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Structural Relationship Between a Normal Chicken DNA Locus and the Transforming Gene of the Avian Acute Leukemia Virus MC29

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We screened a recombinant chicken DNA/λ phage library for sequences homologous to the transformation-specific sequences of the avian acute leukemia virus MC29 by hybridization with molecularly cloned MC29 proviral DNA. Three cellular DNA clones were found and compared with each other and with the viral genome by physical mapping with restriction endonucleases and by heteroduplex analysis. These experiments indicated that the three cellular clones overlap and represent a single cellular locus. The RNA genome of MC29 and normal cell DNA share a homologous region of 1.6 kilobases which is interrupted in the cellular DNA by 1.0 kilobase of sequences not present in the viral genome. Hybridization of the cloned cellular DNA to viral RNA and analysis of the protected viral RNA by fingerprinting techniques indicated that there is extensive sequence homology between the helper virus-unrelated *mcv* sequences of the viral RNA and the cellular DNA, with only minor base differences. The cellular *mcv* locus, however, lacks all helper virus-related sequences of MC29, including those of the partial viral *gag* gene which, together with *mcv*, encodes the probable transforming protein of MC29. We conclude that although the *mcv* locus of the normal cell does not represent a complete structural homolog to the *onc* gene of MC29, it is probably the precursor to the *onc*-specific sequence in the virus.

Avian myelocytomatosis virus MC29 is a highly oncogenic, replication-defective retrovirus that causes acute leukemia, carcinoma, and sarcomas (4, 10). The 5.7-kilobase (kb) RNA of MC29 contains an internal transformation- or *onc*-specific sequence of 1.6 kb, termed *mcv*, that is flanked by a partial Δ*gag* gene at the 5′ end and a Δ*env* gene at the 3′ end (15) (*gag* and *env* genes encode internal and envelope proteins in nondefective avian retroviruses). The *mcv* sequence is expressed as part of a hybrid protein which includes Δ*gag* and encodes a nonstructural phosphoprotein with probable transforming function (5, 15).

On the basis of distinct transformation-specific sequences, acutely transforming avian retroviruses can be classified into different subgroups. Thus, MC29, Rous sarcoma virus, avian erythroblastosis virus, avian myeloblastosis virus, Fujinami sarcoma virus, and Y73 sarcoma virus are each prototypic members of such subgroups (10). The MC29 subgroup includes three additional independent viral isolates, MH2, CMII, and OK10, each with specific sequences related to that of MC29 (4). All viruses of the MC29 subgroup have similar oncogenic spectra and similar, but distinct, genetic structures and code for Δ*gag-mcv* hybrid proteins (4, 6).

Molecular hybridization studies have indicated that *mcv* (17, 21) and the transformation-specific sequences of other avian (3, 24) and mammalian viruses (11, 18, 19) have homologous sequences in normal, uninfected avian or mammalian cells, which have been termed proto-*onc* genes (3, 25). The presence in normal cells of sequences related to the transforming genes of retroviruses has raised the question of whether viral *onc* genes or structural relatives of viral *onc* genes with possibly different functions are detected in normal cells. In accord with the former possibility, it has been proposed recently that enhanced expression of the cellular *mcv*-related sequence caused by adjacently integrated leukosis viruses without *onc* genes is the cause of some viral lymphomas in chickens (12).

To compare directly the cellular *mcv*-related DNA sequence with the genome of MC29, we selected clones of chicken DNA homologous to the *mcv* sequence of MC29 from a recombinant chicken DNA/λ phage library. We compared these cellular DNA clones with viral RNA and with molecularly cloned viral DNA by the map-

ping of common restriction endonuclease sites, heteroduplex analyses, and the fingerprinting of viral RNA hybridized to cloned cellular *mcv*-related DNA. We found that the primary sequence of the chicken locus related to *mcv*, termed proto-*mcv* (21), is nearly identical to the *mcv* sequence in the viral RNA. However, the cellular *mcv*-related sequence is not linked to *gag* or other retroviral sequences outside *mcv*, and it is interrupted by a 1.0-kb region of nonhomology which is not present in the viral genome. A preliminary report of these results has been presented (9).

MATERIALS AND METHODS

Chicken DNA/λ phage library. The Charon 4A/ recombinant chicken DNA phage library was described by Dodgson et al. (8) and was generously supplied by D. Engel. The library was propagated on *Escherichia coli* strain BHB-2600 and screened by the methods described by Benton and Davis (2) and Davis et al. (7). Approximately 5×10^5 phage were plated on a total of five large petri dishes (diameter, 150 mm), and the plaques from each plate were adsorbed to two duplicate nitrocellulose filters (Millipore Corp.). The filters were hybridized with ^{32}P-labeled (see below) proviral MC29 DNA cloned in the bacterial plasmid pBR322 (13), and plaques which were positive on both filters were picked and subjected to two or three subsequent rounds of screening by hybridization at lowered dilutions until every plaque hybridized (7).

Southern blots. Restriction endonuclease digestions of phage DNAs were performed in the appropriate buffers described by their commercial suppliers. Approximately 1 μg of each digest was electrophoresed in a 1% agarose (Seakem) gel in E buffer (40 mM Tris) [pH 8.0], 5 mM sodium acetate, 1 mM EDTA). The DNA fragments were visualized under UV light after staining the gel in ethidium bromide (0.5 μg/ml). The DNA fragments were transferred to nitrocellulose filters essentially by the method described by Southern (23), incorporating the acid pretreatment step of Wahl et al. (27).

Filter hybridizations. Nitrocellulose filters were first prehybridized in 20 ml of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7)–10× Denhardt solution (0.2% Ficoll, 0.2% bovine serum albumin, and 0.2% polyvinylpyrrolidone [Sigma Chemical Co.]) at 68°C for at least 4 h. The solution was replaced with 10 to 20 ml of 6× SSC–10× Denhardt solution–0.1% sodium dodecyl sulfate containing 0.1 to 0.2 μg of ^{32}P-labeled DNA and hybridized at 68°C for 20 h (7). The DNA probes were labeled with ^{32}P by nick translation to a specific activity of 10^8 cpm/μg by the procedure of Maniatis et al. (14) with [α-^{32}P]dCTP (New England Nuclear Corp.). The filters were washed two to three times for 30 min at 68°C in 100 ml of 2× SSC–0.5% sodium dodecyl sulfate, air dried, and exposed to X-ray film (Kodak) at −70°C with intensifying screens (Du Pont Co.).

RNA-DNA hybridizations. MC29 (ring-necked pheasant virus) ^{32}P-labeled 70S viral RNA was prepared as described previously (15). DNA-RNA hybrids were formed in 10 μl of 70% formamide–2× SSC–10 mM sodium phosphate (pH 7.0) at 40°C for 8 h. Each reaction contained 0.25 μg (10^6 cpm) of ^{32}P-labeled MC29 (ring-necked pheasant virus) RNA and 25 μg of the appropriate DNA which had been degraded for 10 min at 95 to 100°C in 0.3 N NaOH. The hybrid was then diluted into 200 μl of 2× SSC and treated with either RNase T_1 (50 U/ml) (Calbiochem) or RNase A (5 U/ml) (Worthington Diagnostics), T_1 (50 U/ml), and T_2 (10 U/ml) (Sigma) at 40°C for 30 min. The hybrid which remained was isolated by gel exclusion chromatography on a column (15 by 1 cm) of P-150 (Bio-Rad Laboratories) in 0.1 M NaCl–10 mM Tris (pH 7)–1 mM EDTA–0.1% sodium dodecyl sulfate. The hybrid was melted by boiling for 2 min, digested with RNase T_1, and subjected to fingerprint analysis (15).

Heteroduplex analysis. Heteroduplexes were formed by mixing equal amounts of each purified DNA, denaturing with alkali, and renaturing in 50% (vol/vol) formamide–0.1 M Tris (pH 8.5)–1 mM EDTA for 1 h at 35°C, essentially as described previously (13). DNA was mounted for electron microscopy at room temperature by the formamide technique (13).

RESULTS

Selection of a cellular *mcv*-related DNA sequence from a recombinant chicken DNA library. The recombinant chicken DNA/λ phage library used in these experiments has been described previously (8). The library was constructed by partial digestion of chicken DNA with the restriction enzymes *Alu*I and *Hae*III to generate random 15- to 20-kb fragments which were then inserted into the phage Charon 4A vector system with *Eco*RI linkers. This library was initially screened with an electrophoretically purified (7) *Bam*HI-resistant 3.0-kb fragment of cloned proviral DNA (Fig. 1). We have shown here (see below and Fig. 4) that this fragment contains all of *mcv* and some *gag* and *env* sequences by hybridizing viral [^{32}P]RNA to this cloned proviral DNA fragment and analyzing the large RNase T_1-resistant oligonucleotides of the viral RNA which are protected in the hybrid. The MC29 DNA was labeled with ^{32}P by nick translation and used as the probe for the screening of the chicken DNA library and the subsequent plaque purification of positive DNA clones.

From the initial screening of 5×10^5 phage, a total of 6 phage were isolated which contained cellular DNA inserts that hybridized to the MC29 DNA probe. This is consistent with the cellular MC29-related DNA locus being a single-copy gene in the haploid chicken genome, assuming random chicken DNA inserts of 15 to 20 kb in the phage vectors and assuming the complexity of chicken DNA to be 1.4×10^6 kb. Preliminary analysis of these six isolates showed that there were three unique cellular DNA inserts represented.

Restriction mapping and orientation of the cellular proto-*mcv* locus. The endonuclease *Bam*HI-

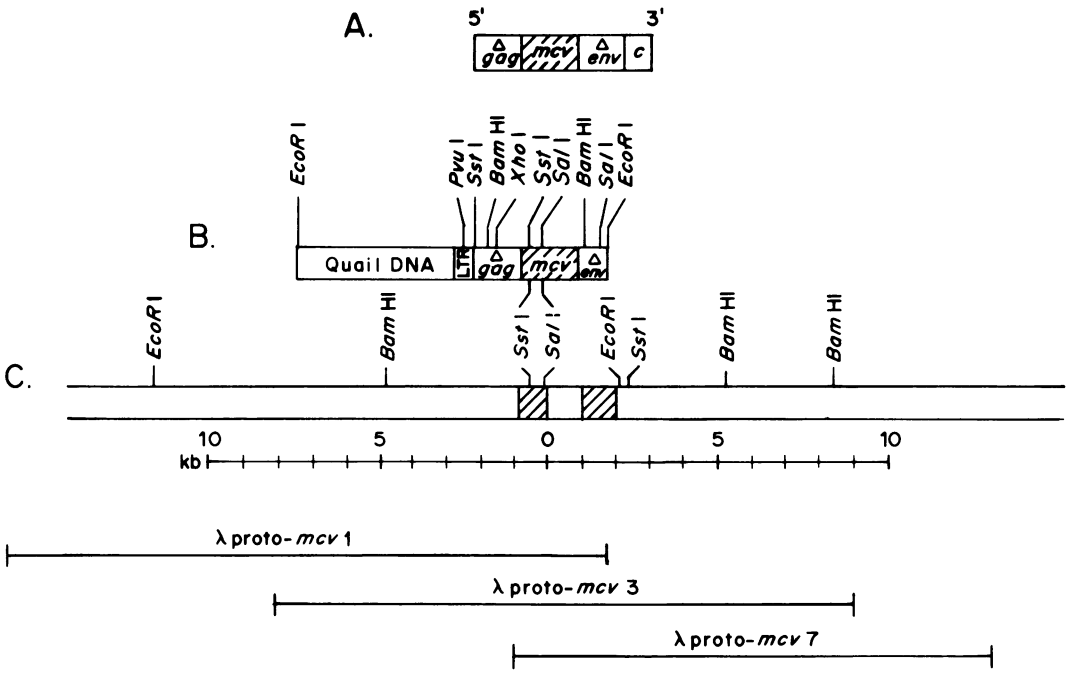

FIG. 1. Physical maps of proviral DNA and the cellular proto-*mcv* locus. (A) Genetic structure of the 5.7-kb RNA genome of MC29 (4, 15). (B) *Eco*RI DNA fragment (9.2 kb) of integrated MC29 proviral DNA and adjacent quail cell DNA (13). Only restriction sites present in the proviral DNA portion are indicated. (C) Restriction map of the chicken cellular proto-*mcv* locus. The endpoints of the cloned cellular DNA inserts in the *mcv*-related region were determined from hybridization experiments shown in Fig. 2. The maps are drawn around a common *Sal*I site shared by both cellular and viral *mcv*-related DNAs.

resistant fragment of MC29 DNA used in the initial screening was further subdivided into two separate fragments by digestion at the *Sal*I site in the *mcv*-specific region (Fig. 1). The availability of these separate regions of the *mcv* sequence allowed us to determine the orientation of the cellular sequence with respect to the proviral DNA clone. We refer to these as the 5′ and 3′ *mcv*-specific DNA probes.

Restriction endonuclease *Bam*HI-, *Sal*I-, and *Eco*RI-sensitive sites of three phage, termed proto-*mcv* 1, 3, and 7, which contained cellular *mcv*-related DNA were determined and mapped by electrophoretic analysis of DNA digests. MC29-related DNA fragments were identified and sized by hybridization with the 5′ and 3′ *mcv*-specific probes (Fig. 2). The results indicate that there is no *Bam*HI- or *Eco*RI-sensitive site within the cellular *mcv*-related locus since a single fragment was detected with either the 5′ or 3′ *mcv*-specific probe. When the proto-*mcv* DNA clones are digested with *Sal*I, the 5′ and 3′ *mcv*-specific probes each hybridize to a different DNA fragment. Therefore, we can deduce that *Sal*I divides viral-*mcv* and proto-*mcv* into homologous segments.

Additional mapping of restriction enzyme sites suggests that both the *Sst*I and *Sal*I sites are present at the same relative locations in both the cellular and viral *mcv* sequences, since double digests of both the proviral DNA clone and the proto-*mcv* clone generated a comigrating 400-base pair fragment which hybridized to the 5′ *mcv*-specific probe (data not shown) (Fig. 1). The results that relative to their internal *Sal*I sites both viral and cellular *mcv*-related sequences have the same polarity and that they share an apparently colinear *Sst*I- and *Sal*I-resistant fragment suggest that in this region the viral-*mcv* and proto-*mcv* are similar (Fig. 1).

The restriction maps of the three proto-*mcv* phage show that they are overlapping with regard to their cellular inserts and together span a region of appoximately 10 kb on either side of the *mcv*-related sequence defined by the *Bam*HI and *Eco*RI sites with either the 5′ or 3′ *mcv*-specific probe (Fig. 1 and 2). Since the two probes contain the entire *mcv* sequences of the virus, we concluded that elements of both probes of viral *mcv* are present in the cellular locus.

Heteroduplex analysis of related viral and cellular *mcv* sequences. To determine whether the viral *mcv* sequence and the cellular *mcv*-related

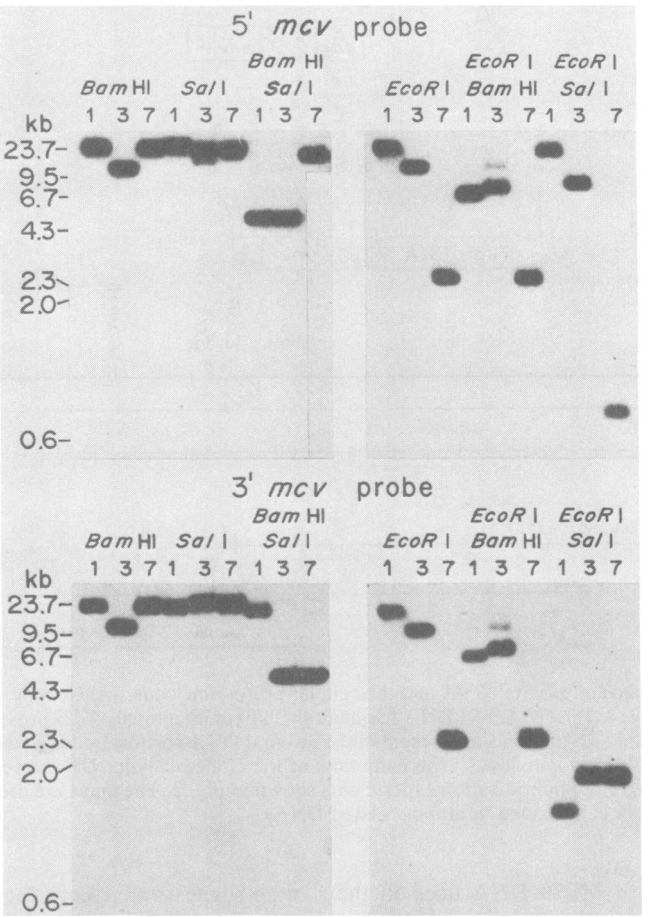

FIG. 2. Restriction enzyme analysis of the recombinant λ phage carrying overlapping segments of proto-*mcv*. DNA (1 μg) from λ proto-*mcv* 1, 3, or 7 was digested with the restriction enzyme(s) indicated, electrophoresed in a 1% agarose gel, and transferred to nitrocellulose filters as described in the text. The DNA filter was first hybridized to the 5′ *mcv*-specific probe (see text) which had been labeled with ^{32}P by nick translation. The first hybrid was removed by rinsing the filter in 0.1 N NaOH at room temperature for 10 min. The filter was then rehybridized to the 3′ *mcv*-specific probe which had also been labeled with ^{32}P by nick translation. The sizes of DNA fragments resulting from *Hin*dIII digestion of λ*c*I857*s*7 which served as molecular size standards are indicated.

sequence are colinear, heteroduplexes were formed between λ proto-*mcv* 3 DNA and two different DNA fragments of the MC29 proviral DNA clone. In the first case, the 9.2-kb *Eco*RI MC29 proviral DNA fragment contains, besides Δ*gag* and *mcv*, 5.0 kb of quail cell DNA linked to the 5′ end of the provirus as well as 0.8 kb of *env* sequences. In the second case, the 3.0-kb *Bam*HI MC29 proviral DNA fragment contains, besides *mcv*, approximately 1.2 kb of *gag* sequences at the 5′ end. The heteroduplexes shown in Fig. 3 indicate that proto-*mcv* is interrupted by a single 1.0-kb region of nonhomology. In both types of heteroduplex molecules formed, the 5′-to-3′orientation of proto-*mcv* relative to the proviral DNA clone could be identified unambiguously with the 5′ quail and Δ*gag* sequences or the Δ*gag* sequences of proviral MC29 DNAs as markers. The *env* sequences which are detected in the RNA hybridization experiments with the 3.0-kb *Bam*HI fragment (Fig. 4) are not apparent. We estimate that the 5′ region (0.8 kb) of proto-*mcv*, homologous to viral-*mcv*, is slightly smaller than the 3′ region (0.9 kb) and that the sum is 1.7 kb. This is in good agreement with previous estimates of 1.6 kb for the size of viral-*mcv* determined by mapping viral RNA or proviral DNA (13, 15).

Partial sequence comparison of proto-*mcv* DNA and MC29 viral RNA. To determine the extent of sequence homology between the proto-*mcv* DNA clone and MC29 RNA, the following strat-

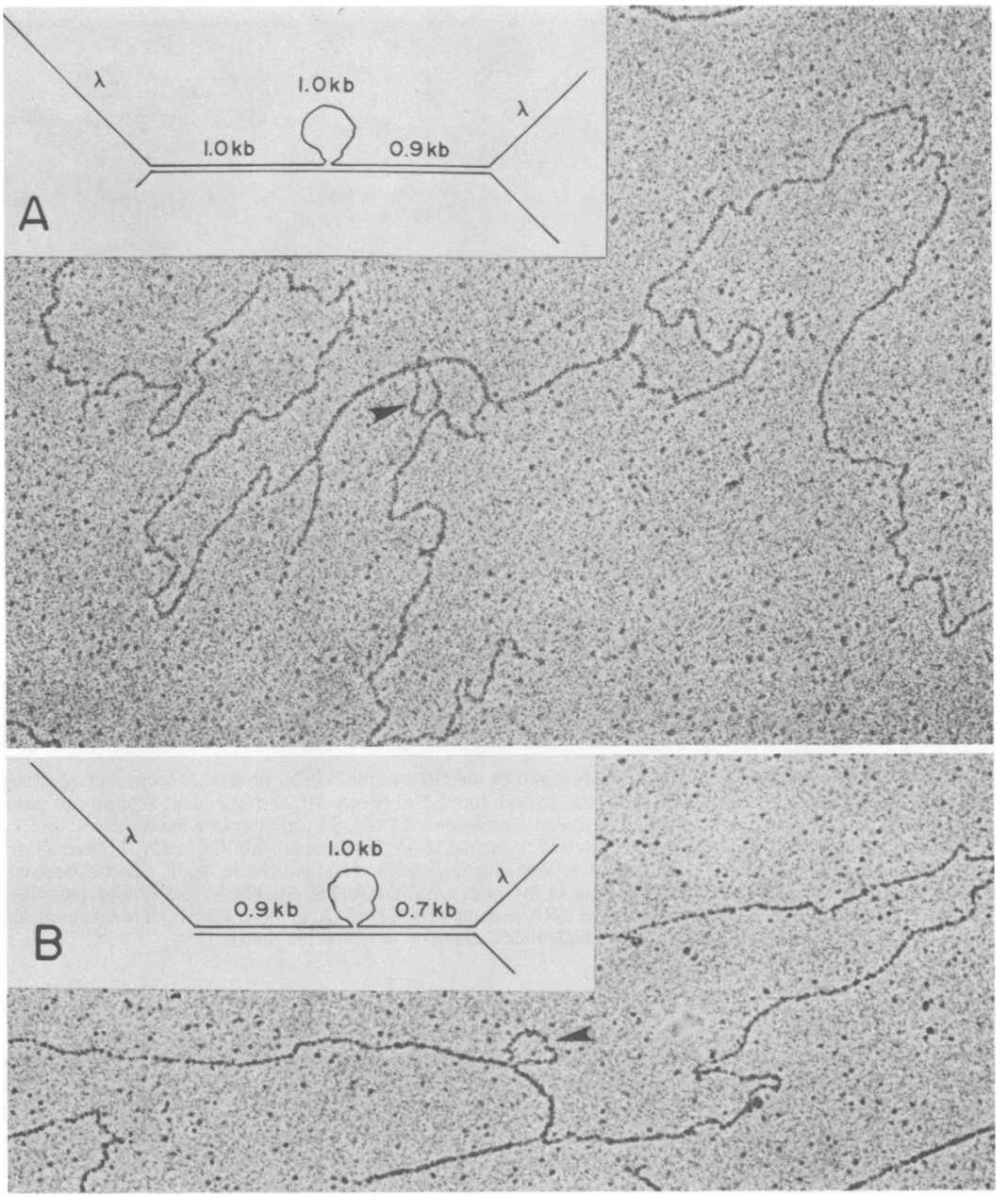

FIG. 3. Heteroduplex analysis of the cellular proto-*mcv* locus. Heteroduplex molecules were formed between λ proto-*mcv* 3 and either (A) a 9.2-kb *Eco*RI fragment or (B) the 3.0-kb *Bam*HI fragment from the cloned integrated MC29 proviral DNA (Fig. 1). The arrow indicates the 1.0-kb sequence of nonhomology that interrupts the *mcv*-related cellular sequence. Mean contour length measurements of 10 to 15 molecules of each type were used to determine the size of the various segments shown in the schematic representations. The two size estimates for the proto-*mcv* region, either 1.6 or 1.9 kb, are in good agreement with previous estimates of 1.6 kb for the viral *mcv* counterpart (15).

egy was employed. DNA from λ proto-*mcv* 3 was hybridized to MC29 [^{32}P]RNA. After digestion of unhybridized RNA with RNase T_1, the hybrid was separated from the hydrolyzed RNA by gel filtration chromatography. The [^{32}P]RNA which remained in the hybrid was then analyzed by fingerprinting RNase T_1-resistant oligonucleotides and compared with fingerprints of MC29 RNA. All eight *mcv*-specific oligonucleotides of MC29 RNA (i.e., no. 1, 3, 6, 7b, 8b, 15, 26, and

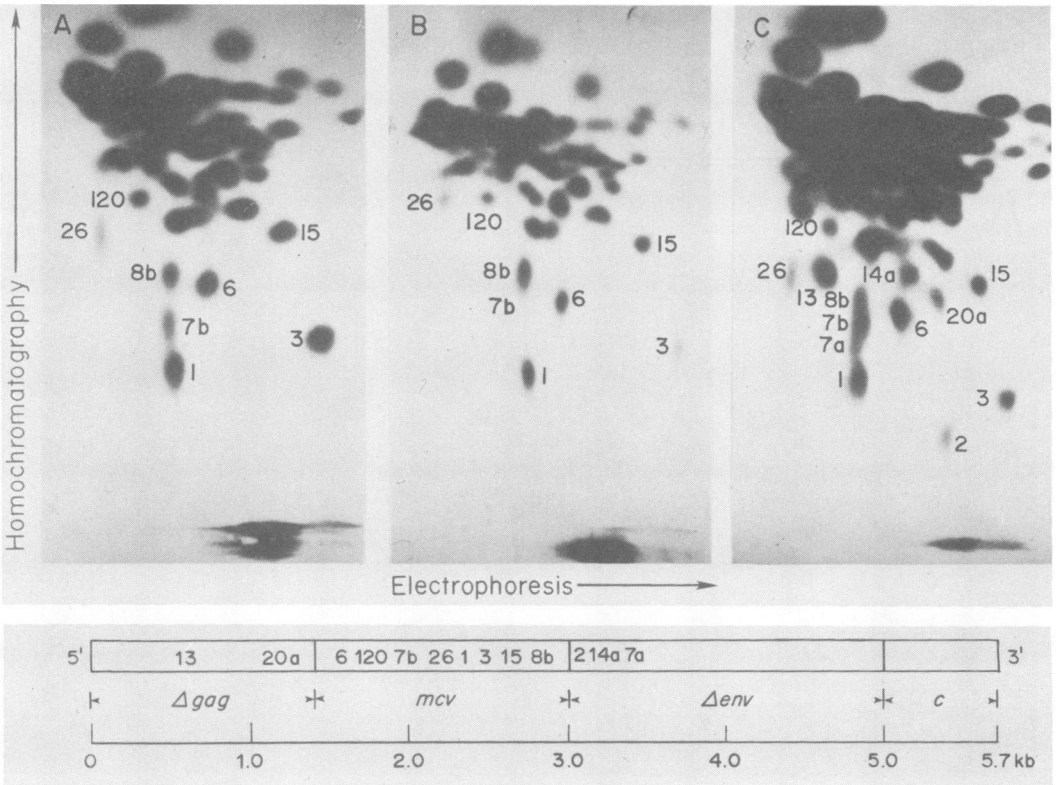

FIG. 4. MC29-related oligonucleotides hybridized by the DNA of the cellular proto-*mcv* locus and by cloned MC29 proviral DNA. (A) Fingerprint of the hybrid formed between MC29 (ring-necked pheasant virus) [^{32}P]RNA (0.25 μg or 10^6 cpm) and alkali-degraded λ proto-*mcv* 3 DNA (25 μg) after treatment with RNase T_1. (B) *mcv* oligonucleotides which remain in a hybrid formed as in (A) but treated with RNases A, T_1, and T_2. (C) *mcv* oligonucleotides and some *gag*- and *env*-related oligonucleotides from an RNase A-, T_1-, and T_2-resistant hybrid formed with a 3.0-kb *Bam*HI fragment of MC29 proviral DNA (Fig. 1). The hybrid was prepared and treated as in (B). The genetic structure of the RNA genome of MC29 (5′ Δ*gag mcv* Δ*env c* 3′) is schematically represented relative to the order of all oligonucleotides identified here and previously (15).

120; Fig. 4A) defined previously (6, 15) were present in the RNase T_1-resistant hybrid, indicating that the sequence and complexity of proto-*mcv* and of viral-*mcv* are essentially the same.

If the cellular and viral *mcv* sequences were related but diverged significantly, the viral RNA–proto-*mcv* DNA hybrid would be mismatched. Most oligonucleotides of such a hybrid would be cut within the hybrid or lost from the hybrid when challenged with RNases A, T_1, and T_2. In a perfect hybrid, all ribonucleotides would be protected from digestion with RNases A, T_1, and T_2. With the exception of oligonucleotides 3 and 7b, which are recovered at less than equimolar amounts, all *mcv* oligonucleotides previously identified are protected from challenge with RNases A, T_1, and T_2, indicating that the nucleotide sequence of the *mcv*-related part of the cellular DNA clone is very similar to that of the virus (Fig. 4B). Control experiments were performed with a *Bam*HI fragment of a cloned proviral MC29 DNA since this should be completely homologous to the viral RNA from which it was derived. All oligonucleotides of the viral RNA corresponding to the proviral DNA fragment were indeed retained when hybrids formed with this DNA under the above conditions were treated with RNases A, T_1, and T_2 (Fig. 4C).

It is important to note that although the RNA used in these experiments contains both helper virus RNA and MC29 RNA, no helper virus-related oligonucleotides have been identified in the proto-*mcv* DNA hybrids. Also, if helper virus RNA alone which does not contain *mcv* sequences was hybridized to proto-*mcv* DNA, no hybridization was detected (data not shown). These results show that there is extensive sequence homology between the cellular proto-*mcv* DNA and MC29 RNA in only the region previously identified as being specific to MC29 and that there are no structural or regulatory

gene sequences of retroviruses adjacent to proto-*mcv* detectable by this method.

DISCUSSION

We have molecularly cloned and analyzed a chicken cellular locus which is homologous to the *onc*-specific sequence of MC29 virus RNA. This is probably the only MC29-related locus in the chicken genome since the restriction map of this DNA locus can account for all MC29-related restriction fragments of chicken DNA identified previously by hybridization with MC29-specific cDNA (21). The three cellular DNA clones which hybridize to both 5′ and 3′ probes of the viral-*mcv* sequence cover a region of approximately 30 kb of chicken DNA. Since all *mcv*-specific RNase T_1-resistant oligonucleotides identified previously in the viral RNA can be hybridized by our clone of cellular *mcv*-related DNA, the cellular locus appears to contain a complete complement of *mcv*. With the exception of *mcv* oligonucleotides 3 and 7b, which are recovered in nonequimolar amounts, all other *mcv* oligonucleotides of MC29 viral RNA hybridized to λ proto-*mcv* 3 DNA are protected from digestion with RNases A, T_1, and T_2. The failure of proto-*mcv*-related DNA to protect these oligonucleotides against RNases A and T_2 in the hybrid probably reflects single base differences.

It is important to note that the *mcv* sequences of OK10, CMII, and MH2, other members of the MC29 subgroup, also differ from each other in one or several *mcv* oligonucleotides (4), although the oncogenic properties of these viruses are very closely related. It follows that the variations observed here between the *mcv* sequence of MC29 and cellular proto-*mcv* DNA do not necessarily reflect functional differences. A close sequence homology has also been described to exist between Moloney sarcoma virus *onc*-specific RNA sequences and the cellular DNA sequence homologous to it in the normal mouse genome (16, 26). In this case, transforming function has been demonstrated for the cellular DNA after ligation to terminal sequences of Moloney sarcoma virus DNA (16). However, similar experiments carried out with λ proto-*mcv* 3 DNA in collaboration with G. Vande Woude have not as yet been successful (unpublished data), whereas the cloned proviral DNA of MC29 was shown to transform NIH 3T3 cells (13).

Heteroduplex analysis between proto-*mcv* and MC29 proviral DNA has revealed that proto-*mcv* is interrupted by a single 1.0-kb region that is not homologous to MC29 proviral DNA. This sequence may be an intervening sequence of a cellular gene which may encode a product similar to that encoded by MC29, but it may also be a coding sequence, in which case it would encode a product that is different from any viral gene product. In this respect the cellular *mcv*-related DNA is analogous to the chicken cellular locus homologous to the *src* gene of Rous sarcoma virus, which contains six regions of nonhomology not present in viral *src* (20). By contrast, the mouse cellular sequence related to the specific sequence of Moloney sarcoma virus and its viral counterpart are colinear (16).

We have also determined that there are no viral structural or regulatory sequences adjacent to proto-*mcv*. This observation implies that the gene product of proto-*mcv*, which has yet to be identified, cannot formally be the same as the Δ*gag mcv* proteins encoded by the viruses of the MC29 subgroup. The functional significance of this observation depends on whether the Δ*gag* portion of the viral transforming protein is required for transformation. To date there is no direct evidence which demonstrates or excludes a functional role for the Δ*gag* portion of the viral transforming protein. However, the Δ*gag* element is part of the probable transforming genes of all viruses of the MC29 subgroup as well as of the transforming genes of other avian (5, 10) and mammalian (1, 22) retroviruses.

The structural similarities which we have shown between the viral transformation-specific *mcv* sequence and its cellular counterpart, proto-*mcv*, strongly suggest that they have a common evolutionary origin. On the other hand, the rare occurrence and isolation of acute transforming viruses with *mcv* or other specific sequences argue against a simple mechanism leading to the incorporation of these sequences into a retrovirus genome. With the example of MC29, our data suggest a rational basis for the infrequent formation of such viruses starting from proto-*mcv* and a retrovirus without *onc* genes since this mechanism must involve (i) deletion of viral sequences, (ii) deletion of cellular sequences, and (iii) illegitimate recombinational events. Such a multistep pathway is unlikely to occur frequently in nature.

Our data cannot determine whether the viral Δ*gag-mcv* hybrid proteins are functionally similar to the putative product of the cellular proto-*mcv* locus. However, since they share a common evolutionary origin, the possibility exists that under rare circumstances this cellular locus may have the potential to transform cells. Recent evidence from Hayward et al. (12) has shown that integration of an avian leukosis virus adjacent to the proto-*mcv* locus is associated with increased transcription of the locus in some avian B-cell lymphomas. It was proposed that the neoplasia was caused by increased levels of the gene product of the proto-*mcv* locus. If,

indeed, the proto-*mcv* gene product and the MC29 viral Δ*gag-mcv* protein have the same function, why has the proto-*mcv* gene not been observed to cause tumors typical of MC29 viral infections? The answer to this paradox may be relevant to the question of functional similarities between the viral transforming proteins and their cellular homologs.

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