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Avian Acute Leukemia Virus OK 10: Analysis of Its *myc* Oncogene by Molecular Cloning

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Several DNAs representing the genome of the avian acute leukemia virus OK 10 were isolated by molecular cloning from a transformed quail cell line, 9C, which contained at least six OK 10 proviruses. Recombinant lambda phages harboring the OK 10 genome and additional flanking cellular DNA sequences were studied by restriction endonuclease mapping and hybridization to viral cDNA probes. Six of the clones represented complete proviruses with similar, if not identical, viral sequences integrated at different positions in the host DNA. The organization of the OK 10 genome was determined by electron-microscopic analysis of heteroduplexes formed between the cloned OK 10 DNA and DNAs representing the c-*myc* gene and the genomes of two other avian retroviruses, Rous-associated virus-1 and MC29. The results indicated that the OK 10 proviral DNA is about 7.5 kilobases in size with the following structure: 5′-LTR-*gag*-Δ*pol*-*myc*-Δ*env*-LTR-3′, where LTR indicates a long terminal repeat. The oncogene of OK 10, v-*myc*$^{OK\ 10}$, forms a continuous DNA segment of around 1.7 kilobases between *pol* and *env*. It is similar in structure and length to the v-*myc* gene of MC29, as demonstrated by restriction endonuclease and heteroduplex analyses. Two of the OK 10 proviruses were tested in transfection experiments; both DNAs gave rise to virus with the transforming capacities of OK 10 when Rous-associated virus-1 was used to provide helper virus functions.

OK 10 is a defective avian leukemia virus of the MC29 group and induces a wide spectrum of tumors in chickens, such as carcinomas and solid tumors consisting of myeloid hematopoetic cells (10, 16, 21). In cell culture, the virus transforms both hematopoietic cells and fibroblasts (2, 12, 21).

The oncogenic capacity of OK 10 is thought to be encoded by v-*myc*$^{OK\ 10}$, which is homologous to the oncogenes of the other members of the MC29 group, i.e., the related but distinct retroviruses MC29, CMII, and MH2 (19, 28, 35). Like all other defective leukemia viruses, OK 10 is thought to have arisen by recombination between an avian leukosis virus and the cellular homolog of the oncogene, c-*myc*, present and conserved in several vertebrate species (28, 31).

The v-*myc* gene of OK 10 comprises 1,500 to 2,000 nucleotides and is inserted between the *pol* and *env* regions in the genome. The *gag* gene is intact, and the *pol* gene is defective in its 3′ end, whereas the *env* gene has suffered considerable deletions in the 5′ region (4, 23; S. Saule, J. Coll, M. Righi, C. Lagrou, M. B. Raes, and D. Stehelin, EMBD J., in press). Because of the deletions in structural genes, OK 10, like all other acute leukemia viruses, is defective in replication and requires a helper virus for the production of infective particles (3, 11, 13).

v-*myc* of OK 10 potentially can be translated into two different proteins: (i) a 200,000-molecular-weight hybrid protein with *gag*, *pol*, and *myc* domains, by using a genome-sized mRNA as template (23–25); and (ii) a still unidentified polypeptide, via a 3.6-kilobase (kb) subgenomic, *myc*-specific mRNA (5; Saule et al., in press). The exact roles in cell transformation of the 200,000-dalton protein and the putative protein encoded by the subgenomic mRNA have not been determined. A similar dual mode of v-*myc* expression has been found in certain MH2-infected cells (Saule et al., in press). We have isolated several proviruses of OK 10 by molecular cloning to further dissect the structure and functions of v-*myc*$^{OK\ 10}$. In this communication we present evidence that the clones encode OK 10 genomes with the transforming capacities of the parental virus. In addition, we show by

heteroduplex analysis and restriction mapping that v-*myc*$^{OK\ 10}$ is similar in structure to the v-*myc* gene of MC29.

MATERIALS AND METHODS

Cells and virus. The OK 10-transformed quail nonproducer cell line OK 10 QDP 9C, (here denoted as 9C) has been described in detail elsewhere (24). Chicken embryo fibroblasts were grown as described previously (22, 34). Cultures of chicken embryo yolk sac macrophages were prepared according to the method of Moscovici et al. (20).

Molecular cloning. High-molecular-weight DNA from the 9C cell line was prepared essentially as previously described (17). Purified DNA was partially cleaved with increasing amounts of the restriction endonuclease *Mbo*I; the partial cleavage products were pooled, and fragments 15 to 20 kb in size were isolated by centrifugation in sucrose gradients (10 to 40% [wt/wt] sucrose in 10 mM Tris-hydrochloride [pH 7.4], 20 mM EDTA, and 0.5 M NaCl, at 30,000 rpm for 20 h at 15°C). The fragments were ligated to sucrose gradient-purified *Bam*HI arms of the lambda phage Charon 28. The vector was obtained from F. R. Blattner. Ligation was performed with a vector arm/insert ratio of 2:1 and a final DNA concentration of 250 μg/ml at 4°C for 16 h with T4 DNA ligase (New England Biolabs) in 20 mM Tris-hydrochloride (pH 7.4)–10 mM dithiothreitol–6 mM $MgCl_2$–1 mM ATP. The chimeric DNA was packaged in vitro into the capsids of lambda phages according to the protocol of Hohn (15). The phages were plated on *Escherichia coli* DP50 *supF* at a density of 3×10^4 per 15-cm petri dish. About 250,000 plaques per μg of ligated DNA were obtained. Plaques formed by the recombinant phages were detected as described by Benton and Davies (1), with a ^{32}P-labeled probe derived from the *myc* oncogene of MC29. Nitrocellulose filters with phage DNA were hybridized overnight at 41°C with 5×10^5 cpm of probe per filter in a buffer containing 50% (vol/vol) formamide and 10% dextran sulfate as described by Shank et al. (30). Positive plaques were picked and plaque purified until pure recombinant phage was obtained. Phage DNA was isolated by digestion of virus particles with proteinase K in the presence of sodium dodecyl sulfate, followed by repeated extractions with phenol-chloroform as described by Dodgson et al. (8).

Subcloning into plasmid vectors was done with pBR322 as the cloning vehicle and *E. coli* HB101 as the host bacterium.

Hybridization reagents. Subgenomic fragments of avian sarcoma virus (ASV) and MC29 DNA were obtained by cleaving clones of ASV (7), MC29 (35), or subclones thereof, followed by electrophoretic purification of the fragments in agarose or polyacrylamide gels. DNA fragments to be labeled in vitro were denatured by boiling and subsequently used as templates for avian myeloblastosis virus reverse transcriptase, with oligomers of calf thymus DNA as primers (17, 30). Alternatively, ^{32}P-labeled probes were prepared by nick translation (26).

cDNA *gag* was prepared with a 1.35-kb *Bam*HI fragment from cloned ASV DNA (ASV-SRA-2). This fragment maps between 0.5 and 1.8 kb from the 5′ end of the 9.4-kb ASV genome and represents the 5′-terminal part of the *gag* gene (7).

cDNA *pol* was synthesized by using as template a 900-base-pair *Xba*I-*Bgl*II fragment from cloned ASV DNA. This fragment comprises the middle region of the *pol* gene and is located between 3.2 and 4.1 kb from the 5′ end of the ASV genome (9).

cDNA *env* 3′ was prepared from a cloned 1.6-kb *Pvu*II fragment of ASV DNA. This *env* probe spans the junction between *env* and *src* and is positioned between 6.2 and 7.8 kb from the 5′ end of the ASV genome (7, 9).

cDNA long terminal repeat (LTR) was synthesized by using as template a 330-base-pair *Eco*RI fragment from an ASV/*Pvu*II D-G subclone (7) which contains two LTRs in tandem. The probe thus contains sequences from both the U3 and the U5 regions.

cDNA *myc* was prepared with a cloned 1.5-kb *Pst*I fragment of MC29 DNA that maps approximately between 2.3 and 3.8 kb from the 5′ end of the MC29 genome. The fragment contains all but around 200 bases from the 5′ end of the *myc* gene and has in addition a small number of *env* sequences (32).

Restriction endonuclease mapping. DNA from recombinant phage was digested with restriction endonucleases in the buffers recommended by the supplier (New England Biolabs). Digested DNA was analyzed by agarose gel electrophoresis as described previously (14). Lambda phage DNA cleaved with *Hin*dIII was used as a molecular weight marker.

DNA fragments were transferred to nitrocellulose filters according to the method of Southern (33) and hybridized for 3 to 5 h at 65°C with 10^5 cpm of ^{32}P-labeled cDNA probes per ml in a buffer containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M Na citrate), 500 μg of denatured calf thymus DNA per ml, 0.5% sodium dodecyl sulfate, 5 mM EDTA, and 1× Denhardt solution (0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll). The filters were washed and subjected to autoradiography (30).

Heteroduplex analysis and electron microscopy. Heteroduplexes between intact recombinant phage DNAs and cloned Rous-associated virus-1 (RAV-1) DNA (a gift from M. Bishop), cloned MC29 DNA (pMCV38) (35), or cloned c-*myc* (36) were prepared as described by Davies et al. (6). Heteroduplexes were spread from 45% formamide under isodenaturing conditions and mounted for electron microscopy. ϕX174 single- and double-stranded circular DNAs were used as size standards.

Transfection of chicken cells with cloned OK 10 DNA. Chicken embryo fibroblasts were prepared from SPAFAS embryos obtained from Lohmann, GmbH, Cuxhaven, Federal Republic of Germany. Transfections were carried out with 1 μg of λ OK 401 or λ OK 601 DNA and 200 ng of RAV-1 DNA (which was excised from plasmid vector DNA and ligated to form tandem arrays of complete genomes) as described previously (8a). Transfected cells were passaged several times, and the medium was harvested and used for focus assays when the reverse transcriptase activity in the medium parallelled that of fully transformed control cultures. Foci from λ OK 401 transfected cultures were isolated and used as the source of virus in macrophage transformation assays (12, 20).

Analysis of viral proteins in transformed cells. Fibroblasts fully transformed by virus obtained after trans-

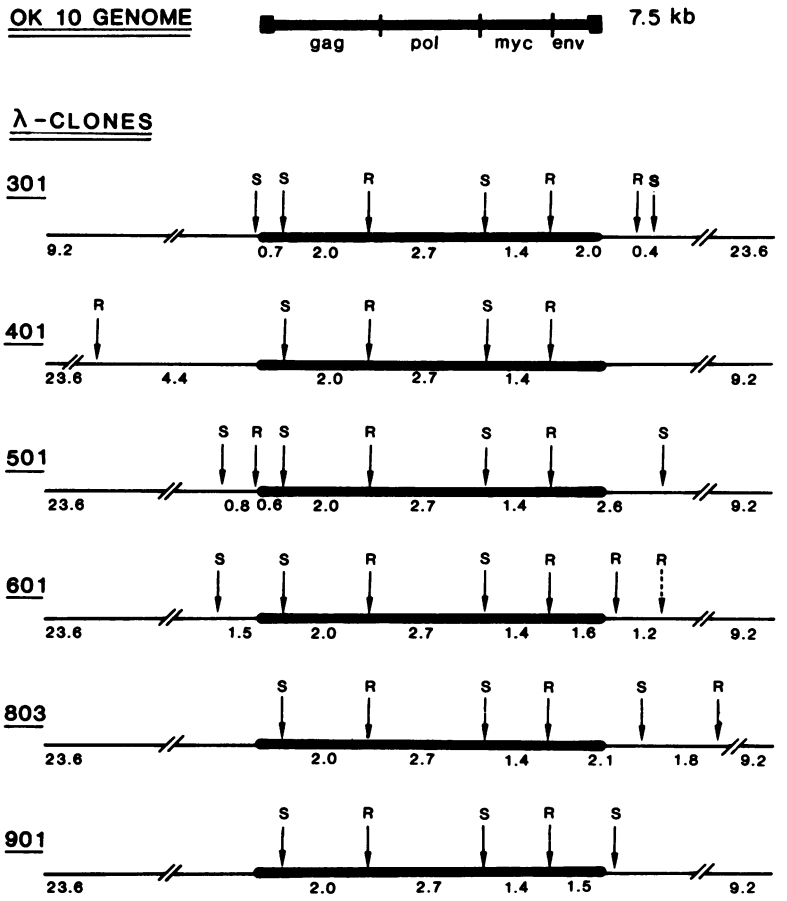

FIG. 1. Physical maps of six λ OK 10 clones. The data are a summary of those for six clones, analyzed by Southern filter hybridizations as described in the legend to Fig. 2. OK 10 proviral inserts are indicated by heavy lines, and flanking host cellular DNA sequences joining the phage CH 28 long (23.6 kb) and short (9.2 kb) arms are indicated by light lines. Pertinent restriction endonuclease cleavage sites are indicated by solid arrows. Abbreviations: S, *SacI*; R, *Eco*RI. The sizes of fragments are in kilobase pairs.

fection with λ OK 401 DNA were labeled with [^{35}S]methionine (Radiochemical Centre, Amersham, England), and virus-specific proteins were immunoprecipitated as described (S. Pfeifer et al., manuscript in preparation), with rabbit anti-p27 serum (a generous gift from K. Moelling) or with a tumor antiserum (no. 689) obtained after injecting OK 10-transformed bone marrow cells into chickens (Pfeifer et al., in preparation). Precipitated proteins were analyzed in sodium dodecyl sulfate-polyacrylamide gradient gels as described by Laemmli (18).

RESULTS

Molecular cloning and restriction mapping of OK 10 DNA. A complete genomic DNA library in λ phage Charon 28 was established by using DNA from the quail nonproducer cell line 9C that contains six to seven OK 10 proviruses (Pfeifer et al., in preparation). The library was screened with a *myc*-specific probe, and plaques that hybridized with the probe were subsequently purified. Six clones with complete OK 10 proviruses integrated into different sites in cellular DNA were further analyzed by restriction endonuclease cleavage and Southern filter hybridization with probes specific for viral structural genes. All six proviruses had a similar if not identical structure; differing only in their integration site in host DNA (Fig. 1). The result of a hybridization analysis of one representative λ OK 10 clone is shown in Fig. 2. A 4.7-kb *Sac*I fragment that hybridized to cDNA *gag* and to cDNA *pol* (Fig. 2B, lanes b and c) and a 4.1-kb *Eco*RI *pol-myc* fragment (Fig. 2A, lanes c and d) were identified in all of the clones. Digestion of the λ OK 10 clones with both *Eco*RI and *Sac*I was used to further analyze the internal structure of the OK 10 proviruses. In every clone we

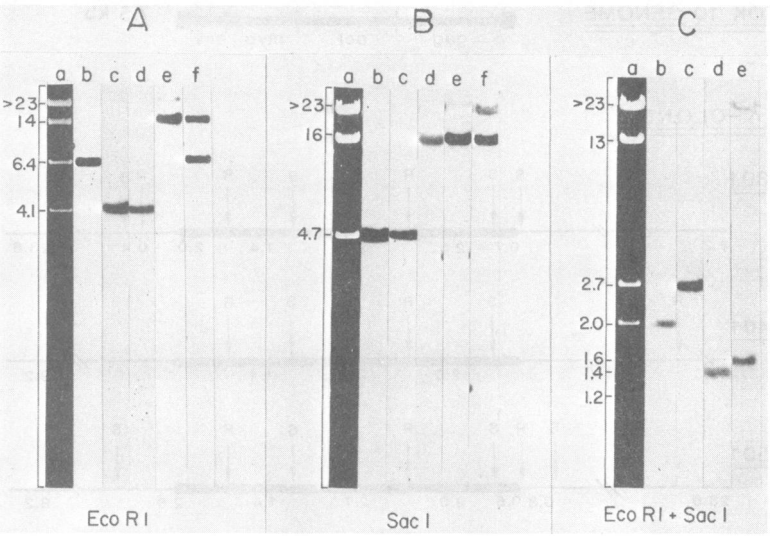

FIG. 2. Southern filter hybridization analysis of a λ clone (λ OK 401) that contains a complete OK 10 provirus. λ OK 401 DNA was cut with restriction endonuclease *Eco*RI (A), *Sac*I (B), or both enzymes simultaneously (C). Fragments were subjected to electrophoresis in a 0.8% agarose gel, transferred to nitrocellulose filters, and hybridized to the following ^{32}P-labeled viral probes: cDNA *gag* (lane b), cDNA *pol* (lane c), cDNA *myc* (lane d), cDNA *env* 3′ (lane e), and cDNA LTR (lane f), except in the double digest (C), where lane e represents hybridization to cDNA LTR. Lane a shows ethidium bromide-stained DNA. Fragments of λ DNA digested with *Hin*dIII were used as molecular weight standards.

identified a 2.0-kb fragment hybridizing to cDNA *gag* but not to other probes (Fig. 2C, lane b); similarly, a 2.7-kb *pol*-specific fragment (Fig. 2C, lane c) and a 1.4-kb *myc*-specific fragment (Fig. 2C, lane d) were detected. OK 10-specific fragments of the same sizes have also been found by Southern filter hybridization analysis of chromosomal DNA from the 9C cell line (Pfeifer et al., in preparation). The LTRs of OK 10 differ in sequence from the LTRs of both RAV-1 and MC29, since the OK 10 LTRs lacked cleavage sites for the restriction enzymes *Eco*RI (cleaves RAV-1, LTRs), *Pvu*I (cleaves RAV-1 and MC29 LTRs), and *Kpn*I (cleaves MC29 LTRs).

To further compare the structure of v-*myc*$^{OK\ 10}$ to the *myc* gene of MC29, a 4.1-kb *Eco*RI fragment of λ OK 501 that contained both the *pol* and *myc* sequences was subcloned in pBR322. The insert was isolated from the plasmid and cleaved with a number of restriction enzymes known to cleave the *myc* gene of MC29 (35). The results are summarized in Fig. 3B. In conclusion, the restriction enzymes tested, *Sac*I, *Pst*I, *Pvu*II, *Sal*I and *Cla*I, cleave at similar, possibly identical, sites in the v-*myc* sequences of OK 10 and MC29.

Heteroduplex analysis of OK 10 proviral DNA. DNA from clone λ OK 401 was annealed to three different cloned DNAs and mounted for electron microscopy. Heteroduplex molecules formed between λ OK 401 DNA and subcloned *Bam*HI fragments of c-*myc* consisted of two duplex regions of 0.77 ± 0.05 and 0.8 ± 0.07 kb interrupted by single-stranded loop of 1.06 ± 0.1 kb (Fig. 4A, D, and G [segments 2, 4, and 3]). This structure is similar to that of heteroduplexes between c-*myc* and cloned MC29 DNA (28, 38), and thus the 1.06-kb single-stranded loop is likely to represent the intron-like sequence specific for c-*myc*. Consequently, the OK 10 v-*myc* region appears to be similar to the v-*myc* region of MC29. This conclusion is further supported by analysis of heteroduplexes between λ OK 401 and unintegrated circular MC29 DNA that was cloned by using a unique *Eco*RI site located in the *env* sequence (Fig. 4C, F, and I). Two double-stranded regions of 1.7 ± 0.1 and 1.4 ± 0.1 kb representing *myc* and *gag* sequences, respectively (Fig. 4I, segments 12 and 14), were interrupted by a single-stranded loop of 3.3 ± 0.2 kb that represents *gag* and *pol* in OK 10 (Fig. 4I, segment 13). From these results we estimate that v-*myc*$^{OK\ 10}$ comprises a continuous DNA segment of 1.6 to 1.7 kb in the OK 10 genome.

The length of the region between the 5′ LTR and the end of *pol* in OK 10 was estimated in two ways. First, OK 10 DNA forms a double-stranded region of 4.5 ± 0.2 kb with RAV-1 DNA (Fig. 4B, E, and H [segment 6]) that starts at the *Sac*I site in RAV-1 DNA (located at around 170 base

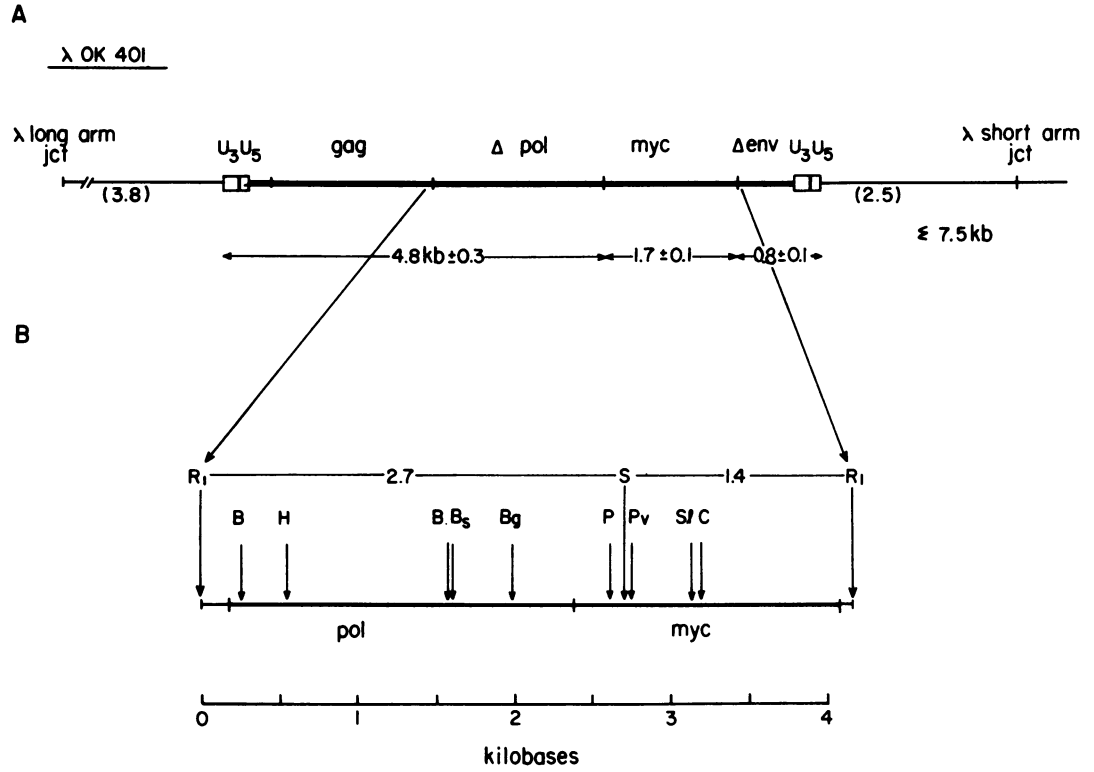

FIG. 3. Organization of the OK 10 provirus. (A) Summary of heteroduplex analysis and restriction enzyme mapping of cloned OK 10 proviral DNA (λ OK 401). (B) Restriction endonuclease cleavage map of a 4.1-kb *Eco*RI fragment from λ OK 501. The fragment, which encodes *pol* and *myc* sequences, was subcloned in pBR322 and analyzed by hybridization with the probes shown in Fig. 2. Abbreviations: R, *Eco*RI; S, *Sac*I; Sl, *Sal*I; B, *Bam*HI; Bs, *Bst*EII; Bg, *Bgl*II; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; C, *Cla*I; jct, junction.

pairs after U5 in RAV-1) and ends at the start of v-*myc*$^{OK\ 10}$ (Fig. 4H, segment 7). Second, this region of OK 10 DNA forms with MC29 DNA (Fig 4C, F, and I) a double-stranded region (1.4 kb; Fig. 4I, segment 14) and a single-stranded region (3.3 ± 0.2 kb; Fig. 4I, segment 13); the former represents LTR and *gag* sequences common to both DNAs, and the latter represents *gag* and *pol* sequences unique to OK 10 DNA. Taken together, the data indicate that U5, L (the untranslated segment between U5 and the AUG codon for *gag*), *gag*, and *pol* in OK 10 comprise around 4.7 kb; this is around 0.5 kb shorter than the corresponding sequence in the Rous sarcoma virus genome (29) and confirms the observations made by us and others that OK 10 is defective in *pol* (4; Saule et al., in press). The *env* and 3′ LTR regions in OK 10 form a duplex of 0.8 ± 0.1 kb with RAV-1 DNA (Fig. 4B, E, and H [segment 9]), suggesting that the *env* region in OK 10 consists of 0.4 to 0.5 kb.

A summary of the heteroduplex data is presented in Fig. 3A. The provirus in the λ OK 401 clone is around 7.5 kb in size and has the following structure: 5′-LTR-*gag*-Δ*pol*-*myc*-Δ*env*-LTR-3′.

Biological activity of cloned OK 10 DNA. The genetic integrity of the cloned OK 10 DNA was demonstrated by transfection and recovery of virus with the biological properties of parental OK 10 virus. Chicken embryo fibroblasts were cotransfected with the DNAs of λ OK 401 or λ OK 601, with RAV-1 DNA since OK 10 is defective in replication and requires a helper virus for replication. Recipient cells were passaged until high titers of virus was detectable in the culture medium as measured by reverse transcriptase activity. The virus released from the transfected cells transformed chicken fibroblasts and macrophages isolated from chicken embryo yolk sacs. The titer for the λ OK 401-derived virus was 4×10^4 and 2×10^3 focus-forming units per ml in chicken embryo fibroblasts and cultures of chicken embryo macrophages, respectively. The transformed fibroblasts and macrophages had a morphology identical to that of cells transformed by the parental OK 10 virus (data not shown).

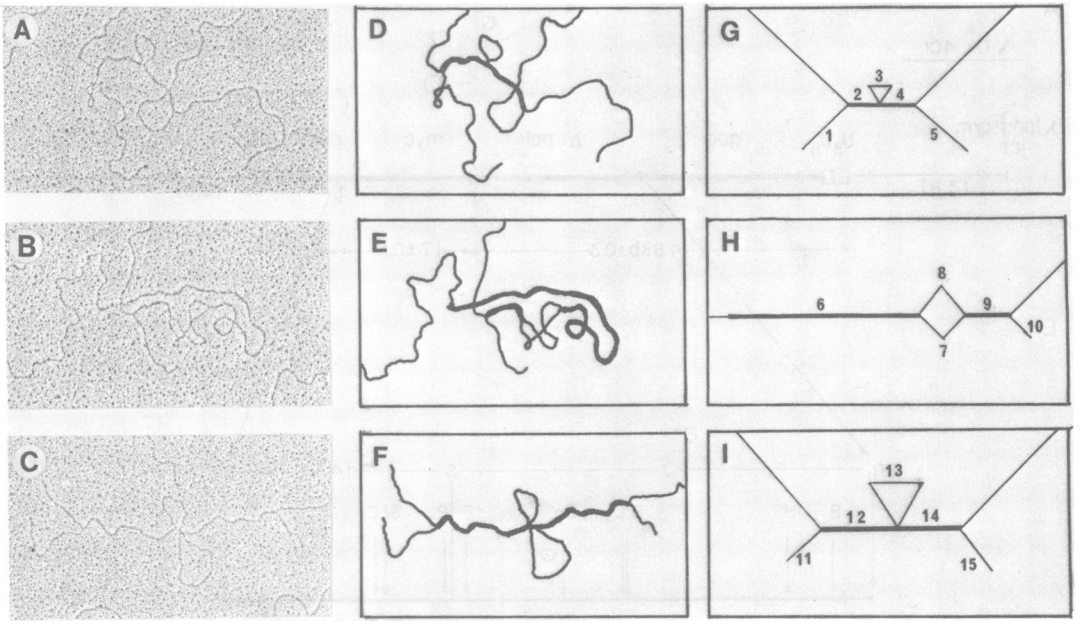

FIG. 4. Heteroduplex analysis of OK 10 DNA in the recombinant phage λ OK 401. The recombinant phage DNA was denatured and annealed to three different cloned DNAs and renatured DNAs under heteroduplex conditions. (A) Heteroduplex between λ OK 401 and the subcloned 9.4-kb *Bam*HI fragment of c-*myc* which had been subcloned in pBR322 and excised before use. (B) Heteroduplex with unintegrated circular RAV-1 DNA that had been cloned at a unique *Sac*I site present at around 270 nucleotides after the 5′ end of the genome; consequently, the linear DNA used in this analysis is permuted. (C) Heteroduplex between λ OK 401 and unintegrated circular MC29 DNA cloned at a unique *Eco*RI site present in the envelope gene. (D, E, and F) Line drawings of the heteroduplexes in panels A, B, and C, respectively. (G, H, and I) Schematic drawings with the individual measured segments numbered. The sizes (in kilobases) of the segments are as follows: 1, 2.7 ± 0.7; 2, 0.77 ± 0.05; 3, 1.06 ± 0.1; 4, 0.80 ± 0.07; 5, 3.1 ± 0.2; 6, 4.5 ± 0.2; 7, 1.7 ± 0.2; 8, 1.4 ± 0.2; 9, 0.81 ± 0.11; 10, 1.2 ± 0.1; 11, 1.06 ± 0.2; 12, 1.7 ± 0.1; 13, 3.3 ± 0.2; 14, 1.4 ± 0.1; 15, 1.2 ± 0.1. Eight to ten heteroduplexes of each type were measured.

From the transfected cells, virus-specific proteins were immunoprecipitated with two antisera, one specific for *gag* (anti-p27) and one tumor antiserum. p200, one of the putative transforming proteins of OK 10, could be detected in the transformed cells with either antiserum (Fig. 5). In our hands, the viral p200 migrates just below the 200,000-dalton myosin marker.

The analysis of the transforming capacities of the virus recovered after the transfection experiments and the protein analysis confirmed that the recombinant clones encoded authentic OK 10 proviral DNAs.

DISCUSSION

This paper describes the molecular cloning of several OK 10 proviruses. The following findings support the conclusion that the clones represent complete and biologically active proviruses: (i) our restriction enzyme analysis of the clones demonstrated that they could be propagated in procaryotic vectors without undergoing gross rearrangements; (ii) two of the clones (λ OK 401 and λ OK 601) gave rise to a virus with transforming activities typical of the parental OK 10 virus upon transfection in conjunction with the DNA of a helper virus; and (iii) we were able to detect the OK 10-specific p200 *gag-pol-myc* protein in fibroblasts transformed by virus recovered after transfection.

We determined the structure of the OK 10 proviral DNA by electron-microscopic heteroduplex analysis and by restriction mapping in conjunction with hybridization with probes specific for the genes of avian retroviruses. The results showed that the *myc* gene has been incorporated at around 500 nucleotides upstream from the end of *pol*, i.e., around 4.8 kb from the 5′ end of the OK 10 genome. We did not discover any other deletions in the OK 10 *pol* sequence, as was reported previously by other investigators (30); these discrepancies are possibly due to differences in the strains of virus used. By our length estimates, the *env* sequence in λ OK 401 comprises around 400 nucleotides, suggesting that *env* in λ OK 401 is more exten-

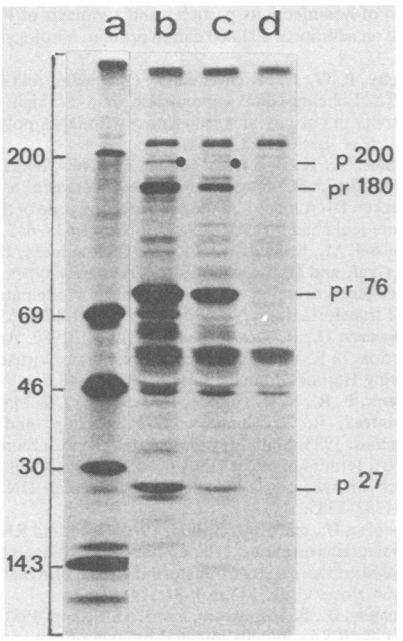

FIG. 5. Immunoprecipitation of proteins from cells transfected with cloned OK 10 proviral DNA. The transfected and transformed fibroblast cultures were metabolically labeled with [^{35}S]methionine, and cellular proteins were immunoprecipitated with rabbit anti-p27 (lane b), chicken OK 10 tumor 689 antiserum (lane c), or normal chicken serum (lane d). Precipitated proteins were analyzed by electrophoresis in sodium dodecyl sulfate-polyacrylamide gradient (5 to 16%) slab gels. A mixture of ^{14}C-labeled proteins (Amersham Corp.) was used for molecular weight standards (lane a). Molecular weights are in kilodaltons. The dots indicate the viral p200 protein.

sively deleted than the *env* sequence in the strain of OK 10 used by others, in which as much as 45% of *env* is preserved (4; Saule et al., in press). These results may explain the differences in the RNA oligonucleotide maps and differences in restriction sites in the *env* gene of the proviruses in the 9C and B5 cell lines (4, 24; Pfeifer et al., in preparation). It should be borne in mind, however, that possible sequence divergence between the *env* regions of OK 10 and RAV-1 may allow mismatches in this region of the heteroduplex molecules, leading to false interpretations of the heteroduplexes.

The v-*myc* gene of OK 10 is similar to the v-*myc* gene of MC29 in both length and structure. Both genes encode the two exons present in c-*myc* while lacking the intron-like sequence present in c-*myc*, suggesting that the capture events involved similar, if not identical, segments of c-*myc*. We cannot at present identify those properties of v-*myc*$^{OK\ 10}$ (or adjacent viral sequences) that mediate *myc* expression via the subgenomic mRNA. However, the availability of molecularly cloned v-*myc*$^{OK\ 10}$ DNA permits a future detailed study of its structure. The expression and function of v-*myc* independent of *gag* sequences can now also be studied, making it possible to clarify the role of p200 and the protein coded by the OK 10 subgenomic messenger RNA.

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