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Molecular Cloning, Genomic Analysis, and Biological Properties of Rat Leukemia Virus and the *onc* Sequences of Rasheed Rat Sarcoma Virus

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Rasheed rat sarcoma virus (RaSV) has been shown to code for a protein of 29,000 M_r not present in replication-competent rat type C helper virus (RaLV)-infected cells. This protein is a fused gene product consisting of a portion of the RaLV p15 *gag* protein and the transformation-specific 21,000 M_r (p21) *ras* protein, which is also found in Harvey murine sarcoma virus. We now report the molecular cloning of both the SD-1 (Sprague-Dawley) strain of RaLV and the transforming *ras* sequences of RaSV. Heteroduplex analysis of these cloned DNAs demonstrated that the RaSV *ras* gene (v-Ra-*ras*) was inserted into the rat type C viral genome with a small deletion of RaLV genetic information in the 5′ region of the *gag* gene and that the v-Ra-*ras* gene (0.72 kilobase pair) is homologous to and colinear with the p21 *ras* gene of Harvey murine sarcoma virus (v-Ha-*ras*). Restriction enzyme mapping confirmed the homology demonstrated by heteroduplex mapping, showing strong site conservation of restriction endonucleases known to cleave v-Ha-*ras*. Cloned v-Ra-*ras* DNA transformed NIH 3T3 cells, inducing the synthesis of the p29 RaSV$^{gag\text{-}ras}$ protein.

There are three independent isolates of transforming retroviruses derived from rats. Two of these, the Harvey and Kirsten murine sarcoma viruses (Ha-MuSV and Ki-MuSV, respectively), were isolated after animal passage (14, 18). Both code for an immunologically related protein of 21,000 M_r (p21) which binds both GDP and GTP (29) and whose synthesis correlates strongly with cellular transformation (30, 31). However, heteroduplex analysis of the Harvey and Kirsten p21-transforming genes (*onc*) (3) under stringent conditions indicated only a limited region of homology (9). In addition, rat genomic DNA has been found to contain two genes homologous to the *onc* gene of Ha-MuSV (v-Ha-*ras*); these are designated c-Ha-*ras* 1 and 2 and are distinct from the Ki-MuSV-related p21 gene (v-Ki-*ras*) (5). One of the Ha-MuSV-related p21 cellular genes, rat c-Ha-*ras* 2, appears to be completely colinear with the v-Ha-*ras* gene as determined by heteroduplex analysis and restriction enzyme mapping (5). The second Ha-MuSV-related p21 cellular gene (rat c-Ha-*ras* I) has been shown to have a distinct restriction enzyme map, and by heteroduplex analysis this cellular gene shows three intervening sequences, dividing rat c-Ha-*ras* I into four homologous stretches that can be aligned to the common regions in the restriction enzyme map of v-Ha-*ras* and rat c-Ha-*ras* 2. It thus appears that the second Ha-MuSV p21 cellular gene contains three introns, although further analysis of the mRNA of this gene needs to be performed to make this observation conclusive (2a, 5). The v-Ki-*ras* gene also has homology to normal rat cellular DNA, although the absolute number of different Ki-MuSV-related cellular p21 genes (rat c-Ki-*ras*) has not yet been determined (9).

The third transforming retrovirus containing rat genetic information, Rasheed rat sarcoma virus (RaSV), derived by in vitro cocultivation of transformed rat cells and a rat leukemia virus (RaLV)-producing cell line (23), contains a p21-transforming gene more closely related to the v-Ha-*ras* p21 gene (38). In contrast to Ha-MuSV and Ki-MuSV, which contain sequences apparently generated by a double recombinational event involving Moloney or Kirsten murine leukemia virus, endogenous rat 30S virus-like sequences, and rat cellular p21 genes, RaSV has

been shown to contain only rat type C virus (RaLV) genetic information in addition to the rat p21 *onc* gene (5, 9, 37, 39).

We now report that the p21 gene of RaSV (v-Ra-*ras*) has extensive sequence homology to the v-Ha-*ras* gene as determined by heteroduplex analysis and restriction enzyme mapping. In addition, further analysis of v-Ra-*ras* DNA cloned from transformed murine NIH 3T3 cells indicates that RaLV genetic information lies both proximal and distal to the p21 gene and that the p21 gene appears to have been inserted into the RaLV genome with only a small loss of RaLV genetic information. Furthermore, cloned v-Ra-*ras* DNA is fully capable of transforming NIH 3T3 cells, and the resulting transformants synthesize the RaSV $p29^{gag\text{-}ras}$ gene product.

MATERIALS AND METHODS

Viral stocks and cells. Rat SD-IT and normal rat kidney (NRK) cells have been described previously (8, 24, 38). NIH 3T3 cells were obtained from Donald Blair, Laboratory of Viral Carcinogenesis, National Cancer Institute (NCI). RaSV-transformed NIH 3T3 cells were obtained after infection with RaSV from nonproducer RaSV-transformed Fischer rat embryo cells rescued with Friend murine leukemia virus. The virus stock was obtained courtesy of Edward Scolnick, NCI. NIH 3T3 cells were infected with serial dilutions of the RaSV (Friend murine leukemia virus)/Fischer rat embryo supernatant, with foci appearing 7 to 10 days later. Selected foci were then subcloned in soft agar, and the cloned transformed cultures were assayed for virus production. All subclones were shown to produce Friend murine leukemia virus as determined by XC plaque assay (performed by A. Rein, NCI-Frederick Cancer Research Facility). SD-1 RaLV was propagated in NRK cells after infection of these cells with supernatant from RaLV-producing SD-IT cells as described below.

All cells were grown in Dulbecco modified minimal essential medium containing 5 or 10% fetal calf serum at 37°C in an atmosphere of 8% CO_2. Cells were routinely checked for mycoplasma contamination by Richard Del Giudice, NCI-Frederick Cancer Research Facility, and all cultures were found to be negative.

Restriction endonucleases and cloning vectors. Restriction endonucleases were obtained from either Bethesda Research Laboratories or New England Biolabs and used according to the manufacturer's specifications. T4 DNA ligase and pBR322 were obtained from Bethesda Research Laboratories. *Eco*RI arms of λgtWES · λB were obtained from Ron Hiebsch (NCI-Frederick Cancer Research Facility).

Preparation and cloning of RaLV proviral DNA. The RaLV retroviral genome SD-1 was cloned at the *Sst*I (*Sac*I) site of the vector λgtWES · λB exactly as described for the cloning of the rat 30S genome (37). As with the rat 30S genome, RaLV DNA was obtained from a Hirt supernatant of NRK cells 18 h after infection of these cells with supernatant from RaLV-producing SD-IT cells. After Hirt extraction (15), unintegrated RaLV DNA was further purified by CsCl-ethidium bromide density gradient centrifugation as described elsewhere (13). Radioactive probes used to identify RaLV clones were obtained by reverse transcription of SD-1 RaLV 70S viral RNA, using calf thymus DNA fragments for primer (33).

Preparation of integrated RaSV DNA. DNA obtained from RaSV-transformed NIH 3T3 cells was purified by the standard phenol-chloroform extraction method described elsewhere (12). Approximately 150 μg of DNA was digested with 40 U of *Eco*RI at 37°C for 18 h. This DNA was then phenol-chloroform extracted and layered onto an 11-ml sucrose gradient containing 10 mM Tris (pH 7.4)-1 M NaCl-1 mM EDTA-5 to 20% sucrose. After centrifugation at 16,000 rpm for 20 h at 15°C in a Beckman SW41 rotor, 0.4-ml fractions were collected and a portion of each fraction was analyzed on a 0.7% agarose gel. Those fractions containing DNA of 4 to 9 kilobase pairs (kbp) in length were pooled, 2 volumes of room temperature ethanol was added, and the mixture was placed at −20°C for 16 h. After centrifugation, the precipitated DNA pellet was resuspended in sterile 10 mM Tris (pH 7.4)-1 mM EDTA.

Cloning of RaSV DNA. A 400-ng amount of RaSV/NIH 3T3 cellular DNA was ligated to 800 ng of λgtWES · λB *Eco*RI arms (21), using 2 U of T4 DNA ligase, at 9°C for 18 h. The ligated DNA was then packaged into lambda particles by the method of Hohn and Murray (16). RaSV-positive clones were detected by using a ^{32}P-labeled DNA probe derived by nick translation (27) of a DNA clone, BS-9, obtained from the endogenous Ha-MuSV-related p21 gene specific for Ha-MuSV and not Ki-MuSV (a gift from R. Ellis, NCI [5, 9]).

Subcloning RaLV and RaSV in pBR322. After cloning RaSV into λgtWES · λB, the recombinant DNA was digested with *Eco*RI and the resulting fragment (6.8 kbp) was subcloned into the *Eco*RI site of pBR322. The RaLV insert from λgtWES · λB was cleaved with *Sac*I and ligated into the *Sac*I site of a pBR322 clone containing a 650-bp fragment representing the long terminal repeat (LTR) of Ha-MuSV (a gift from E. H. Chang, NCI [2a]). This pBR322 (Ha-MuSV/LTR) clone contains a single *Sac*I site. The subcloned RaLV genome was further used for restriction enzyme mapping and heteroduplex studies.

Restriction endonuclease analysis of DNAs. Cloned DNAs were digested with restriction endonucleases under conditions suggested by the manufacturers (Bethesda Research Laboratories or New England Biolabs). The amount of DNA per sample varied from 0.1 to 2 μg. Digested DNAs were subjected to electrophoresis through gels of 0.8% agarose (Seakem) and transfer of DNA fragments to nitrocellulose for blotting as described by Southern (32). When nick-translated ^{32}P-labeled DNA was used, filters were prehybridized for 3 h at 42°C 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), herring sperm DNA at 50 μg/ml, and 0.1% sodium dodecyl sulfate. Hybridization was carried out at 42°C for 24 h in the above solution with deionized formamide adjusted to 50% and 10^6 to 10^7 cpm of ^{32}P-labeled DNA per ml. Filters were washed with 1× SSC at 55°C. After washing, filters were dried and exposed to Kodak XAR film at −70°C, using an intensifying screen (DuPont Lightning Plus) when needed.

Transfection. pBR322 recombinants containing RaSV DNA were grown, and the plasmid was extracted. CsCl banded, and concentrated. The recombinant plasmid was then digested with *Eco*RI, and the insert fragment (6.8 kbp) was separated from the pBR322 by electrophoresis into a 0.5% low-temperature melting agarose gel (Sigma Chemical Co.), staining of the agarose gel with 1 μg of ethidium bromide per ml, and cutting out the 6.8-kbp band. The gel containing the 6.8-kbp band was heated to 67°C, extracted with butanol followed by 0.2 M NaCl to separate DNA from ethidium bromide. The DNA solution was further extracted with chloroform and ethanol precipitated according to previously published methods (20). This DNA was then transfected onto NIH 3T3 cells by the $CaCl_2$ precipitation method of Graham and van der Eb (11). Individual foci were picked and placed into soft agar. Rapidly growing colonies were then selected from the agar, put into 30-mm petri dishes, and utilized for immunoprecipitation experiments.

Immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and autoradiography. Immunoprecipitation, gel electrophoresis, and autoradiography of [^{35}S]methionine-labeled, RaSV-transfected NIH 3T3 cell extracts were performed exactly as described in previous publications (38, 39). All labeled cell extracts were precleaned with 100 μl of a 10% suspension of heat-killed *Staphylococcus aureus* to remove nonspecific binding proteins before immunoprecipitation as described by Richert et al. (26).

Heteroduplex analysis. Heteroduplex analysis of cloned DNAs was performed as previously described (37). Briefly, a mixture of linear DNA molecules (each at a concentration of 1.0 μg/ml) was denatured by incubation in 0.1 N NaOH for 10 min at 37°C. The solution was neutralized by the addition of 0.2 volume of 1 M Tris-hydrochloride, pH 7.0. Deionized formamide was added to a final concentration of 50%, and renaturation was permitted by incubation at 22°C for 20 to 30 min. Heteroduplexes were mounted for electron microscopy, using cytochrome *c* (30 μg/ml) as the carrier protein, in spreads from a hyperphase of 60% formamide–0.1 M TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]–0.01 M EDTA (pH 8.5) onto a hypophase of 16% formamide containing 1/10 the electrolyte (4).

RESULTS

Cloning and molecular characterization of RaLV. The RaLV genome was cloned into the vector λgtWES · λB from a Hirt supernatant of RaLV-producing, SD-IT-infected NRK cells. The viral DNA had been previously determined to contain an *Sst*I (*Sac*I) site at each end of the genomic DNA and was approximately 8.2 kbp in length. A restriction enzyme map of this genome has been determined and can be seen in Fig. 1. The DNA was oriented with respect to the 5′ and 3′ ends of viral RNA by utilizing a ^{32}P-labeled oligodeoxythymidylate-primed cDNA made from polyadenylate-containing SD-IT RaLV RNA. This orientation was determined after initial analysis of the genome with a calf thymus DNA fragment-primed, ^{32}P-labeled cDNA (33) synthesized from the SD-IT viral RNA. The nitrocellulose membrane which initially was hybridized with the random SD-IT cDNA probe was heated to 85°C for 15 min, washed extensively with hybridization buffer (32), and rehybridized with the oligodeoxythymidylate-primed, ^{32}P-labeled cDNA. This technique, along with separate hybridization experiments using oligodeoxythymidylate-primed, ^{32}P-labeled cDNA alone (data not shown), confirmed the apparent 5′-to-3′ orientation of the cloned RaLV DNA (Fig. 1).

Cloning of RaSV from cellular DNA. Due to the low virus titers obtained when trying to rescue RaSV from nonproducer cells with various type C helper viruses, it was not possible to obtain enough double-stranded viral DNA intermediates from Hirt supernatants, and attempts were made to clone the integrated RaSV DNA from RaSV-transformed NIH 3T3 cells. It had previously been shown that when a ^{32}P-labeled DNA probe (BS-9) specific for the v-Ha-*ras* is used, an RaSV-specific band approximately 6 to 7 kbp can be detected in DNA extracted from RaSV-transformed NIH 3T3 cells treated with *Eco*RI (9). Although the RaLV genome contains three *Eco*RI cleavage sites (Fig. 1), these sites were 3′ to the p21 gene insert in RaSV. Since the Ha-MuSV p21 gene does not contain an *Eco*RI site (5, 9), attempts were made to clone this 6- to 7-kbp RaSV-specific DNA from the integrated RaSV-transformed NIH 3T3 cellular DNA. *Eco*RI-cleaved, RaSV-transformed NIH 3T3 cellular DNA partially enriched by sucrose gradient centrifugation was ligated into λgtWES · λB *Eco*RI arms. Approximately 2.5×10^5 recombinant plaques were obtained and scanned, using the ^{32}P-labeled BS-9 probe. A number of potentially positive plaques were selected and subcloned, but further analysis revealed only a single recombinant to be highly specific for the ^{32}P-labeled BS-9 v-Ha-*ras* gene probe. One liter of this recombinant phage was grown, the DNA was extracted and digested with *Eco*RI, and the recombinant DNA was found to contain an insert 6.8 kbp in length which showed strong homology to both a ^{32}P-labeled DNA probe synthesized from RaLV-cloned DNA and Ha-MuSV ^{32}P-labeled DNA probes representative of the entire exogenous and endogenous p21 genes (rat c-Ha-*ras* 1 and 2), as well as the BS-9 probe.

Fine structural comparison of RaLV and RaSV. To compare RaLV- and RaSV-cloned genomes, both restriction endonuclease and heteroduplex mapping were used. Because of the strategy used to clone the *ras* sequences, a genome lacking 3′ RaLV sequences was obtained. The segment of integrated RaSV obtained will be referred to as v-Ra-*ras*. This

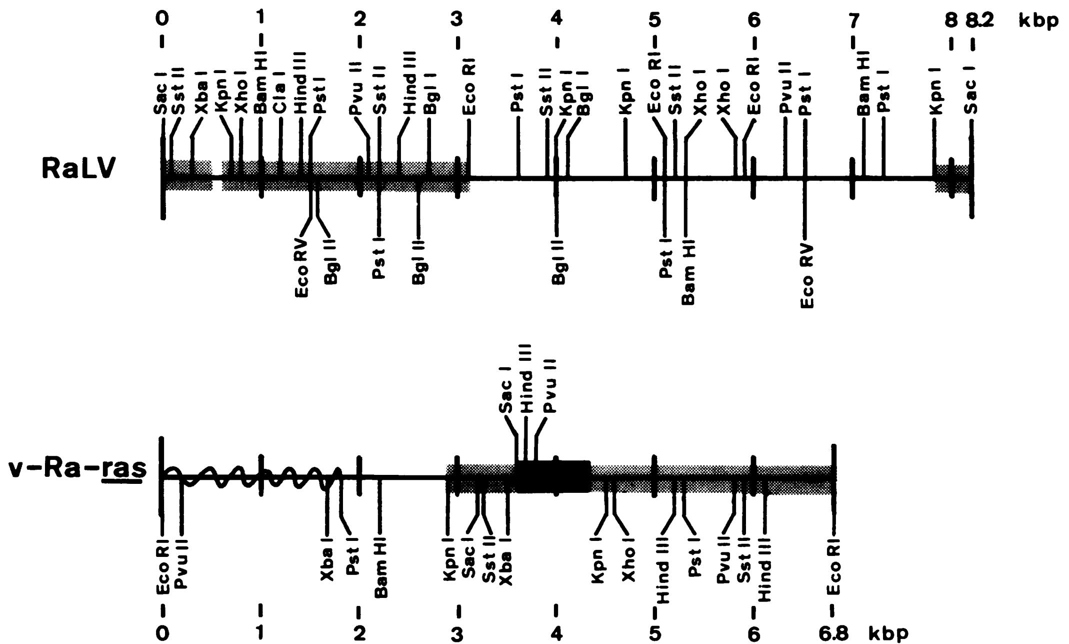

FIG. 1. Structural relationship of cloned RaLV and v-Ra-*ras* sequences obtained from restriction endonuclease maps. RaLV was cloned in the LTR sequences with *Sst*I (*Sac*I) from unintegrated closed circular double-stranded viral DNA. The v-Ra-*ras* clone was obtained from an *Eco*RI fragment of integrated RaSV sequences from NIH 3T3 cells nonproductively infected with RaSV. Restriction site placements are accurate to ±50 bp. As deduced from both Southern blot analyses and heteroduplex mapping, the homologous RaLV sequences represented in the v-Ra-*ras* clone are noted by the shaded bar. The *onc* sequences of the v-Ra-*ras* clone homologous to v-Ha-*ras* not represented in the RaLV clone are highlighted by the solid bar on the v-Ra-*ras* clone. The region of flanking mouse cellular DNA is indicated by the wavy line in the v-Ra-*ras* *Eco*RI clone. Restriction endonucleases *Sac*I, *Sst*II, *Pvu*II, *Hin*dIII, *Xho*I, *Xba*I, *Pst*I, *Kpn*I, *Eco*RI, and *Bam*HI were used to map both RaLV and v-Ra-*ras*. Additional restriction endonuclease sites for *Bgl*I, *Bgl*II, and *Eco*RV were mapped for RaLV.

terminology is justified since this clone is biologically active, as described later. A restriction endonuclease map of the 6.8-kbp cloned v-Ra-*ras* DNA was developed and compared with RaLV (Fig. 1). Two portions of v-Ra-*ras* DNA have no homology with RaLV DNA. One region is upstream of the 5′ terminus of RaLV, whereas the other resides within the RaLV genome towards the 5′ end of RaLV-specific sequences.

Heteroduplex mapping of cloned RaLV and v-Ra-*ras* DNA generated a circular molecular with a single 0.7-kilobase (kb) substitution and a 2.6-kb single-stranded DNA tail (Fig. 2), confirming the presence of two non-RaLV sequences noted in the restriction enzyme map generated for v-Ra-*ras* (Fig. 1). The presence of a circular DNA molecule after heteroduplex analysis can be explained by the fact that *Sac*I cleaves within the LTR of RaLV, and thus the ends of RaLV would contain a portion of the viral LTR at the 5′ and 3′ termini which come together at the single intact LTR of the cloned RaSV genome. Two homologous regions of DNA are observed 5′ and 3′ from the ends of the 0.7-kb substitution in the heteroduplex molecule (Fig. 2C, regions b and d). These regions are representative of the RaLV sequences present in v-Ra-*ras* and account for 3.8 kbp of the clone. A small portion of RaLV information has been deleted at the 0.7-kb substitution in v-Ra-*ras* (Fig. 2C, region e) as noted by the presence of a 0.13-kb region of single-stranded DNA. From the heteroduplex data it is also apparent that greater than half of the RaLV genome at the 3′ terminus is not represented in the v-Ra-*ras* clone at all, as marked by the presence of a single-stranded DNA region of 4.23 kb (Fig. 2C, region f). These data further substantiate the absence of RaLV information to the 3′ terminus of RaSV as shown in the restriction endonuclease map presented in Fig. 1.

The 2.6-kb single-stranded DNA tail (Fig. 2C, region a) probably represents mouse DNA flanking sequences to the outside of the v-Ra-*ras* 5′ LTR. This was deduced from Southern blot analysis of digested v-Ra-*ras*, using an entire

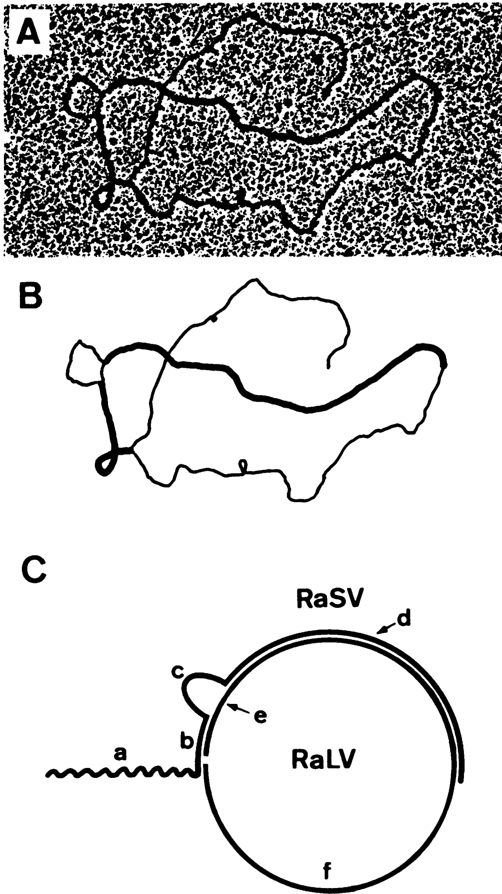

FIG. 2. Heteroduplex analysis of the genomic relationship of RaLV- and v-Ra-*ras*-cloned DNA molecules. Heteroduplexes were prepared from genomic inserts freed from their cloning vectors by restriction endonuclease digestion and preparative gel electrophoresis. (A) Actual heteroduplex; (B) interpretive drawing; (C) schematic representation. The dimensions given in (C) are based on measurements of over 25 molecules. Contour lengths, measured from actual photographs with a digital length calculator, were as follows (in kilobases): a = 2.49 ± 0.09; b = 1.19 ± 0.05; c = 0.72 ± 0.08; d = 2.56 ± 0.09; e = 0.13 ± 0.04; f = 4.23 ± 0.33. Measurements for cloned RaLV = b, d, e, and f, and those for v-Ra-*ras* = a, b, c, and d. The wavy line (a) represents mouse cellular flanking DNA in the v-Ra-*ras* clone.

^{32}P-labeled RaLV nick-translated DNA probe and a ^{32}P-labeled DNA probe representative of the entire v-Ha-*ras* gene.

Identification of v-Ra-*ras*-specific sequences. The 0.7-kb nonhomologous (to RaLV) region within the middle of the v-Ra-*ras* clone (Fig. 1) shows conservation of *Sac*I, *Hin*dIII, and *Pvu*II restriction sites when compared with v-Ha-*ras*, the endogenous colinear rat c-Ha-*ras* 2, and the intervening sequences containing c-Ha-*ras* I (2a, 5). This observation strongly suggested that the p21-coding portion of v-Ra-*ras* was identical to the p21 *ras* gene represented in Ha-MuSV.

To further test this hypothesis, heteroduplex analyses were performed between v-Ra-*ras* and cloned DNAs representing v-Ha-*ras* and rat c-Ha-*ras* I. Figure 3 shows a heteroduplex molecule between v-Ra-*ras* and v-Ha-*ras*. A colinear region of homology of 0.7 kbp exists at approximately the middle of the v-Ra-*ras* genome in the region of the internal substitution. Figure 4 shows a heteroduplex between v-Ra-*ras* and rat c-Ha-*ras* I. The duplexed molecule shows a homologous stretch of DNA again approximately in the middle of the v-Ra-*ras* clone of 0.7 kbp. However, the homologous stretch is segmented by three intervening sequences of the same size and dimensions as those reported for heterodu-

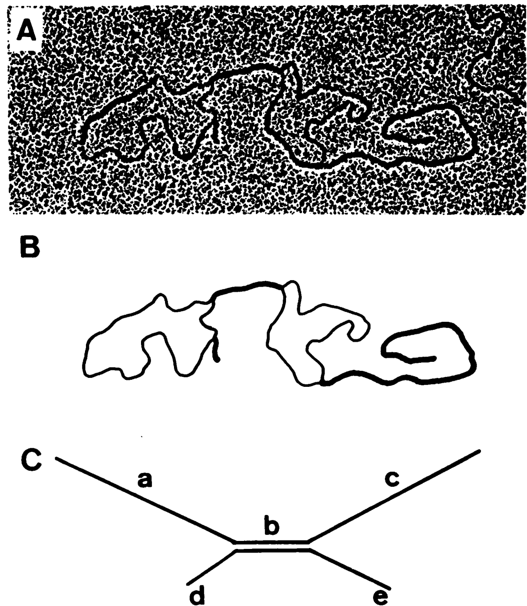

FIG. 3. Heteroduplex analysis of the genomic relationship of the v-Ra-*ras* clone and clone HB-11 (a *Bam*HI to *Eco*RI, 2.3-kb clone of Ha-MuSV). The HB-11 clone is representative of the entire v-Ha-*ras* sequences of Ha-MuSV. Heteroduplexes were prepared with cloned inserts in their cloning vectors (pBR322) cut with *Sal*I. (A) Actual heteroduplex; (B) interpretive drawing; (C) schematic representation of the inserts free of their vectors. The dimensions given in (C) are based on measurements of over 25 molecules. Contour lengths were as follows (in kilobases): a = 3.90 ± 0.27; b = 0.68 ± 0.05; c = 2.74 ± 0.15; d = 0.65 ± 0.02; e = 1.01 ± 0.08. The v-Ra-*ras* clone is represented by a, b, and c. The HB-11 clone is represented by d, b, and e. Segment b represents the homologous *onc* sequences of v-Ra-*ras* and v-Ha-*ras*.

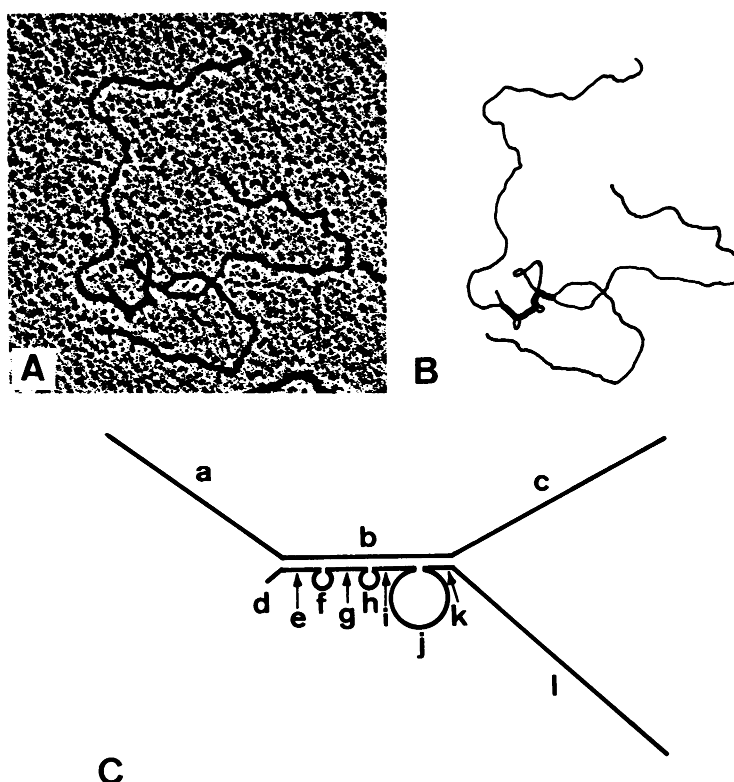

FIG. 4. Heteroduplex analysis of the genomic relationship of v-Ra-*ras*- and rat c-Ha-*ras* I-cloned DNA molecules. Heteroduplexes were prepared from genomic inserts freed from their cloning vectors by restriction endonuclease digestion and preparative gel electrophoresis. (A) Actual heteroduplex; (B) interpretive drawing; (C) schematic representation. The dimensions given in (C), based on measurements from actual photographs with a digital length calculator, were as follows (in kilobases): a = 3.72 ± 0.21; b = 0.73 ± 0.06; c = 2.52 ± 0.15; d = 0.06 ± 0.01; e = 0.19 ± 0.03; f = 0.22 ± 0.02; g = 0.22 ± 0.02; h = 0.19 ± 0.02; i = 0.17 ± 0.03; j = 0.71 ± 0.06; k = 0.16 ± 0.01; l = 3.18 ± 0.16. Measurements for v-Ra-*ras* = a to c; those for rat c-Ha-*ras* I = d to l.

plexes between v-Ha-*ras* and rat c-Ha-*ras* I (5). Thus, both restriction enzyme mapping and heteroduplex analyses indicate that the RaSV *ras* gene is essentially identical to v-Ha-*ras* with qualifications as described below.

First, the closely aligned (50 to 100 bases) *Sac*II, *Bgl*I, and *Sma*I sites located (5′ to 3′) at the 5′ terminus of v-Ha-*ras* are not present in the v-Ra-*ras* gene. Second, in heteroduplexes the homologous region observed between v-Ra-*ras* and both v-Ha-*ras* and rat c-Ha-*ras* I is approximately 200 bases shorter than similar regions seen when the latter two are heteroduplexed. Analysis of the heteroduplexes between v-Ra-*ras* and rat c-Ha-*ras* I show that most of the "deletion" is from the 3′ terminus of the v-Ra-*ras* gene, i.e., localized to the 3′-terminal exon. Analysis of restriction enzyme digests (Fig. 5), using RaLV- or v-Ha-*ras*-cleaving enzymes *Sac*II, *Bgl*I, *Sma*I, *Sac*I, *Hin*dIII, and *Pvu*II (these sites are oriented 5′ to 3′ in the v-Ha-*ras* map) to map the v-Ra-*ras*, showed that only *Hin*dIII and *Pvu*II cleave the *ras* gene of v-Ra-*ras* when hybridized with a ^{32}P-labeled HB-11 clone of Ha-MuSV (*Bam*HI to *Eco*RI, 2.3, kbp, which contains all of v-Ha-*ras* flanked by rat 30S sequences [5, 9]). In light of its position relative to *Hin*dIII and *Pvu*II, it is possible that the *Sac*I site is the v-Ha-*ras* homolog and that the apparent inability of *Sac*I to cleave v-Ra-*ras* is due to the presence of too little *ras* DNA to the 5′ side of *Sac*I to give an efficient hybridization signal. Thus, the p21 *ras* gene represented in the v-Ra-*ras* clone starts at or after the *Sac*I site and ends 0.7 kbp downstream.

Biological activity of the cloned v-Ra-*ras* genome. v-Ra-*ras* DNA was purified from pBR322 after *Eco*RI digestion, and the purified DNA was then transfected onto NIH 3T3 cells by the calcium phosphate precipitation method (11). Approximately 100 transformed foci were observed per microgram of transfected DNA.

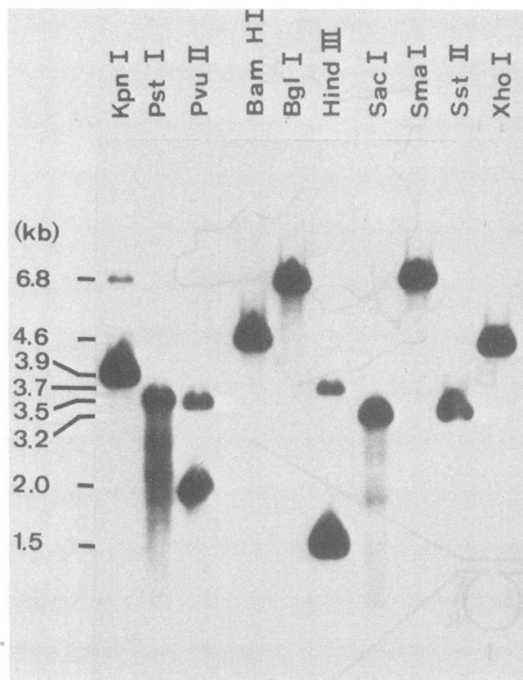

FIG. 5. Southern blot analyses of the v-Ra-*ras* clone, using restriction enzymes known to cut RaLV and v-Ha-*ras*. The ^{32}P-labeled HB-11 DNA clone representative of the entire v-Ha-*ras* sequences was used to hybridize to homologous v-Ra-*ras* sequences. From these data only *Hin*dIII and *Pvu*II cut the *onc* sequences in v-Ra-*ras*. The *Kpn*I and *Sst*II lanes are partial digests. The *Kpn*I 3.9-kbp band is further cleaved into a 1.6-kbp band which contains the *ras* gene and a 2.3-kbp band which contains RaLV sequences. For *Sst*II, the 3.5-kbp band is further cleaved into a 2.6-kbp band which contains the *ras* gene and a 0.9-kbp band which contains RaLV sequences (see Fig. 1).

Three of these colonies, designated cl A, E, and F, were selected and purified by growth in soft agar.

Immunoprecipitation of v-Ra-*ras* p29. Rapidly growing agar colonies were plated into 30-mm petri dishes pulse-labeled with [^{35}S]methionine, and the labeled proteins were immunoprecipitated with p21 antiserum as described previously (38, 39). The transformed clones contained p29 (Fig. 6), indicating that the v-Ra-*ras* DNA clone contained the entire RaSV-transforming gene as well as the necessary promotors for transcription. The p29 band in the v-Ra-*ras*-transformed cells is a doublet representing the phosphorylated and nonphosphorylated forms of the protein (39). In addition to the p29 band, a p21 band was also precipitated by this antiserum. It was found in the v-Ra-*ras*-infected cells (Fig. 6, lanes 5, 6, and 7) and in uninfected control cells (Fig. 6, lane 8). The p21 probably represents the cellular homolog of v-Ha-*ras* p21 present in many species of vertebrate cells, although at much lower levels than cells transformed by Ha-MuSV (19). The p29 was also immunoprecipitated by RaLV p15 antiserum (data not shown), indicating that the v-Ra-*ras* clone does indeed contain the expected p29$^{gag\text{-}ras}$ fused gene product (39).

Cloned v-Ra-*ras* contains redundant sequences. During the heteroduplex mapping experiments between v-Ra-*ras* and RaLV genomic inserts, many single-stranded DNA molecules were observed which contained regions of intramolecular hybridization forming a single-stranded DNA circle with a double-stranded DNA tail and a single-stranded DNA segment at its terminus (Fig. 7). We sought to determine whether these sequences responsible for intramolecular hybridization were present in the RaLV or v-Ra-*ras* clone; therefore, we denatured, annealed, and spread RaLV and v-Ra-*ras* independent of one another. In these experiments, only the v-Ra-*ras* clone demonstrated the intramolecular hybridization as described above. From measurements of these molecules the single-stranded region of the tail appeared to be the cellular flanking sequences not represented in the RaLV clone. The single-stranded DNA circle gave measurements corresponding to the RaSV genomic information. The inverted sequences re-

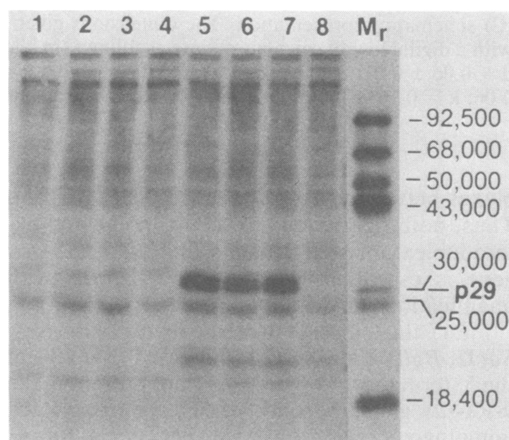

FIG. 6. Immunoprecipitation of RaSV p29 from clones of NIH 3T3 cells transfected with the v-Ra-*ras* clone free of its vector. Immunoprecipitation of [^{35}S]methionine-labeled cellular extracts with rat antiserum with reactivity to Ha-MuSV p21 were carried out as previously described (39). Lanes 1 to 4 were precipitated with normal rat serum. Lanes 5 to 8 were precipitated with rat antiserum to Ha-MuSV p21. (Lanes 1 and 5) Clone A; (lanes 2 and 6) clone E; (lanes 3 and 7) clone F; (lanes 4 and 8) normal uninfected NIH 3T3 cells.

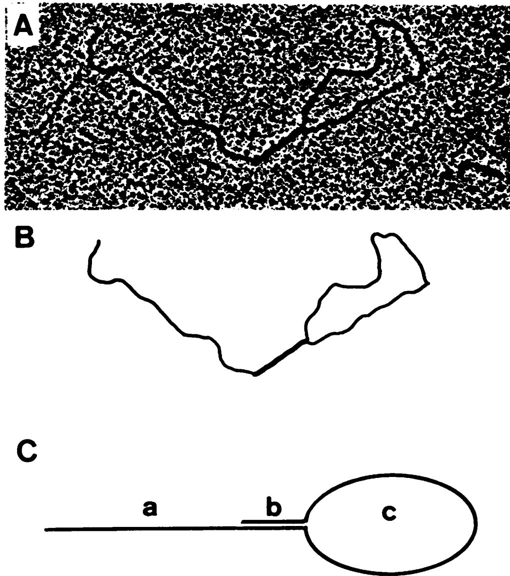

FIG. 7. Electron micrograph of intramolecular base pairing of the v-Ra-*ras* insert. (A) Actual electron micrograph; (B) interpretive drawing; (C) schematic representation. The dimensions given in (C) are based on measurements of over 40 molecules. Contour lengths, measured from actual photographs, were as follows (in kilobases): a = 1.99 ± 0.09; b = 0.50 ± 0.07; c = 3.82 ± 0.09.

sponsible for intramolecular hybridization (0.5 kbp) are approximately the size of the LTR. Restriction enzyme mapping of this region of the v-Ra-*ras* clone did not yield any insight into this homology. The reason for this anomaly remains to be determined.

DISCUSSION

The transforming mammalian retroviruses owe their oncogenic potential to genetic information derived from a cellular sequence (c-*onc*) inserted into a type C viral genome (3, 5, 7, 9, 10, 22). For the c-*onc* sequences of rat origin the term *ras* is used (3). In many cases, the insertion of this cellular gene occurs near the 5′ end of the viral genome, leading to the formation of a *gag-onc* gene fusion product which appears to be essential for transformation (17, 25, 28, 36). An insertion of this type might be advantageous for gene expression because the necessary transcriptional control sequences would be supplied by the LTR of the viral genome (6).

The cellular p21 gene of Ha-MuSV and Ki-MuSV was also inserted near the LTR at the 5′ end of the viral genome (5, 9, 35, 37). In the case of Ha-MuSV and Ki-MuSV, some impact on transcription might be attributable to the virus-like 30S genome since it is actively transcribed in many cells and tumors (34, 40) and 30S sequences are present proximal to the p21 gene (5, 9, 35, 37). The endogenous rat p21 genes (c-*ras*) are capable of elevated levels of transformation when they are linked to specific viral sequences present in the LTR (2, 5). The structure of the RaSV genome is consistent with this model. RaSV appears to have arisen through the insertion of a cellular p21 gene into the *gag* gene region of the RaLV genome with concomitant loss of only a small amount of RaLV *gag* gene information. Those mammalian sarcoma viruses which code for a *gag-onc* fusion protein appear to have lost the *gag* portion of the genome distal to the insertion site, and thus substitution of a cellular gene for viral genes, rather than simple insertion, is the general rule (1, 7, 10, 25, 28, 36). Such a substitution is also evident from the heteroduplex analysis of Ha-MuSV and 30S DNA (37); in contrast, Ki-MuSV shows little or no loss of the corresponding 30S sequences (9).

The p21 *ras* genes of RaSV and Ha-MuSV are essentially identical except that the v-Ra-*ras* insert is approximately 200 bases shorter than v-Ha-*ras*. Sequences have been lost from the 5′ and 3′ ends of v-Ra-*ras*, as noted by the loss of v-Ha-*ras* restriction endonuclease sites and measurements from heteroduplexes with Ha-MuSV viral and cellular *ras* genes. Indeed, sequence analysis of v-Ha-*ras* suggests that the p21 coding region of *ras* starts to the 3′ side of the *Sac*I site and only *Hin*dIII and *Pvu*II sites are actually represented in the p21 coding region (E. M. Scolnick, personal communication).

We observed, during heteroduplex analysis, that a region of RaSV hybridized to itself, suggesting a redundancy of a portion of the DNA in this clone. This redundant DNA did not arise during cloning since the restriction endonuclease maps of both cloned and integrated RaSV DNA were identical (data not shown). The location of this redundancy (approximately 2.5 kbp from the 5′ end of the 6.8-kbp DNA) suggests that it may occur near the terminal redundancy (LTR). One hypothesis is that integration of RaSV into NIH 3T3 cells occurred within a murine viral or 30S virus-like genome which might share some homology with RaLV. At present, the exact nature of this redundant DNA sequence remains uncertain, and the hypothesis as to its integration site is under study.

Despite the fact that an intact, functional RaSV-transforming gene has now been isolated, the structure of the entire RaSV genome has not yet been determined. Based on the restriction map of RaLV (Fig. 1), a large portion of the 3′ end of the RaLV genome would not be present in the DNA clone due to the choice of *Eco*RI as a cloning site. This fact is consistent with the observation that attempts to rescue RaSV from

NIH 3T3 cells transfected with v-Ra-*ras* have been unsuccessful (A. Rein, unpublished data). Detailed analysis of RaSV RNA will be required to answer this question.

It is evident that RaSV arose through recombination of an endogenous p21 gene and the RaLV genome. It is not clear, however, why the virus was detected only after the cocultivation of two transformed rat cells and an RaLV-producing cell and whether the high levels of 30S RNA present in both cells are relevant to this process. In the process of these studies it was observed that the cloned 30S and RaLV DNAs have homology at the ends of the respective DNAs, and, in fact, both were cloned in a 5′-to-3′ orientation and contain an *Sst*I (*Sac*I) site at each end of the DNA. The 30S genome may have acted as an intermediate in the generation of RaSV; thus, a two-step mechanism of recombination can be hypothesized. In the first round, recombination may have taken place between the *ras* gene and the 30S genome (as in Ha-MuSV and Ki-MuSV), and then a second round between the 30S *ras* gene and RaLV may have occurred. Cocultivation of the two cells may be required because infection of one of the rat cells with RaLV would result in DNA intermediates which could then undergo the proposed recombination. This hypothesis is testable since recreation of RaSV can be achieved reproducibly, although at low frequency (38).

Another hypothesis is that one of the two rat cells contains a masked RaSV genome and that cocultivation somehow activates that genome. Whereas this cannot be totally ruled out, we have been unable to activate RaSV from either of the two cells by 5-azacytidine, 5-bromodeoxyuridine, anaerobic stress, or superinfection of both cells with heterologous virus (H. Young, unpublished data). In addition, analysis of the DNA extracted from the two cells used for cocultivation indicates that only two p21 genes appear to be present after *Eco*RI digestion (data not shown). Thus, the overall cell population does not contain an RaSV-specific genome and resembles normal rat DNA as previously reported (40).

Finally, to assure that the portion of RaSV which was subcloned into pBR322 contained the total transforming region of the virus, the purified DNA was transfected into NIH 3T3 cells, observed to cause transformation (focus formation) of the NIH 3T3 cells, and these transformed cells were found to contain RaSV-specific p29. Thus, this DNA clone contains all of the necessary sequences for promotion and transcription of the full v-Ra-*ras* p29 gene. The relationship of the sequences responsible for promotion and initiation of p29 gene transcription to other known retroviral promoters will await further sequence analysis of the cloned RaSV DNA.

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