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Molecular Cloning of the Avian Myelocytomatosis Virus Genome and Recovery of Infectious Virus by Transfection of Chicken Cells

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The avian retrovirus myelocytomatosis virus 19 (MCV) possesses an interesting diversity of oncogenic potentials, but the virus has proven difficult to study because of its inability to replicate without the assistance of a helper virus. We have therefore isolated and amplified the genome of MCV by molecular cloning in a procaryotic vector. The topography of the cloned DNA was explored by the use of restriction endonucleases and radioactive complementary DNAs representing specific domains in avian retrovirus genomes. The cloned DNA appeared to be an authentic representation of the MCV genome: the size and genetic topography of the DNA were comparable to those of MCV, and transfection of the cloned DNA into chicken cells (in company with the DNA of a suitable helper virus) gave rise to virus with the genome and transforming potentials of MCV. The availability of cloned MCV DNA should facilitate a variety of genetic and biochemical manipulations directed at elucidating the mechanism of oncogenesis by MCV.

Myelocytomatosis virus 29 (MCV) is the prototype for a group of avian retroviruses that induce a variety of tumors in chickens, including carcinomas, sarcomas, and solid tumors composed of myeloid hematopoietic cells (8, 16). The same viruses induce morphological transformation of fibroblasts, epithelial cells, and macrophages in cell culture (8). The haploid genome of MCV is a single-stranded RNA of ca. 5.5 kilobases (kb) (7, 21). A nucleotide sequence of ca. 1.5 kb located near the middle of the MCV genome is thought to carry the oncogenic potential of the virus (7, 13, 15, 20, 21). This putative oncogene distinguishes MCV (and viruses with tumorigenic capacities similar to those of MCV) from other retroviruses and was apparently derived from the genome of vertebrate cells (17, 19). The genome of MCV has sustained a large deletion which affects all three of the replicative genes of retroviruses, leaving only a portion of *env* and *gag* and none of *pol* (13, 15). As a consequence, MCV can replicate only when complemented by a helper virus and is therefore a "defective leukemia virus" (DLV) (8). It is hypothesized that the deletion occurred during the acquisition of an oncogene by MCV (17, 19).

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The versatile oncogenicity of MCV gives the virus substantial interest but has been difficult to analyze in detail because of the replicative defectiveness of the virus. We therefore turned to the use of molecular cloning to isolate the genome of MCV in a form that would be suitable for genetic and biochemical manipulation. Our work exploited the fact that during acute infection of cells by retroviruses, the viral RNA genome is transcribed into DNA by reverse transcriptase (23). The first stable product of viral DNA synthesis is a linear duplex that is coextensive with the viral genome, but that has, in addition, terminal redundancies composed of three domains: U3, encoded at the 3′ end of the viral RNA; R, a small redundancy encoded at each end of the viral RNA; and U5, encoded at the 5′ end of viral RNA (4). These domains are arranged in the order 5′-U3-R-U5-3′ (12, 18) to give a structure that is denoted LTR (for long terminal redundancy). The linear duplexes are then formed into closed circular molecules of two sorts: those containing one copy of the LTR, and those that contain two copies. The circular forms of viral DNA can be isolated from acutely infected cells and used for molecular cloning of the MCV genome.

We exploited our previously described strategy, in which Hirt fractionation and extraction

with phenol at pH 4.0 are combined to isolate closed circular DNA from cells acutely infected with retroviruses (6). For the cloning of DLV genomes, it is convienient to first eliminate the DNA of the helper virus. We accomplish this by identifying a restriction endonuclease that cleaves the DNA of the helper virus but not that of the DLV. The cleaved DNA can then be separated from the unaffected, closed circular DNA of the DLV by extraction with phenol at pH 4.0 (24, 25). The isolated DLV DNA is cloned into an appropriate procaryotic vector. Using this strategy, we were able to clone the complete genome of MCV into the phage vector λgtWES·λB (15). The cloned DNA was used to map sites of cleavage by a number of restriction endonucleases on the MCV genome. The genetic integrity of the cloned DNA was documented by demonstrating that virus recovered from transfection with the DNA displayed the characteristic transforming potentials of MCV. The molecular clones described here should find many applications in the genetical and biochemical analysis of MCV.

MATERIALS AND METHODS

Cells and viruses. Uninfected and infected cultures of chicken embryo fibroblasts were prepared and propagated as before (3). QT-6 cells (explanted from a fibrosarcoma induced in quail with methylcholanthrene) were obtained from C. Moscovici and subsequently cloned in our laboratory (10). We have described the genesis of our standard stock of MCV (MCAV) (21) (MCAV is the helper virus in field isolates of MCV). Recently cloned Rous-associated virus 1 (RAV-1) was a kind gift from L. Crittenden.

Preparation and analysis of DNA. We extracted DNA from acutely infected cells by Hirt fractionation (11), as described previously (6, 10). Closed circular DNA was isolated from the supernatant of this fractionation by extraction with phenol at pH 4.0 (25). Restriction endonucleases and T4 phage DNA ligase were obtained from Biolabs Inc. and used as described previously (6, 24). DNA was fractionated by electrophoresis in gels of agarose, transferred to sheets of nitrocellulose, and detected by hybridization with specific complementary DNAs (cDNA's) (6, 24). Reference standards in the electrophoretic analyses included the supercoiled DNAs of pBR313 and simian virus 40 and the DNAs of lambda phage and simian virus 40 after cleavage with the restriction endonuclease *Hin*dIII.

Preparation of cDNA's. The preparation and specificity of all of the cDNA's used in the present study have been summarized recently (7a). $cDNA_{rep}$ was transcribed from the entire genome of the Schmidt-Ruppin strain (subgroup A) or the Prague strain (subgroup C) of Rous sarcoma virus (RSV). $cDNA_{MCV}$ was specific for the allegedly oncogenic domain of the MCV genome and was prepared from a 1.5-kilobase pair (kbp) fragment of DNA derived from the cloned genome of MCV by cleavage with the restriction endonuclease *Pst*I (see Results). $cDNA_{env}$ was prepared with a 0.75-kbp *Pvu*II fragment of DNA derived from within the *env* gene of RSV, and $cDNA_{env/src}$ was prepared with a 1.6-kbp *Pvu*II fragment that overlaps the junction between *env* and *src* on the RSV genome (6); the positions of both of these cDNA fragments have been verified from the nucleotide sequence of the relevant region of the RSV genome (5). $cDNA_{3'}$ represented a large portion of the U3 domain of the LTR of RSV.

Molecular cloning of DNA. MCV DNA was cloned into the *Eco*RI site, and DNA of RAV-1 was cloned into the *Sac*I site, of the phage vector λgtWES·λB. The cloned DNA of RAV-1 was a generous gift of G. Payne; its preparation will be described elsewhere (manuscript in preparation). Our procedures for molecular cloning have been described in detail previously (6) and conformed to the guidelines of the National Institutes of Health Committee on Recombinant DNA.

Fractionation of viral RNA by electrophoresis in agarose gels. RNA was fractionated by electrophoresis through gels of 1.2% agarose containing 10 mM methyl mercury hydroxide (2). The RNA was transferred from the gels to dimethylbenzyloxymethyl paper, and viral RNAs were then located by molecular hybridization to specific cDNA's (1).

Transfection of chicken cells with cloned DNA. We have previously described the strategy and details of our procedure for transfecting cloned DNA of DLV into permissive chicken embryo fibroblasts (24). Briefly, cloned DNA of MCV was excised from the chimeric form, purified by electrophoresis in agarose gels, first ligated into tandem arrays and then joined to tandem arrays of cloned DNA of RAV-1 by means of a fragment (3.8 kbp) of lambda phage DNA, and introduced into chicken embryo fibroblasts by the use of calcium phosphate and dimethyl sulfoxide (9, 22). The procedure is designed to facilitate the simultaneous transfection of individual cells by both MCV DNA and the necessary helper virus DNA (24).

RESULTS

Preparation of viral DNA for molecular cloning. We isolated DNA from infected cells according to a protocol that provides preparations of highly enriched supercoiled circular molecules (6). Quail cells (QT-6) were infected with MCV(MCAV) at a multiplicity of ca. 1.0. Twenty-four hours later, when the bulk of retrovirus DNA was not yet unintegrated (10), DNA was prepared from the infected cells by Hirt fractionation (11). The supernatant from this fractionation was extracted at pH 4.0 to selectively isolate closed circular DNA (25).

The extracted DNA contained two major classes of high-molecular-weight virus-specific molecules (Fig. 1, lane 1). The electrophoretic mobilities of these DNAs were characteristic of closed circular forms with sizes of 9.0 kbp (corresponding to the length of the MCAV genome) and 5.5 kbp (the length of the MCV genome). Both classes of circular DNA were resolved into

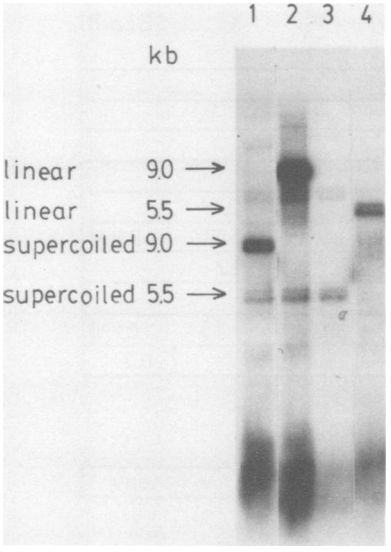

FIG. 1. *Preparation of MCV DNA for molecular cloning. QT-6 cells were infected with MCV(MCAV) at a multiplicity of ca. 1. Twenty-four hours later, DNA was prepared from the cells by Hirt fractionation, followed by extraction of the supernatant fraction with phenol at pH 4.0. After electrophoresis through a 0.8% agarose gel, DNA was transferred to nitrocellulose paper and hybridizated with $cDNA_{rep}$. Lane 1, DNA after extraction from infected cells; lane 2, DNA after cleavage with restriction endonuclease HpaI; lane 3, DNA after cleavage with HpaI and subsequent extraction with phenol at pH 4.0; lane 4, DNA prepared as in lane 3 and then cleaved with EcoRI.*

two populations (more evident for the 5.5-kbp class in lanes 1, 2, and 3 of Fig. 1) that represent circular molecules containing either one to two copies of the LTR (4, 12, 18, 23). Heterogeneous smaller viral DNA was also present, presumably because low-molecular-weight linear DNA is not completely extracted by phenol at pH 4.0 (6). As anticipated from our previous experience (6, 24), the preparation contained no detectable full-length linear forms of viral DNA and was therefore suitable for the subsequent manipulations in our cloning strategy.

Cleavage of the circular DNAs with the restriction endonuclease *Hpa*I converted the 9.0-kbp form to linear molecules but had no effect on the 5.5-kbp viral DNA (and little or no effect on the low-molecular-weight viral DNA) (Fig. 1, lane 2). Extraction of the cleaved DNA with phenol at pH 4.0 removed all of the linear 9.0-kbp viral DNA, as intended, and much of the remaining low-molecular-weight viral DNA (Fig. 1, lane 3). The remaining viral DNA consisted mainly of 5.5-kbp closed circular molecules and a smaller amount of ostensibly larger DNA which we presume to be relaxed duplex circles (form II) of MCV DNA generated from supercoiled DNA during the extraction with phenol at pH 4.0.

A preliminary survey had indicated to us that MCV DNA has a single site of cleavage by the restriction endonuclease *Eco*RI (data not shown). Thus, cleavage with this enzyme converted the circular viral DNA to 5.5-kbp linear molecules (Fig. 1, lane 4) suitable for cloning into the phage vector λgtWES·λB.

Molecular cloning and restriction mapping of MCV DNA. Supercoiled MCV DNA was cleaved with *Eco*RI and then cloned into λgtWES·λB. We obtained a number of chimeric isolates that appeared to have inserts of full-length (i.e., 5.5-kbp) MCV DNA. One of these was denoted λMCV38 and subjected to detailed analysis. The positions of cleavage sites for a number of restriction endonucleases were mapped on the DNA of λMCV38, using single and double digestions in conventional procedures (6, 24). Of the endonucleases tested, *Hin*dIII, *Xba*I, and *Hpa*I failed to cleave the DNA of λMCV38. A map of the sites for a number of other endonucleases is shown in Fig. 2, with the ends of the DNA being demarcated by the single site for *Eco*RI.

Mapping the genetic coordinates of λMCV38 DNA. We used hybridization with specific cDNA's to align the restriction map of λMCV38 DNA with the genome and provirus of MCV. The MCV insert in λMCV38 was excised by cleavage with *Eco*RI and then separated from the DNA of the phage vector by electrophoresis in an agarose gel. The purified MCV DNA was cut with *Pst*I, the cleavage sites for which are distributed at convenient intervals throughout the DNA (Fig. 2). The resulting restriction fragments were fractionated by electrophoresis and identified by hybridization with specific cDNA's.

Four of the five *Pst*I fragments were detected with $cDNA_{rep}$ (Fig. 3, lane 4); the fifth fragment (1.5 kbp) is located almost entirely within the specific locus of the MCV genome (see Fig. 2 and below) and therefore did not react detectably with $cDNA_{rep}$. The presence of the 1.5-kbp fragment on the filter was demonstrated by hybridization with a cDNA prepared from the subclone of the fragment described below (data not illustrated).

Two fragments (0.7 and 0.4 kbp) hybridized with $cDNA_{env}$ (Fig. 3, lane 2). This finding locates the *Eco*RI site in the midst of the portion of *env* found in the MCV genome (see Fig. 2 and references 13 and 15). The 1.1-kbp fragment reacted with $cDNA_{env/src}$ (Fig. 3, lane 3), which detects the boundary between *env* and *src* in the RSV genome and therefore locates the 3′ margin

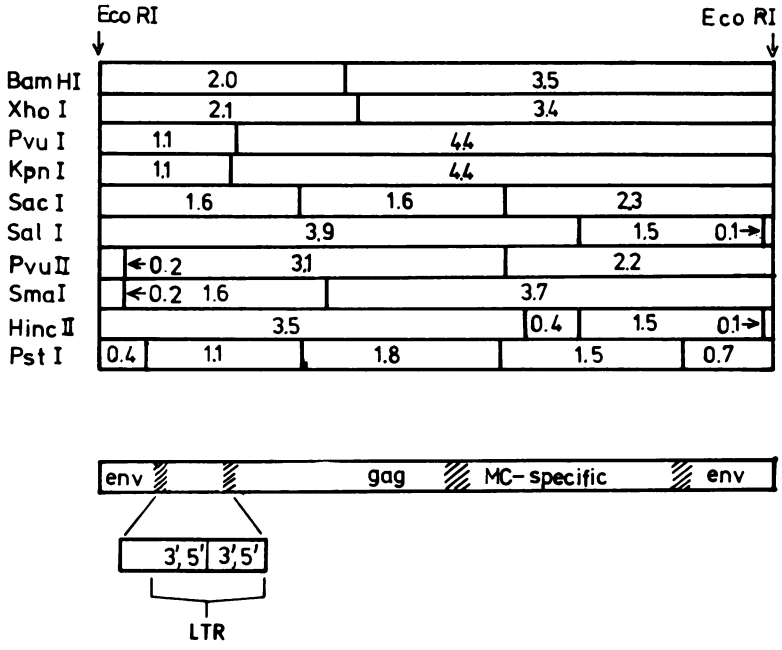

FIG. 2. *Sites of cleavage for restriction endonucleases in λMCV38 DNA. The closed circular DNA of MCV was prepared from infected cells as described in Fig. 1 and then cloned into the EcoRI site of the phage vector λgtWES·λB. One chimeric isolate was selected for further analysis and designated λMCV38. The 5.5-kbp insert of MCV DNA was excised from the chimeric DNA with EcoRI and separated from the vector DNA by electrophoresis in agarose gels. The purified viral DNA was mapped with restriction endonucleases according to conventional procedures. Details are available from Björn Vennström on request. The DNA of λMCV38 was aligned with the genome and provirus of MCV as described in the text. Ambiguities in the locations of junctions between separate genetic domains are indicated by diagonal shading. The block labeled 3',5'/3',5' denotes the approximate location of the LTRs, whose fusion generates the circular form of viral DNA (12, 18).*

of the *env* component in MCV (Fig. 2).

A single *Pst*I fragment (1.1 kbp) hybridized with $cDNA_{3'}$ (Fig. 3, lane 1). This finding provided an approximate location for the U3 domain of the LTR on the physical map of λMCV38. We substantiated this conclusion by using $cDNA_{3'}$ to identify the U3 sequences in nine separate isolates of cloned MCV DNA. Four of these (λMCV38 included) yielded two 1.6-kbp fragments when cleaved with *Sac*I (Fig. 2); the DNA of the remaining five isolates has one 1.6-kbp *Sac*I fragment and one 1.3-kbp fragment; the latter replaces the leftward 1.6-kbp *Sac*I fragment illustrated in Fig. 2. We found that $cDNA_{3'}$ hybridized with either the 1.3-kbp fragment or the leftward 1.6-kbp fragment (i.e., the fragment that overlaps the 1.1-kbp *Pst*I fragment); representative data are shown in Fig. 4. Since the LTR of RSV (12, 18) and DLV (24) is approximately 0.3 kbp in size, we conclude that λMCV38 and its three similar isolates probably contain two copies of the LTR, whereas the other five isolates studied contain only one copy.

The preceding data and previous descriptions of the MCV genome (13, 15) facilitated an approximate alignment of the physical map of λMCV38 with the genome and provirus of MCV (Fig. 2). The location of MCV-specific nucleotide sequences (i.e., the putative oncogenic domain) on MCV38 DNA was deduced from previous descriptions of the MCV genome and substantiated as follows. We predicted that the 1.5-kbp *Pst*I fragment would derive entirely from the MCV-specific region of the genome and would represent the bulk of that region. We therefore subcloned the fragment into pBR322, used the subcloned DNA to prepare radioactive cDNA, and tested the specificity of the cDNA for MCV nucleic acids. When hybridized with the filter illustrated by Fig. 1, the cDNA reacted with MCV but not with helper virus DNAs (data not illustrated). Similarly, hybridizations with viral RNAs after fractionation by electrophoresis in agarose gels revealed specific reactions with MCV RNA; representative data are illustrated below (see Fig. 5). We occasionally detected very weak reactions with other RNAs containing the *env* gene and therefore suspect that the 1.5-kbp *Pst*I fragment may overlap slightly into the *env* domain of the MCV genome (see Fig. 2). By

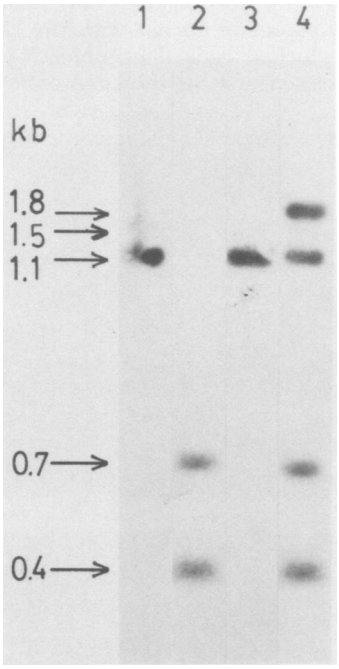

FIG. 3. *Mapping genetic domains on the DNA of λMCV38. The MCV DNA of λMCV38 was purified after excision with EcoRI, as described for Fig. 2, and then cleaved with PstI. The resulting fragments were fractionated by electrophoresis in a gel of 0.8% agarose and identified by molecular hybridization, as described for Fig. 1. Lane 1, $cDNA_{3'}$; lane 2, $CDNA_{env}$; lane 3, $cDNA_{env/src}$; lane 4, $cDNA_{rep}$.*

contrast, we have never observed hybridization between the 1.5-kbp *Pst*I fragment and restriction fragments containing the 3′ domain of *gag*, a finding that is in accord with the map illustrated by Fig. 2.

Biological activity of λMCV38 DNA. We sought to demonstrate the genetic integrity of λMCV38 DNA by using transfection to recover virus with the biological activities of MCV. This strategy appears to require the simultaneous transfection of single permissive chicken cells with cloned DNA of both MCV and a helper virus (24), a requirement that we met by physically linking the two viral DNAs prior to transfection (24).

The DNA of λMCV38 and cloned DNA of RAV-1 were joined by a "neutral" linker of lambda phage DNA (3.8-kbp fragment) as described previously for cloned DNA of avian erythroblastosis virus (24). The linked DNAs were then introduced into chicken cells by the use of calcium phosphate and dimethyl sulfoxide (9, 22). The recipient cells were passaged until infectious virus appeared in the harvest media and then were used to prepare viral RNAs for analysis by electrophoresis in agarose gels under denaturing conditions. We found two species of virus-specific RNA in virions released by the transfected cells: 8.0 and 5.5 kb (Fig. 5, lanes 2 and 4). Both species reacted with $cDNA_{rep}$ (Fig. 5, lanes 3 and 4), whereas only the 5.5-kb species hybridized with $cDNA_{MCV}$ prepared from the 1.5-kbp *Pst*I fragment (Fig. 5, lane 2). The 8.0-kb species migrated with the genome of MCAV (Fig. 5, lanes 3 and 4), and the 5.5-kb species migrated with the genome of MCV (Fig. 5, lanes 1 and 2).

The virus recovered by transfection transformed chick fibroblasts to a characteristic morphology (Fig. 6). The same virus, when introduced into either macrophage or bone marrow cultures, elicited clones in soft agar in a manner characteristic of MCV (not illustrated). The relative titers measured on fibroblasts (2.7×10^4)

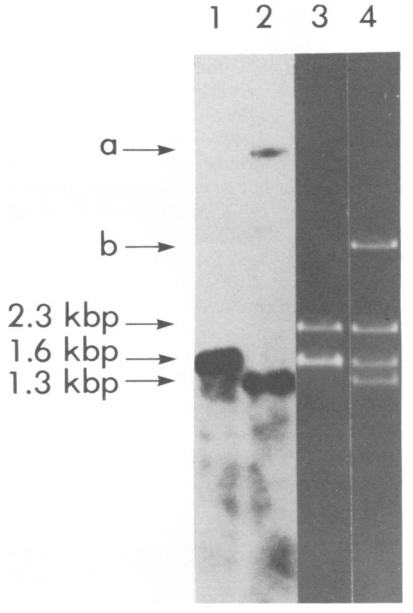

FIG. 4. *Mapping the U3 domain on cloned DNAs of MCV. Two molecular clones of MCV DNA, designated λMCV37 and λMCV38, were employed. The MCV DNA of both clones was isolated after excision with EcoRI, as described for Fig. 2, and then cleaved with SacI. The resulting fragments were fractionated by electrophoresis in a gel of 0.8% agarose, transferred to a nitrocellulose sheet, and hybridized with $cDNA_{3'}$. Lane 1, λMCV38; lane 2, λMCV37. Lanes 3 (λMCV38) and 4 (λMCV37) illustrate ethidium bromide staining of the gel prior to transfer of the DNA to nitrocellulose. The band labeled "a" in lane 1 is a minor product attributable to partial enzymatic digestion of the DNA. The band labeled "b" in lane 4 is an unidentified (but not virus-specific) additional insert in the clone of λMCV37.*

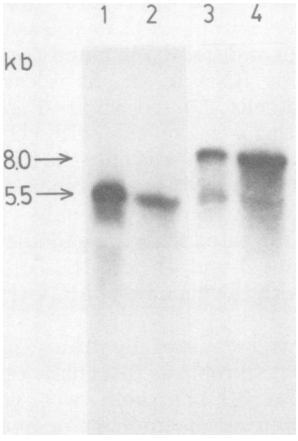

FIG. 5. *The genome of virus recovered by transfection with λMCV38 DNA. The MCV insert in λMCV38 was purified as described for Fig. 2 and then linked to cloned DNA of RAV-1 by means of a fragment from lambda phage (24). The ligated DNA was used to transfect chicken embryo fibroblasts, which were then passaged until infectious virus appeared in harvested medium (generally eight passages). Viral RNA was prepared from medium harvested at 2-h intervals, fractionated by electrophoresis in a gel of 1.2% agarose under denaturing conditions, transferred from the gel to diazobenzyloxymethyl paper, and located by hybridization with either $cDNA_{MCV}$ (lanes 1 and 2) or $cDNA_{rep}$ (lanes 3 and 4). Lanes 1 and 3 contained RNA prepared from our standard stock of MCV (MCAV); lanes 2 and 4 contained RNA from the virus recovered by transfection with λMCV38 DNA.*

and on bone marrow cultures (1.9×10^2) were also typical of MCV. We conclude that the DNA of λMCV38 can give rise to the biologically active genome of MCV, with at least two of the virus's characteristic transforming activities intact.

DISCUSSION

We have used molecular cloning in a procaryotic vector to isolate and amplify the genome of MCV. Our data indicate that the cloning procedure did not appreciably disturb the structure of the viral genome, as follows: (i) the length and topography of the cloned DNA conformed to previous descriptions of the MCV genome; (ii) radioactive cDNA prepared with a suitable restriction fragment that maps within the specific region (or oncogene) of the MCV genome reacted appropriately with the RNA and DNA of MCV; and (iii) transfection with the cloned DNA in conjunction with the DNA of a helper virus gave rise to infectious virus with a genome and transforming potentials characteristic of MCV.

We used a transfection assay developed in our laboratory expressly for use with the DNAs of DLVs in chicken cells. The objective of the protocol is to ensure that the DNA of both DLV

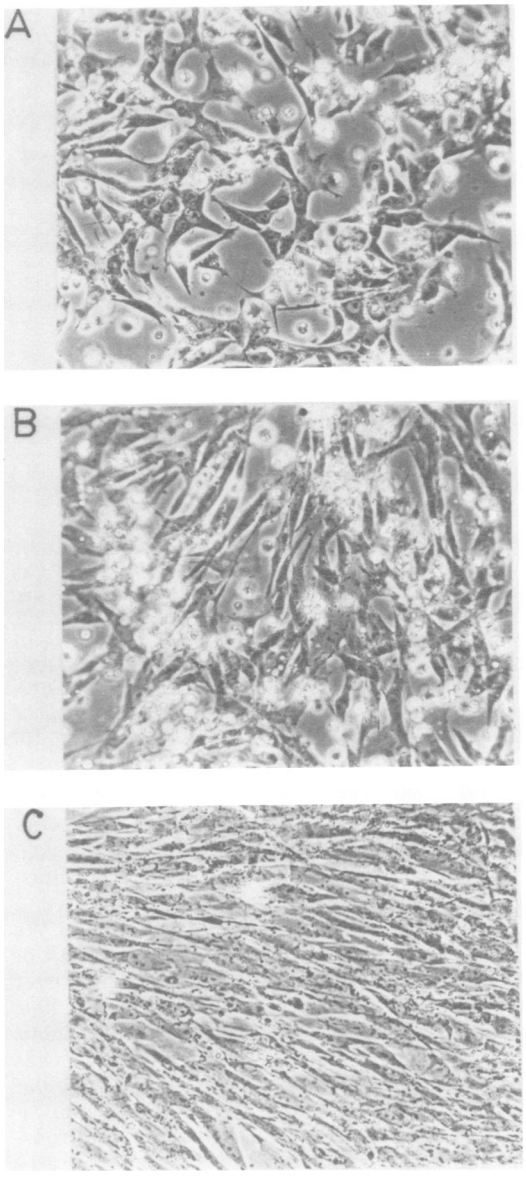

FIG. 6. *Morphological transformation of fibroblasts by virus recovered from λMCV38 DNA. Virus recovered by transfection with λMCV38 DNA (as described for Fig. 5) was used to infect secondary cultures of chicken embryo fibroblasts. Infected and uninfected cells were then passaged in parallel. Photographs were taken three passages after infection. (A) Cells infected with virus recovered from λMCV38 DNA. (B) Cells infected with our standard stock of MCV (MCAV). (C) Uninfected cells.*

and helper virus enter the same cell. In principle, this objective might be achieved simply by transfecting with large amounts of both viral DNAs. In practice, this approach has not succeeded for us, and we have therefore adopted the strategy of linking the DNAs prior to transfection. Even with this assist, the procedure (at least in its present form) proved quite inefficient; it permitted recovery of virus from cloned DNA, but was not amenable to quantitative assays.

The availability of cloned MCV DNA permits a satisfying confirmation of previous identifications of the viral genome: the biological activity of the DNA can be demonstrated by transfection, a test not possible with the RNA form of retrovirus genomes. In addition, the cloned DNA can be propagated in large quantities without the encumbrance of a helper virus. Since the MCV DNA is stable (even to the point of retaining its full activity in transfection) during repeated propagations in procaryotic hosts (unpublished data), it will be possible to employ the cloned DNA in a variety of biochemical and genetic analyses directed at elucidating the mechanism of oncogenesis by MCV.

ACKNOWLEDGMENTS

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