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Molecular cloning of avian myelocytomatosis virus (MC29) transforming sequences

(recombinant DNA/acute leukemia virus/bacteriophage λ/R looping)

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ABSTRACT Avian myelocytomatosis virus (MC29), a defective acute leukemia virus, has a broad oncogenic spectrum *in vivo* and transforms fibroblasts and hematopoietic target cells *in vitro*. We have used recombinant DNA technology to isolate and to characterize the sequences that are essential in the transformation process. Integrated MC29 proviral DNA was isolated from a library of recombinant phage containing DNA from the MC29-transformed nonproducer quail cell line Q5. The cloned DNA was analyzed by Southern blotting of restriction endonuclease digests and by electron microscopic visualization of R loops formed between the cloned DNA and MC29 or helper virus RNA. It was found that the 9.2-kilobase cloned DNA insert contains approximately 4 kilobases of viral sequences and 5.2 kilobases of quail cellular sequences. The viral sequences contain all of the MC29-specific sequences and 5′ helper-related sequences as well as part of the envelope region. The size of the cloned *Eco*RI fragment is the same as that of the major band in *Eco*RI-cleaved Q5 DNA that hybridizes to viral sequences. Transfection of the cloned DNA into NIH 3T3 cells revealed that the MC29-specific sequences are functional in that they induce foci of transformed cells with high efficiency.

Myelocytomatosis virus (MC29) is an avian acute leukemia virus that induces a broad spectrum of malignant diseases including myelocytomas, carcinomas, and sarcomas (1). The virus was isolated from a hen with spontaneous myelocytomatosis (2). The virus is defective in that it lacks functional *gag*, *pol*, or *env* genes and thus requires a helper virus in order to replicate. Nonproducer quail cell lines transformed by MC29 contain a 110,000-dalton protein with viral antigenic determinants (3). This protein is a fusion product of the *gag* genes and MC29-specific sequences. The presence of this protein in these transformed cells indicates that the viral genome is stably maintained in these cells. The size of MC29 genome is 5.7 kilobases (kb), based on oligonucleotide mapping (4–6) and heteroduplex analysis (7). By utilizing these techniques, specific sequences have been identified in the center of the genome that are unique to MC29 and have been implicated in neoplastic transformation.

In this paper, we describe the molecular cloning of a restriction fragment containing most of the integrated proviral genome including all of the MC29-specific sequences. The cloned DNA is functional in transformation assays, which allows us to use it as a tool to study the genetic control of transformation induced by this acute leukemia virus.

MATERIALS AND METHODS

Cells and Viruses. Cell line Q5 (3), an MC29-transformed nonproducer, was obtained from Peter Vogt. From supernatants of Q5 cells infected with Rous associated virus strain 60 (RAV60) (8) we obtained MC29(RAV60) viral pellets.

Nucleic Acids. High molecular weight DNA was prepared from Q5 cells by the method of Hughes *et al.* (9). [^{32}P]cDNA probes of avian myeloblastosis virus(myeloblastosis-associated virus) [AMV(MAV)] and MC29(RAV60) were prepared as described (10) by using oligo(dT) to prime reverse transcription of the 35S RNA template.

Isolation of Cloned Integrated Proviral DNA Sequences. Sucrose gradient-purified restriction fragments from a complete *Eco*RI digestion of Q5 DNA, ranging in size from 5 to 15 kb, were ligated to λgtWES·λB arms (11). After banding in CsCl, phage particles produced from the ligation reaction by *in vitro* packaging (12) were adsorbed to *Escherichia coli* LE392 (13) as described by Blattner *et al.* (14) and spread on 150-cm agar plates at a density of 5000 plaques per plate. Nitrocellulose filters were lifted from the plates (15) and positive plaques were identified by hybridization to an AMV(MAV) [^{32}P]cDNA probe.

Transfection. NIH 3T3 cells were transfected with cloned DNA as described by Lowy *et al.* (16).

Recombinant DNA. All recombinant DNA experiments were carried out under P1-EK1 conditions as outlined by the January 1980 revision of the National Institutes of Health recombinant DNA research guidelines.

RESULTS

Analysis of the Integrated Proviral Genome. A complete *Eco*RI digest of Q5 DNA was size fractionated on an agarose gel and blotted by the method of Southern (17) onto a nitrocellulose filter. When the filter was hybridized to AMV(MAV) cDNA, one major and two minor bands were seen (Fig. 1). The major band had a size of 9.0 kb; the sizes of the minor bands were 6.7 and 5.8 kb.

Cloning and Restriction Enzyme Mapping of Integrated Proviral DNA Sequences. A library of phage was prepared from a size-fractionated digest of Q5 DNA. Two independent plaque-purified phage that hybridized to AMV(MAV) cDNA probe were isolated from the library. Both of these phage contained a 9.2-kb *Eco*RI insert and yielded the same restriction pattern for each of seven enzymes tested. One of these phage, designated λMC29-1, was selected for further study. The restriction patterns for digests of λMC29-1 DNA by several restriction enzymes and for digests of this DNA with these enzymes in the

Abbreviations: ASV, avian sarcoma virus; AMV, avian myeloblastosis virus; MAV, myeloblastosis-associated virus; MC29, avian myelocytomatosis virus strain MC29; RAV60, Rous associated virus strain 60; kb, kilobase(s); bp, base pair(s); LTR, large terminal redundancy.

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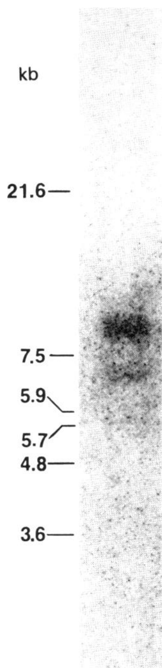

FIG. 1. Integrated MC29 proviral sequences in quail nonproducer cell line Q5 DNA. Q5 DNA (6 μg) was cleaved with *Eco*RI, electrophoresed on a 0.5% agarose gel, and transferred to a nitrocellulose filter by the method of Southern (17). Hybridization to the AMV(MAV) [^{32}P]cDNA probe was as described (9).

presence of *Eco*RI are shown in Fig. 2*A*. Southern blot analysis of these digests with MC29(RAV60) cDNA probes revealed the bands that were of viral origin (Fig. 2*B*). On the basis of these data, restriction maps were constructed (Fig. 3). Those fragments that hybridized to the probe are indicated as open bars. These results indicate that the quail cellular and the proviral sequences are attached to the left and right arms of the phage, respectively. The junction point between the quail and the proviral sequences lies within the 560-bp *Sst* I fragment.

The orientation of the proviral sequences within the cloned DNA insert was established by utilizing a probe representing sequences from the *gag* region of the genome. The restriction fragments that hybridized to this probe are indicated with solid bars in Fig. 3. Because fragments mapping near the quail–virus junction hybridized to this probe, this region represents the 5′ portion of the viral genome. This is also suggested by the presence of four restriction sites (*Pvu* I, *Sst* I, *Bam*HI, and *Xho* I) that are found in the 5′ region of other avian retroviruses (19, 20). Because the size of the proviral sequence in the cloned DNA is less than the reported size of the MC29 genome (4), the cloned fragment lacks a portion of the 3′ end of the sequence.

In a similar experiment, the 0.6-kb *Bam*HI/*Eco*RI fragment at the right end of the insert hybridized to an AMV(MAV) [^{32}P]cDNA probe. Because the AMV-specific sequences present in this probe do not hybridize to MC29-specific sequences (21), this indicates that the proviral end of the cloned insert DNA contains helper-related sequences. Because this fragment maps too far from the region that contains 5′ helper-related sequences, it must have *env*-related sequences. This is consistent with the occurrence of a *Sal* I site about 100 bp from the *Eco*RI site of the viral end of the insert. A *Sal* I site and an *Eco*RI site separated by the same distance have been mapped within the *env* gene of several nondefective avian retroviruses (19, 20).

R-Loop Analysis of Cloned Proviral DNA. MC29(RAV60) RNA was hybridized to the cloned proviral DNA in a formamide concentration high enough to favor RNA·DNA over DNA·DNA hybridization (22). The resulting hybrid molecules were then visualized by electron microscopy. Numerous forked structures were observed (Fig. 4), indicating that the portion of the cloned DNA that is homologous to proviral sequences extends from a point near the center of the insert to the *Eco*RI site at the right end of the fragment. The sizes of the cloned DNA insert and that of the proviral sequences are 9.1 and 4.0 kb, respectively. This is in direct agreement with Southern blot analysis of the restricted cloned DNA insert (Fig. 3). The absence of looped structures within the hybrid region is evidence for colinearity of cloned provirus sequences and viral RNA. We therefore conclude that there is no structural evidence for sequence rearrangement in the proviral sequences of the cloned DNA.

MC29 virus has been shown to contain 5′ helper-related sequences (5, 7). We therefore hybridized MAV RNA to the cloned proviral DNA under conditions favoring R-loop formation. The structures observed by electron microscopy consisted of molecules with a single internal loop (Fig. 5). The displaced sequences ranged from 0.22 to 0.43 fractional contour units from the nearest end. This indicates that the size of the hybrid region is about 1500 bp. The apparent virus–cell junction by using helper RNA (0.428 ± 0.017 fractional contour length) was at a position similar to that found by using MC29 RNA (0.429 ± 0.017 fractional contour length).

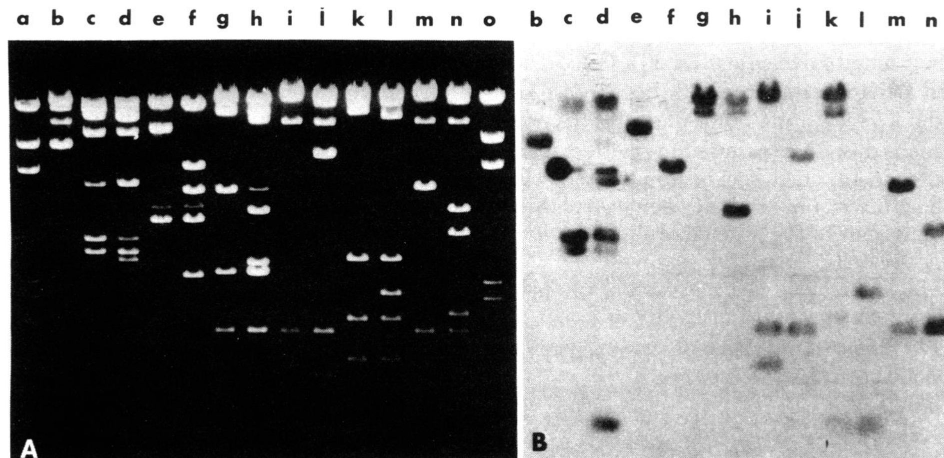

FIG. 2. Ethidium bromide stain patterns (*A*) and Southern blots (*B*) of restriction endonuclease-cleaved λMC29-1. Ethidium bromide stain patterns of *Hin*dIII-cleaved phage λ DNA are shown in lanes a and o. The enzymes used were: b, *Eco*RI; c, *Bam*HI; d, *Bam*HI/*Eco*RI; e, *Hin*dIII; f, *Hin*dIII/*Eco*RI; g, *Kpn* I; h, *Kpn* I/*Eco*RI; i, *Sal* I; j, *Sal* I/*Eco*RI; k, *Sst* I; l, *Sst* I/*Eco*RI; m, *Xho* I; n, *Xho* I/*Eco*RI. The hybridization probe consisted of MC29(RAV60) [^{32}P]cDNA.

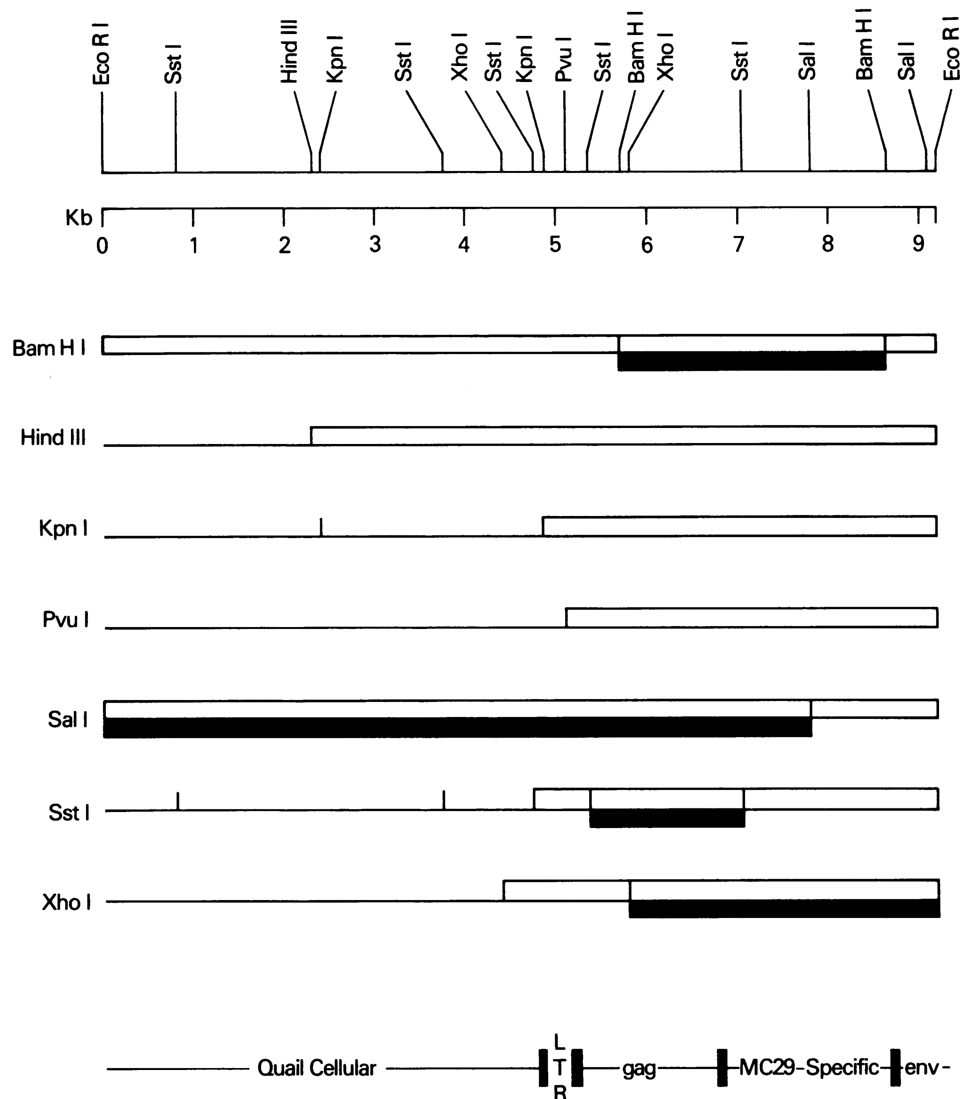

FIG. 3. Restriction endonuclease and biological function map of the 9.2-kb *Eco*RI insert in λMC29-1. The map is oriented so that the side of the insert that adjoins the right arm of λgtWES·λB is on the right. The open bars represent fragments that hybridized to the MC29(RAV60) probe as shown in Fig. 2 except that the *Pvu* I data were determined in a separate experiment. The solid bars represent fragments that hybridized to a ^{32}P-labeled probe containing 5′ helper-related sequences. This probe consisted of a nick-translated 1.5-kb *Bam*HI fragment from AMV proviral DNA clone 11A1-1 (18) located between 0.9 and 2.4 kb from the cell-virus junction of the 5′ large terminal redundancy (LTR).

Transfection of the Cloned DNA into NIH 3T3 Cells. The MC29 proviral cloned DNA was digested with *Eco*RI to separate the insert from the λ vector arms. After phenol extraction and ethanol precipitation, this DNA was used to transfect NIH 3T3 cells. Ten days after transfection, foci of transformed cells were observed with an efficiency of about 10^3 per μg of DNA, which was roughly equivalent to the efficiency observed with Harvey sarcoma virus clone H-1 DNA (24) that was used as a positive control. The MC29-induced foci consisted of compact accumulations of round cells that were distinctly different in appearance from the foci induced by the cloned Harvey sarcoma virus DNA (Fig. 6).

DISCUSSION

We have cloned the integrated transforming sequences of avian myelocytomatosis virus (MC29). The cloned fragment contains about 4 kb of the MC29 genome and 5 kb of quail cellular DNA. The viral portion of the cloned DNA includes two genomic segments characteristic of avian acute leukemia viruses. These are the 5′ helper-related region and the MC29-specific sequences. In addition, the cloned DNA contains part of the 3′ helper-related region. By analogy with the structure of other integrated proviruses (25), the cloned DNA should contain a large terminal redundancy (LTR) between the quail cellular DNA and the 5′ helper-related region.

On the basis of R-loop and restriction endonuclease digestion data, the *Sst* I site at 5350 bp in Fig. 3 is located at or near the junction between the 5′ helper-related sequences and the LTR. Comparison of the restriction maps of the integrated MC29 proviral DNA and avian sarcoma virus (ASV) DNA (19, 20) indicates homology in this region. Both sequences contain four restriction sites (*Pvu* I, *Sst* I, *Bam*HI, and *Xho* I) in the same order separated by the same number of base pairs. The *Pvu* I site of ASV occurs within the U_3 region of the LTR; the other sites in ASV occur at the 5′ end of the nonrepeated sequences homologous to the 5′ portion of the genome. Presumably these sites are in similar functional regions in the MC29 proviral DNA. The *Eco*RI site, present within the U_3 region in other

FIG. 4. Electron microscopic analysis of R-loop structures formed between *Eco*RI-cleaved λMC29-1 DNA and MC29(RAV60) RNA. *Eco*RI-cleaved λMC29-1 DNA and MC29(RAV60) viral RNA were incubated under R-loop-forming conditions, mounted for electron microscopy, and visualized as described (23). 42.9 represents percent contour length.

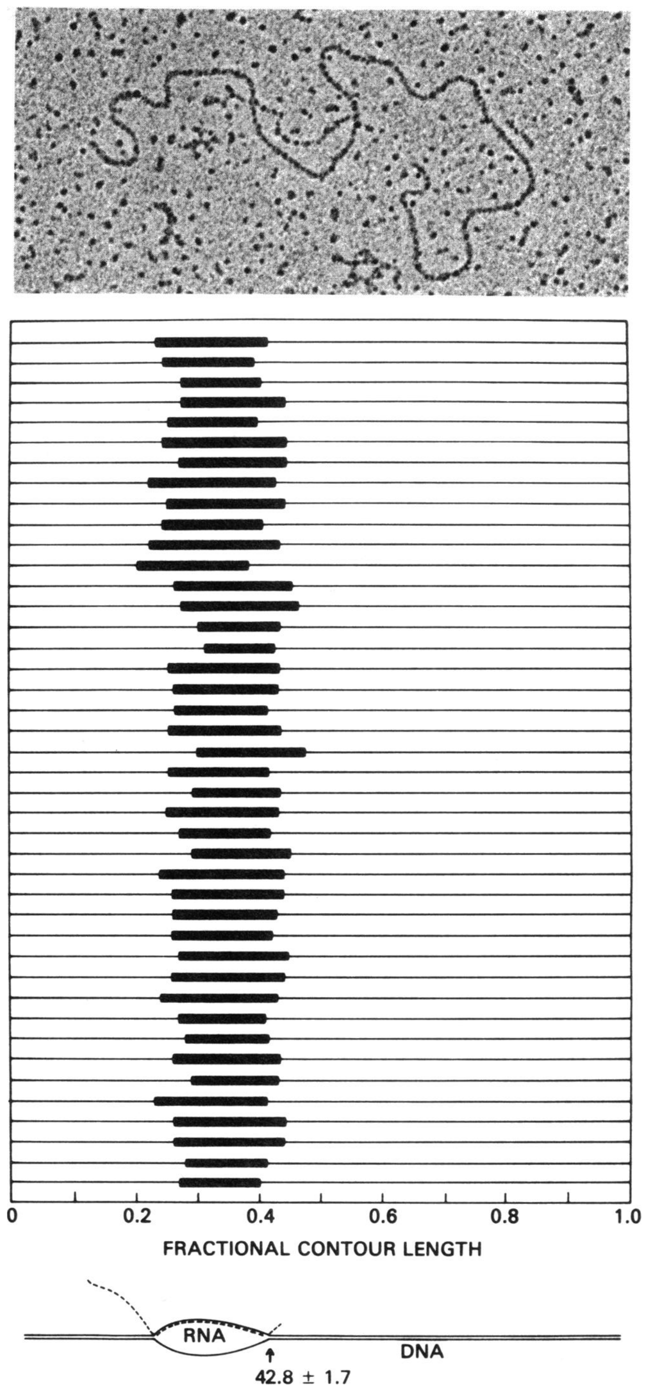

FIG. 5. Electron microscopic analysis of R-loop structures formed between *Eco*RI-cleaved λMC29-1 DNA and MAV RNA. R loops were analyzed as in Fig. 4. 42.8 represents percent contour length.

exogenous avian retroviruses, is absent from MC29. This is in agreement with oligonucleotide pattern data that show distinct differences in the U_3 region between MC29 and other exogenous avian retroviruses (26).

Oligonucleotide analysis of MC29 RNA (4–6) has revealed that the genome consists of helper-related regions at both the 5′ and 3′ ends separated by an MC29-specific domain. Our R-loop data (Fig. 5) and Southern blot analysis using a probe specific for 5′ helper-related sequences indicate that the 5′ helper-related sequences occur between 2.4 and 3.9 kb from the viral end of the cloned DNA insert. Because the 3′ end of the proviral insert hybridizes to helper cDNA probe, the insert also contains some 3′ helper-related sequences. It is therefore concluded that our cloned DNA contains all of the MC29-specific sequences.

Copeland and Cooper (27) found that the DNA of MC29-infected chicken cells can transform NIH 3T3 cells and that the DNA of these transformed cells can in turn transform NIH 3T3 cells. Therefore, the chromosomally integrated form of the MC29 transforming gene can be introduced into the cell in an active state. The ability of the cloned MC29 proviral DNA to induce foci in NIH 3T3 cells by transfection indicates that it

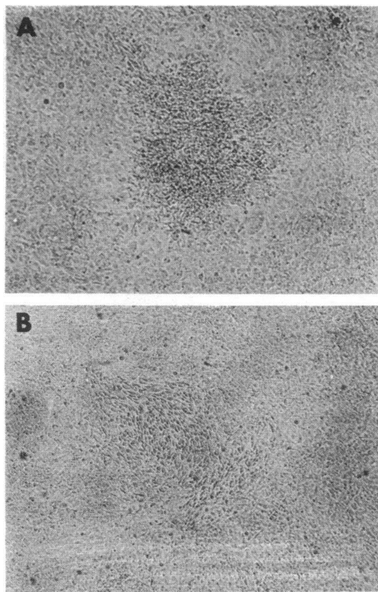

FIG. 6. Foci of transformed NIH 3T3 cells induced by the DNAs of λMC29-1 (*A*) or Harvey sarcoma virus clone H-1 (*B*).

carries a functional transforming gene. In addition, this indicates that, if activation of the transforming gene by a promoter in the U_3 region of the LTR is necessary for transformation, only one LTR is sufficient in order to allow the expression of the transforming function. The ability of this cloned DNA to transform cells in culture offers a valuable tool for analysis of the genetic control of transformation by MC29.

MC29 is a virus that has the ability to transform a wide variety of target cells both in tissue culture and in the intact animal (1). The specific sequences of this virus which probably represent the MC29 transforming gene are highly conserved throughout the evolutionary scale in a way similar to the transforming genes of the other retroviruses (28, 29). It has been suggested that the induction of malignancy by nontransforming leukosis viruses is due to integration of the virus next to a potentially transforming cellular gene which can now be transcribed efficiently from the introduced viral promoter (26, 30). The MC29 transforming gene conceivably can be one of the targets in this process. This has been shown recently to be the case in B-cell lymphoid malignancies induced in chickens by avian leukosis viruses (W. Hayward, B. Meel, and S. Astrin, personal communication). The startling observation that the majority of the leukosis virus-induced B-cell lymphomas express the MC29 transforming gene raises several important questions about the biology of this gene. If MC29 has a wide spectrum of oncogenic potential, why would the exogenous activation of the endogenous gene lead only to development of B-cell lymphoid malignancies? The answers to these questions are not clear from the existing information.

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