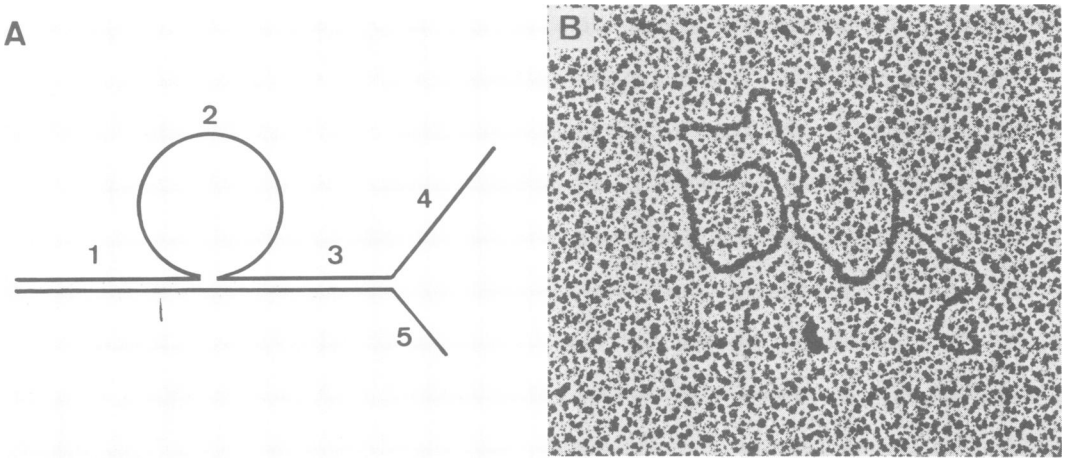

FIG. 6. Heteroduplex of the REV-A 7.3-kb *Sac*I fragment with the REV 3.05-kb *Sac*I fragment. (A) Schematic heteroduplex. (B) Actual heteroduplex. The dimensions of the sections (in kilobases) were as follows: 1, 1.44 ± 0.09; 2, 2.94 ± 0.18; 3, 1.12 ± 0.07; 4, 1.80 ± 0.09; 5, 0.46 ± 0.06. The REV-A fragment was made up of sections 1 through 4 (total, 7.3 kb), and the REV fragment was made up of sections 1, 3, and 5 (total, 3.02 kb).

tion 2.1 to map position 5.0 in REV-A is deleted in REV, that the genomes are again homologous over a distance of 1.1 kb in a region which presumably originates from the *pol* gene or the *env* gene of REV-A or both (map positions 5.0 to 6.1), and that the region unique to REV is about 1.9 kb long.

REV is related to sequences in uninfected chicken DNA. We reported previously (38) that in standard solution hybridization experiments REV(REV-A) cDNA but not REV-A cDNA was able to hybridize to a limited extent (~15%) with DNAs from uninfected chickens and related fowl. Subsequently, we found by using Southern blot experiments that REV(REV-A) cDNA but not REV-A cDNA could hybridize to 6.6- and 1.8-kb *Sac*I fragments in DNA from uninfected chickens. However, since 50 to 70S RNA from REV(REV-A) is often contaminated with rRNA and other cellular species, this result required verification with a cloned probe. Toward this end, the DNA from a pBR322 subclone of the REV 3.05-kb *Sac*I fragment was used. This fragment extends from the *Hin*dIII site at REV map position 2.9 to the *Eco*RI site at map position 3.55. At its 5′ end it contains REV-A-related sequences, and at its 3′ end it contains at least 350 base pairs which are unique to REV.

Figure 7 shows hybridization of this subcloned DNA to DNAs from various types of chicken cells. By virtue of its REV-A-related sequences, this DNA hybridized to the 7.3-kb *Sac*I fragment in DNA from a REV(REV-A)-producing cell line. It also hybridized to the REV-derived 3.05-kb *Sac*I fragment in both producer and nonproducer DNAs. Neither of these fragments is evident in the DNA from uninfected chickens. However, there are sequences in uninfected chicken DNA that are related to this probe, for hybridization to a 6.6-kb *Sac*I fragment was observed. Since this fragment was not detectable when hybridization was carried out with either REV-A cDNA or the cloned REV-A 7.3-kb *Sac*I fragment, we concluded that the hybridizing sequences in chicken DNA are related to the REV-specific region of the genome. Therefore, like the other known acute leukemia viruses, REV appears to be a product of recombination between a replication-competent nontransforming virus (REV-A) and host DNA.

Other defective viral forms in chicken cells producing REV(REV-A). Hybridization of DNAs from several REV(REV-A)-producing cell lines with the nick-translated 7.3-kb *Sac*I fragment of REV-A revealed a number of bands besides those derived from REV-A and REV (Fig. 3 and 7). We examined two of these in some detail. A preparative *Sac*I digest of S3D6 DNA was subjected to electrophoresis, and DNAs about 4 kb and about 3 to 3.5 kb long were purified and cloned into λgtWES·λB. The smaller DNA yielded clones not only of the 3.05-kb *Sac*I fragment of REV, but also of an REV-A-related 3.4-kb fragment, and the larger DNA yielded clones of a 4.1-kb fragment. The structures of both of these were studied by restriction mapping and by heteroduplexing.

The genome from which the 3.4-kb fragment was derived is evidently a deletion mutant of REV-A. Beginning from the 5′ end, the first 1.4 kb of this fragment is extremely similar to the

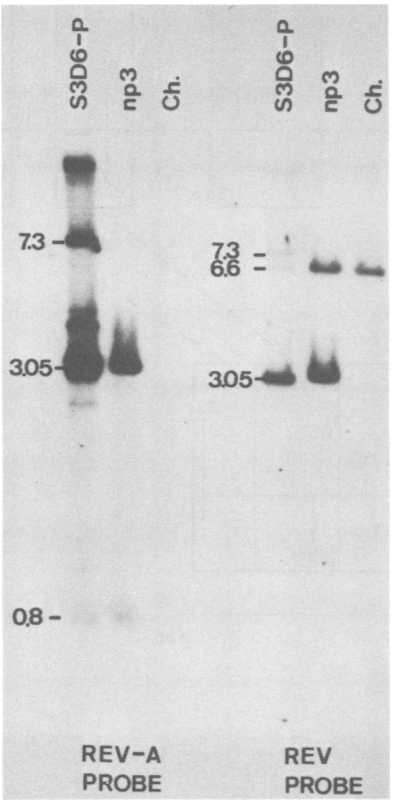

FIG. 7. Hybridization of a cloned fragment of REV with DNA from an uninfected chicken. DNAs from the S3D6 cell line producing REV(REV-A), from a nonproducer Line (np3), and from an uninfected SPAFAS chicken (Ch.) were digested with *Sac*I, electrophoresed, blotted onto nitrocellulose, and hybridized. The amount of DNA per lane was 2.5 μg for S3D6 DNA and 17 μg for np3 and uninfected chicken DNAs. For the REV-A probe hybridization was with a nick-translated 7.3-kb *Sac*I fragment of REV-A, and for the REV probe hybridization was with a nick-translated 0.65-kb fragment of REV, spanning map positions 2.9 to 3.55 (*Hin*dIII to *Eco*RI).

first 1.4 kb of the REV-A 7.3-kb *Sac*I fragment (Fig. 8). This same region is also found in REV (Fig. 2). This is followed by about 0.85 kb, which is derived from the *pol* gene or the *env* gene of REV-A or both (assuming a gene order of *gag-pol-env*). Again, this same region is also found in REV, although there the homology extends over about 1.1 kb. Two more regions (0.6 and 0.4 kb) from within the REV-A *env* gene follow, and the fragment ends with the *Sac*I site marking the beginning of the 3′ LTR. Thus, this genome appears to consist solely of REV-A segments and, as far as can be determined, has no new sequences inserted into it. A viral RNA transcribed from this genome and also containing one copy of the LTR and about 200 bases of polyadenylic acid would be 4.35 kb long (the sum of 3.4, 0.75, and 0.2 kb). In fact, we previously observed an RNA of about this size in REV(REV-A) preparations taken from both bone marrow (13) and S3D6 spleen cell lines. Like the 3.4-kb DNA, the RNA appeared to contain only REV-A-related sequences.

The 4.1-kb *Sac*I fragment is slightly more complicated. Like REV and the 3.4-kb fragment, at least 1.2 kb at the 5′ end of this DNA is derived from REV-A (Fig. 9). As determined by restriction mapping, this region is slightly smaller than it is in REV or the 3.4-kb fragment, for the *Bgl*II, *Xba*I, and *Ava*I restriction sites at REV-A map positions 2.0 to 2.1 are not present. The 4.1-kb fragment is also very similar to the 7.3-kb REV-A *Sac*I fragment at the 3′ terminus, where, as determined by restriction mapping and by heteroduplexing, 1.2 to 1.5 kb is held in common. The origin of the middle region of the 4.1-kb fragment has not been determined with certainty. Heteroduplexes with the REV-A 7.3-kb *Sac*I fragment have shown no pairing in this region, and the restriction map shows a configuration of sites not found in REV-A. Nevertheless, the fragment from *Sma*I to *Bgl*II spanning map positions 1.4 to 2.4 within this region is able to hybridize strongly with REV-A cDNA. Since the sequence of sites *Sma*I, *Ava*I, and *Bgl*II within this region is the exact reverse of the sequence spanning map positions 2.0 to 3.0 in REV-A, we suggest that an inversion event could have given rise to this region of the 4.1-kb fragment. In an attempt to find some difference between the two regions which would rule out this explanation, each of the 14 enzymes which we used to cleave REV-A was also tested on the 4.1-kb fragment; just as none of these enzymes cut within this region in REV-A, none cut the *Sma*I-*Bgl*II region of the 4.1-kb fragment. Although an inversion event is consistent with these data, the failure to find structures with inversion loops in the course of heteroduplexing experiments is puzzling. Thus, definition of this region awaits further work with purified subfragments. We do know that this apparent inversion did not arise during the course of cloning, for it also exists within the 4.1-kb *Sac*I fragment in S3D6 cellular DNA (data not shown).

DISCUSSION

Previous studies by us (13, 38) and others (5, 43) have established that cDNA synthesized from 50 to 70S REV(REV-A) RNA contains both helper-related and helper-unrelated sequences and that the helper-unrelated sequences are able to hybridize with DNAs from uninfected chickens and similar fowl. However, since

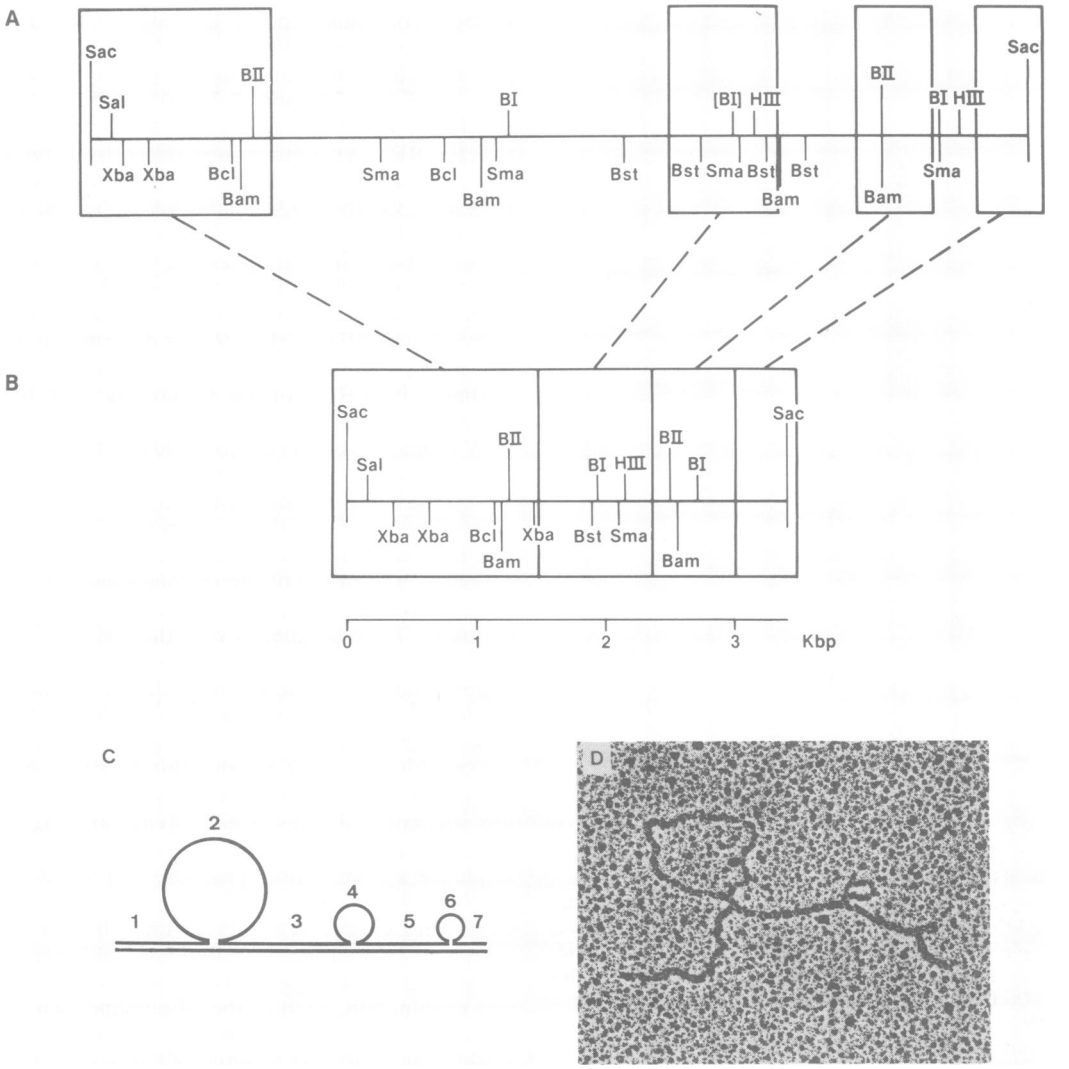

FIG. 8. Structure of an REV-A deletion mutant. A 3.4-kb *Sac*I fragment able to hybridize with REV-A cDNA was cloned out of REV(REV-A)-producing cell line S3D6 into λgtWES·λB. The structure of the cloned fragment was determined by restriction endonuclease mapping according to the general methods described in the text and in the legend to Fig. 1. (A) Restriction sites in the 7.3-kb REV-A *Sac*I fragment for the relevant enzymes. The regions of similarity between the 7.3-kb fragment and the 3.4-kb fragment (B) are enclosed in boxes. The enzyme abbreviations are as described in the legend to Fig. 2. The purified 3.4-kb fragment was also heteroduplexed with the purified 7.3-kb *Sac*I fragment of REV-A. (C) Schematic heteroduplex. (D) Actual heteroduplex. The dimensions of the sections (in kilobases) were as follows: 1, 1.40 ± 0.06; 2, 3.27 ± 0.17; 3, 0.85 ± 0.08; 4, 0.73 ± 0.08; 5, 0.60 ± 0.05; 6, 0.38 ± 0.04; 7, 0.40 ± 0.04. The REV-A fragment was made up of sections 1 through 7 (total 7.6 kb). The deletion mutant was made up of sections 1, 3, 5, and 7 (total, 3.25 kb). The orientation of the molecules was confirmed by heteroduplexing the REV-A 7.3-kb *Sac*I fragment with REV(REV-A) RNA, in which this deleted genome also appears. The RNA molecules had circular simian virus 40 DNA molecules attached to their 3′ ends via polybromodeoxyuridine.

REV(REV-A) RNA from the bone marrow line is a mixture of at least five (and presumably more) different species (8.8, 5.7, and 4.7 kb and 28 and 18S rRNAs) (13), these studies could not unequivocally link the distinctive properties of the cDNA with the transforming (REV) genome. The results described in this paper accomplish this task, for (i) the single viral genome present in nonproducer transformed cells was found to contain both helper-related and helper-unrelated

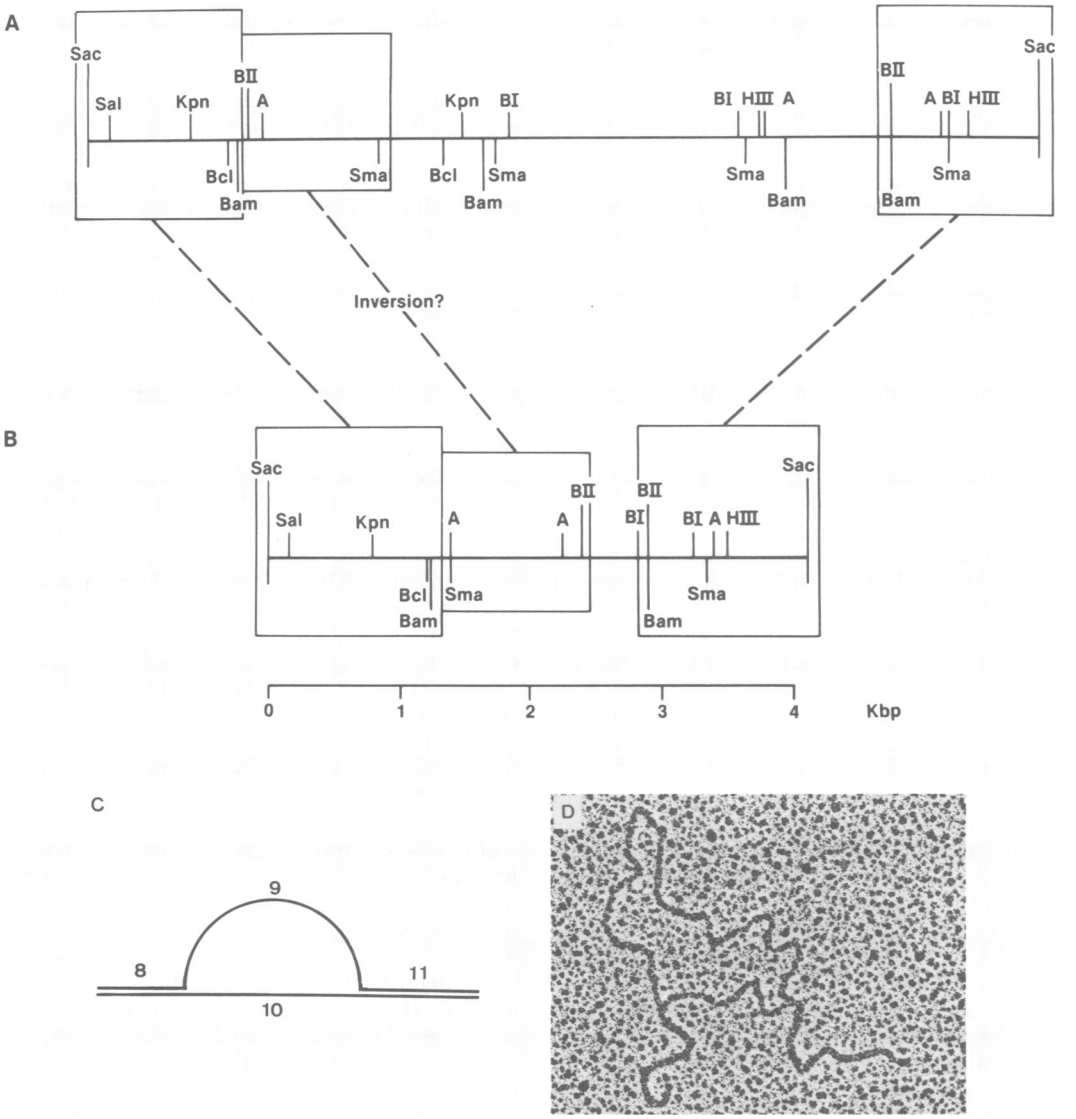

FIG. 9. Structure of a second REV-A-related mutant genome. A 4.1-kb *Sac*I fragment able to hybridize with REV-A cDNA was cloned out of line S3D6-p into λgtWES·λB. The structure of this fragment was compared with that of the REV-A 7.3-kb *Sac*I fragment, as described in the legend to Fig. 8. The dimensions of the sections (in kilobases) were as follows: 8, 1.23 ± 0.05; 9, 4.60 ± 0.20; 10, 1.41 ± 0.05; 11, 1.54 ± 0.07. REV-A was made up of sections 8, 9, and 11 (total, 7.4 kb). The mutant was made up of sections 8, 10, and 11 (total, 4.2 kb). The orientation of the molecules was inferred from the following facts: (i) the 7.3- and 3.4-kb *Sac*I fragments were homologous at their 5′ ends over a distance of 1.4 kb (Fig. 8) and (ii) the 4.1- and 3.4-kb fragments were homologous at their 5′ ends over a distance of 1.24 kb (data not shown). Therefore, the 7.4- and 4.1-kb fragments must be homologous at their 5′ ends over a distance of 1.24 kb (section 8 rather than section 11).

sequences and (ii) a cloned fragment of the transforming genome containing helper-unrelated sequences was able to hybridize with DNAs from uninfected chickens. Each of these points is discussed below.

Like the other known acute leukemia viruses and some sarcoma viruses, REV is replication defective (16); the integrated genome of REV is only about 5.8 kb long and consists of several segments derived from the helper virus (REV-A) and a single new segment not found in the helper. Our mapping and heteroduplexing results established the sizes of these segments and showed that REV corresponds to the type 1

molecules previously described by Hu et al. (19) in a heteroduplexing study of full-length REV-A cDNA with REV(REV-A) RNA. The helper-unrelated segment is presumably responsible for the transforming activity of the complex, for in several viral systems transformation occurs after transfection with subgenomic fragments bearing the helper-unrelated segment (1, 2, 6, 9, 30). In most viruses that have this general structure, the new segment is adjacent to *gag* sequences and is expressed as part of a polyprotein containing *gag* determinants. For example, the MC29 transforming region is preceded (on the 5′ side) by a large section of helper-derived *gag* (18); translation of the resulting mRNA yields a 110,000-dalton fusion protein containing p19, p12, and p27 determinants covalently linked to the transforming protein (33). However, the structure of avian myeloblastosis virus (AMV) is somewhat different. Heteroduplexes have shown that the AMV genome contains all of *gag* and most or all of *pol* and has a new segment inserted within *env* (10, 40). In addition to genome-sized RNA, a 2.3-kb RNA is found in nonproducer cells. Since this RNA contains no *gag*, *pol*, or *env* sequences but does contain AMV-specific sequences, it has been suggested that it could be the mRNA that specifies the AMV transforming protein (7, 14). If this is so, then a fusion protein probably does not exist in the AMV system.

The structure of REV resembles that of AMV rather than that of MC29, for the 1.9-kb presumptive transforming region is adjacent to 1.1 kb derived from the 3′ end of *pol* or the 5′ end of the REV-A *env* gene or both (assuming a gene order in REV-A of 5′-*gag-pol-env*-3′). No *env*-linked transforming protein has been identified yet, despite repeated attempts at precipitation with anti-gp70 sera or antiviral sera (24; H. R. Bose, unpublished data). If a fusion protein does exist, it may contain so small a segment of gp70 that it is immunologically undetectable. On the other hand, the REV transforming protein may not exist as a fusion product at all. In fact, our current sequencing studies of an REV fragment spanning the junction of helper-related and helper-unrelated sequences have shown no open reading frames to the 5′ side of the transforming region (R. Stephens, R. Hiebsch, N. R. Rice, H. R. Bose, Jr., and R. V. Gilden, unpublished data).

REV also contains a large fraction of the presumptive REV-A *gag* gene, for the two genomes are very similar over a distance of 2.1 kb beginning at their 5′ termini. Nevertheless, no *gag* gene products have been found in REV-transformed nonproducer cells (17; H. R. Bose, unpublished data). We have noticed that the *gag* region of the REV genome present in our two nonproducer lines is slightly larger (by about 50 base pairs) than its counterpart in REV-A from the canine line. For instance, the size difference between the fragment from *SalI* to *BclI* in REV and that in REV-A is readily apparent when the two are electrophoresed in neighboring lanes. Therefore, a small insertion might be responsible for either a frame shift or a new termination codon or both. Variation is also possible in the opposite direction, for the REV fragment from *SalI* to *BclI* in the SOC6 producer line is smaller than that of REV-A.

Like the other known acute leukemia viruses and the sarcoma viruses, the transforming region of REV appears to be derived from host DNA. A cloned fragment of REV containing some helper-related sequence and at least several hundred base pairs of helper-unrelated sequence was able to hybridize with a 6.6-kb *SacI* fragment in DNAs from uninfected chickens. Since neither REV-A cDNA nor the cloned 7.3-kb fragment of REV-A is able to hybridize with chicken DNA, this binding must be due to the transforming sequence of REV. We have also observed binding to a 1.7-kb *SacI* fragment in chicken DNA by cDNA enriched for sequences specific for REV, and we presume that this fragment would be detected by other subclones from within the REV transforming region. It is already apparent that hybridizing fragments with sizes predicted from the viral map do not exist within DNAs from uninfected chickens. Therefore, either the cellular gene contains intervening sequences, or it has diverged sufficiently from the viral gene to have a modified restriction map.

As determined by restriction endonuclease analysis, the structure of REV-A is very similar to that previously reported for SNV (21, 28, 29, 36). The nine enzymes which were used to study both of these viruses generated 15 sites in identical or very similar positions in each genome, and only 2 sites which differed. Regarding the latter, REV-A was found to have a *Hin*dIII site at map position 7.5 and a *Kpn*I site at position 1.5, neither of which has been reported for SNV. On the other hand, SNV but not REV-A from the canine cell line has an *Xba*I site at position 2.0 and a *Bam*HI site in the LTR. However, REV from the two nonproducers resembles SNV rather than REV-A in these latter two respects. A high degree of similarity between the SNV and REV-A genomes was expected, based on the known antigenic and nucleic acid relatedness of the two viruses (3, 20, 25); the genomic variations which account for the differing pathogenicities (31, 32) and for the slight differences in neutralizability (31) remain to be elucidated.

In addition to REV-A and REV, we also observed the genomes of two REV-A deletion

mutants in DNAs from two chicken cell clones which produce REV(REV-A). These mutants exhibit striking similarities to each other and to REV. All three genomes contain a substantial portion of the *gag* gene (REV and the 3.4-kb fragment contain very similar *gag* sequences, whereas the *gag* sequences of the 4.1-kb fragment are slightly less extensive), and all lack most or all of *pol* (assuming a gene order of *gag-pol-env*). REV and the 3.4-kb fragment both contain the same segment (about 1 kb) derived from REV-A *pol* or *env* or both, whereas the 3.4- and 4.1-kb fragments share a segment (about 1 kb) derived from the 3′ end of REV-A. The basis for these similarities is not known. One possibility is that one of the mutant forms gave rise to the others via additional recombinations. For example, the 3.4-kb fragment might have arisen as a result of a splicing error (27); REV could then have been generated by recombination of the 3.4-kb genome with host sequences, and the 4.1-kb genome could have been generated by recombination with REV-A sequences. An intriguing alternative possibility is that the three genomes arose independently and that the REV-A genome may have preferred sites of recombination, as suggested for Moloney murine leukemia virus (12). Some combination of these two schemes is also possible.

Whatever the mechanism(s) of origin, the existence of closely related deleted viral forms is not limited to REV-infected cells. Hu et al. (18) have described a deletion mutant in MC29-infected cells which is identical to MC29 for about 1.8 kb at its 3′ end and is very similar to MC29 at its 5′ end. As in the REV-T-producing bone marrow cell line, the transforming genome and the deletion form were transcribed at about equal rates.

Finally, the 4.1-kb fragment contains a 1-kb region which appears to be an inversion of REV-A sequences. Three restriction sites found in this region are present in reverse orientation in REV-A DNA. The region shows no homology with REV-A in heteroduplexes, yet it hybridizes strongly with REV-A cDNA. In the one reported case of a retroviral inversion mutant with which we are familiar, the inverted segment consists of one LTR and adjacent sequences (37). The origin of the mutant was traced convincingly to an abortive integration event, a mechanism which requires inclusion of an LTR in the inverted region. However, there is no LTR involvement in the apparent inversion in the 4.1-kb fragment, and therefore its mechanism of origin remains to be established.

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