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The Transforming Sequences of Avian Myelocytomatosis Virus (MC29)

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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 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 kb cloned DNA insert contains approximately 4 kb of viral sequences and 5.2 kb 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.

Key words: recombinant DNA, acute leukemia virus, bacteriophage λ, R-looping

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

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, kilobases; bp, base pairs; LTR, large terminal redundancy; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.

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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 M29 genome is 5.7 kilobases, based on oligonucleotide mapping [4–6] and heteroduplex analysis [7]. Utilizing these techniques, specific sequences have been identified in the center of the genome that are unique to MC29, and which have been implicated in neoplastic transformation.

The life cycle of avian retroviruses is best understood for the nondefective viruses such as ASV [8]. Following infection, the RNA genome is copied into circular and linear double-stranded DNA forms. One or both of these forms may then integrate into the cellular genome and can then be transcribed by the host into viral messages and new viral genomes. An important property of this integrated form is the presence of large terminal redundancies (LTR) on the 5′ and 3′ flanks of the proviral genome. Analysis of the intracellular location of the defective virus is normally complicated by the presence of excessive amounts of helper virus. To avoid this complication, we have used quail nonproducer cells transformed by MC29. The MC29 proviral DNA in these cells is an excellent cloning target since it lacks exogenous helper and endogenous viral sequences. 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 ask questions regarding 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. MC29 (RAV60) viral pellets were obtained from supernatants of RAV60 [9] infected Q5 cells. AMV (MAV) viral pellets from the plasma of leukemic chickens and AMV reverse transcriptase were obtained through the Virus Cancer Program of the National Cancer Institute.

Nucleic Acids

High molecular weight DNA was prepared from Q5 cells by the method of Hughes et al [10]. Sucrose gradient purified *Eco*RI generated arms from λgtWES•λB [11] were a gift from A. Srinivasan. λcI857S7 DNA used for size markers was purchased from Bethesda Research Laboratories. DNA from λ phage containing viral sequences was prepared by phenol extraction of phage that had been purified by two CsCl bandings. Restriction fragments used to prepare nick-translated probes were electroeluted from agarose gels and concentrated by ethanol precipitation. Viral RNAs from AMV and MC29 (RAV60) pellets were extracted as previously described [12]. The 60–70S RNA fraction was purified on sucrose gradients and stored in 70% ethanol until further use.

Enzyme

Escherichia coli (E coli) DNA polymerase I and bovine pancreatic DNase I used for nick translation were obtained from New England Nuclear, and were used according to the manufacturer's instructions. DNA ligase from T4-infected E coli was from Bethesda Research Laboratories, and restriction endonuclease *Eco*RI was supplied by Boehringer-Mannheim. Other restriction enzymes were either from Bethesda Research Laboratories or New England Biolabs. *Eco*RI reactions were carried out in 100 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, whereas all other restriction enzyme reactions were carried out in 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol.

Radioisotopes

(^{32}P)dGTP and (^{32}P)dCTP were purchased from either Amersham or New England Nuclear. (^{32}P)cDNA probes of AMV(MAV) and MC29(RAV60) were prepared as previously described [12] using oligo-dT to prime reverse transcription of the 35S RNA template.

Isolation of Cloned Integrated Proviral DNA Sequences

After being heated 20 min at 65° to inactivate restriction enzyme, a complete *Eco*RI digest of Q5 DNA was layered on sucrose gradient (10–40%) and sedimented for 18.5 hr at 25,000 rpm in a SW27 rotor in a Beckman L5-75 ultracentrifuge at 20°. Fractions were collected and aliquots were electrophoresed on an agarose slab gel. The fractions ranging from 5 to 15 kilobases were pooled, phenol-extracted, and ethanol-precipitated. DNA was resuspended in 10 mM Tris-Hcl, pH 8.0, 1 mM EDTA, and ligated to λgtWES•λ in a reaction mixture of 0.4 ml containing the following: 5 μg size-fractionated Q5 DNA, 40 μg λgtWES•λB, 80 units of T4 DNA ligase, 66 mM Tris-HCl, pH 7.4, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, and 0.4 mM ATP. The reaction was incubated at 4° for 18 hours. Phage particles produced from the ligation reaction by in vitro packaging [13] were banded in CsCl and dialyzed. These phage were then preabsorbed to E coli LE392 [14] as described by Blattner et al [15] and spread on 150 cm NZYDT agar plates at a density of 5,000 plaques/plate. Nitrocellulose filters were lifted from the plates by the method of Benton and Davis [16], incubated for 1 hr at 65° in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 1× Denhardt's solution (0.02% bovine serum albumin, 0.02% polyvinyl pyrrolidone, 0.02% ficoll), 0.5% SDS, and 25 μg/ml single-stranded calf thymus DNA. (^{32}P) cDNA AMV(MAV) probe was added to the reaction, and incubation was continued under the same conditions for 48 hrs. The filters were washed three times for 1 hr each at 65° in 3× SSC, 1× Denhardt's, 0.2% SDS, and 1 mM EDTA. They were then dried and autoradiographed with Kodak XR-2 film using intensifying screens. A screening of 120,000 plaques yielded two positive plaques. These were replated and rescreened until a stock from a single plaque yielded over 90% positive plaques.

Transfection

NIH 3T3 cells were transfected with cloned DNA as described by Lowy et al [17]. The cloned DNA was mixed with sheared calf thymus DNA in HEPES buffered saline, pH 6.9 and then precipitated with $CaCl_2$. The precipitate was

layered over a 75% confluent monolayer of NIH 3T3 cells. After a 4-hr incubation, the cells were treated with 15% dimethylsulfoxide for 2 min. The cells were then washed with culture media and passaged the next day into 60-mm petri dishes. Foci were scored 10 days after transfection. Harvey sarcoma virus proviral DNA clone H-1 [18] was used as a positive control.

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

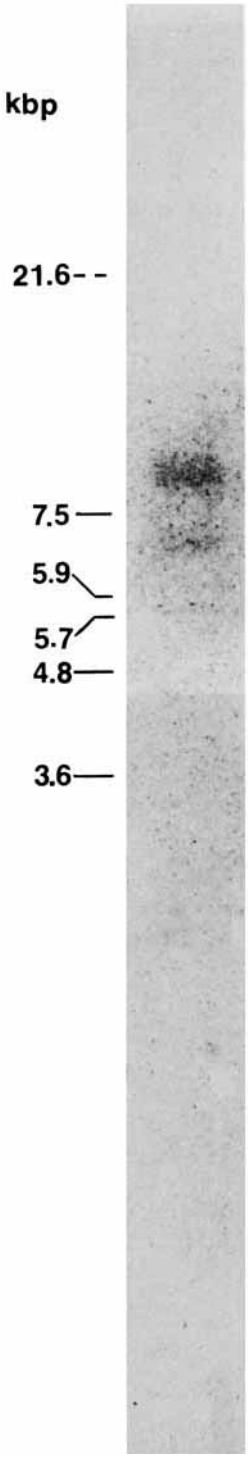
Analysis of the Integrated Proviral Genome

A complete *Eco*RI digest of Q5 DNA was size fractionated on an agarose gel and blotted by the method of Southern [19] 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, and the sizes of the minor bands were 6.7 kb and 5.8 kb. Since most of the sequences in Q5 DNA that hybridized to the viral cDNA probe were contained in a single *Eco*RI fragment, this fragment was selected as a cloning target. The use of a complete digest allowed enrichment of the target sequences by size fractionation.

Cloning and Restriction Enzyme Mapping of Integrated Proviral DNA Sequences

A library of phage was prepared from a size-fractionated digest of Q5 DNA as described in Materials and Methods. 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 λ MC29-1, was selected for further study. The restriction patterns for digests of λ MC29-1 DNA by several restriction enzymes and for digests of this DNA with these enzymes in the presence of *Eco*RI are shown in Figure 2A. Southern blot analysis of these digests with MC29(RAV60) cDNA probes revealed the bands that are of viral origin (Fig. 2B). On the basis of these data, restriction maps were constructed (Fig. 3). Those fragments that hybridized to the probe are indicated as open bars. It is apparent from these results 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.

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 [19]. Hybridization to the AMV(MAV) [^{32}P]cDNA probe was as described [10]. The *Eco*RI digest of phage lambda DNA was used for markers. The sizes indicated are those of Daniels et al [20].


kbp
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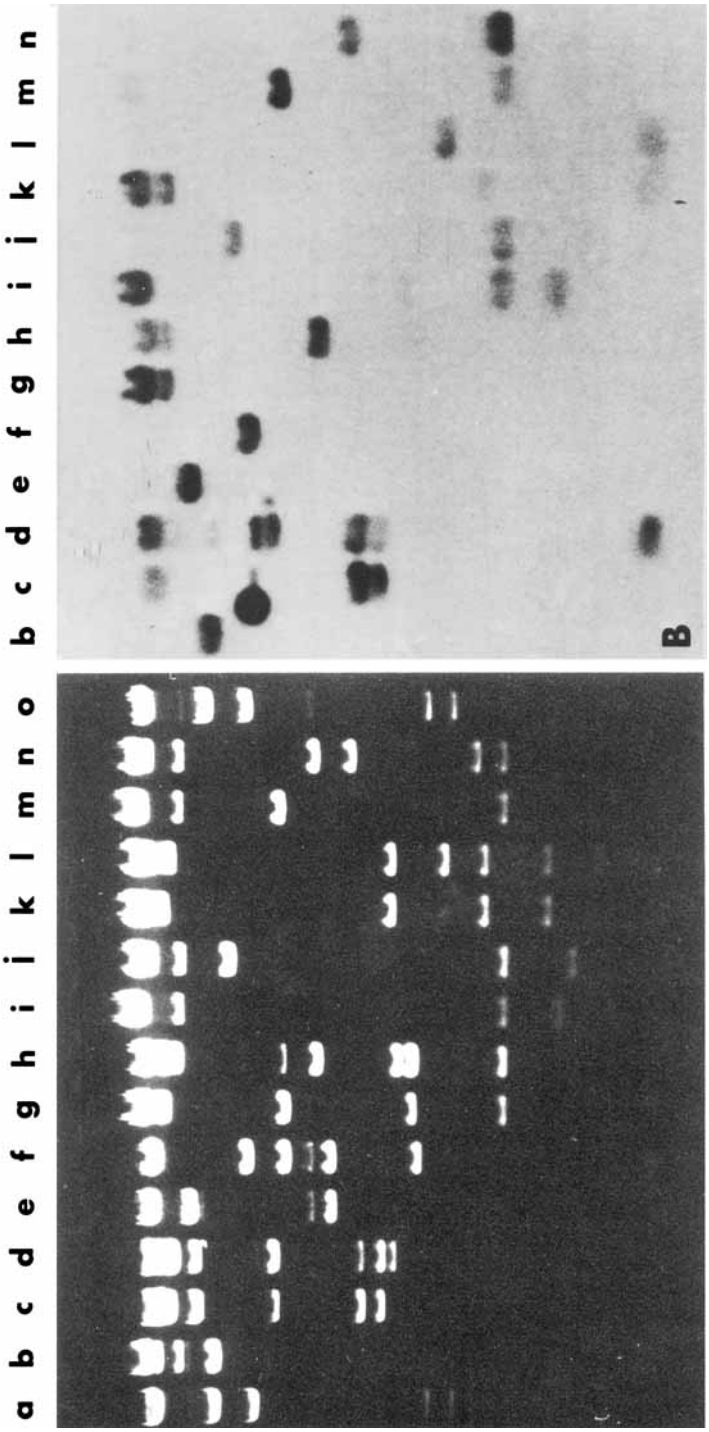

Fig. 2. Ethidium bromide stain patterns (A) and Southern blots (B) of restriction endonuclease-cleaved λMC29-1. Ethidium bromide stain patterns of *Hind*III-cleaved phage λ DNA are shown in lanes a and o. The enzymes used were: b, *Eco*RI; c, *Bam*HI; d, *Bam*HI-*Eco*RI; e, *Hind*III; f, *Hind*III-*Eco*RI; 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. Hybridization to the MC29(RAV-60) (^{32}P)cDNA probe was as described in Materials and Methods.

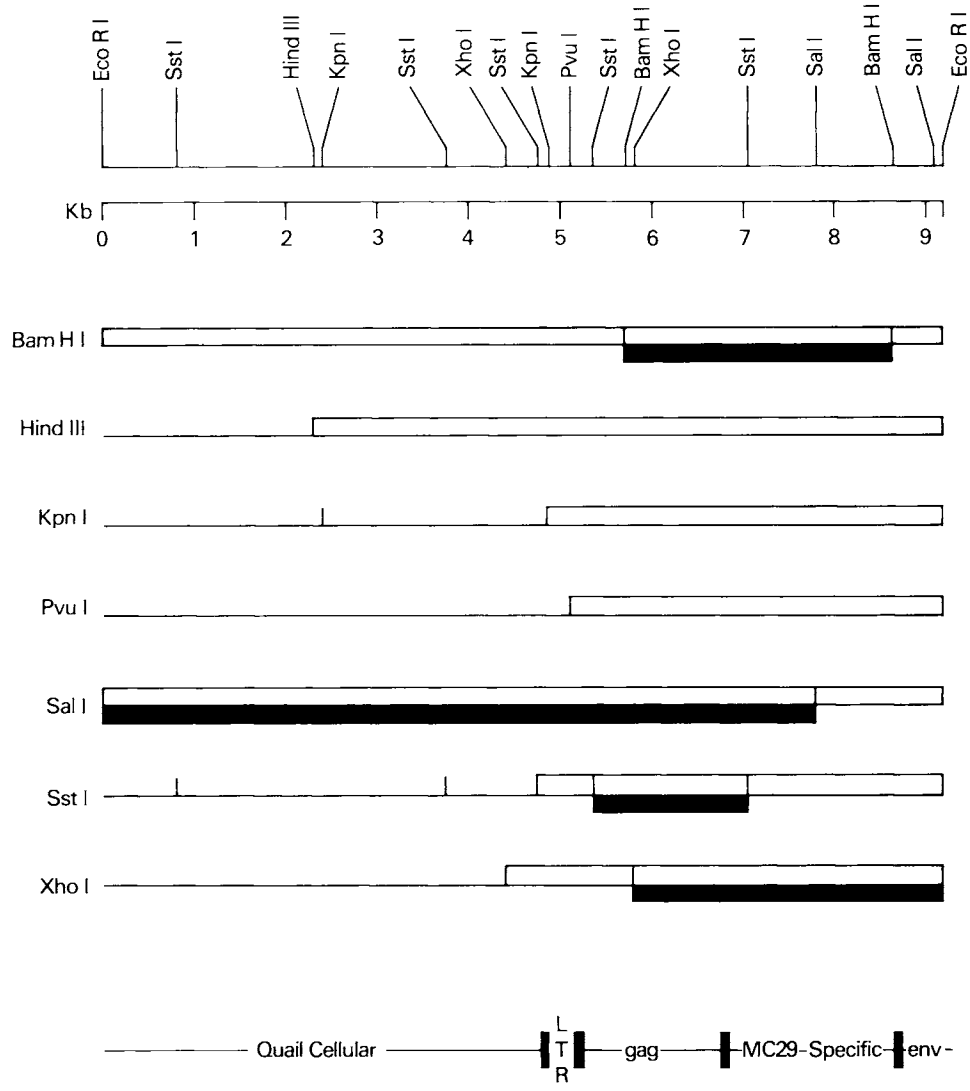

Fig. 3. Restriction endonuclease and biological function map of the 9.2 kb *Eco*RI inset in λMC29-1. The map is oriented so that the side of the insert that adjoins the right arm of λgtWES•λB is on the right. The open bars represent fragments that hybridized to the MC29(RAV-60) probe as shown in Figure 2 except to the *Pvu*I data, which were determined in a separate experiment. The solid bars represent fragments that hybridized to a ^{32}P-labeled probe containing 5′ helper-related sequences as shown in Figure 4.

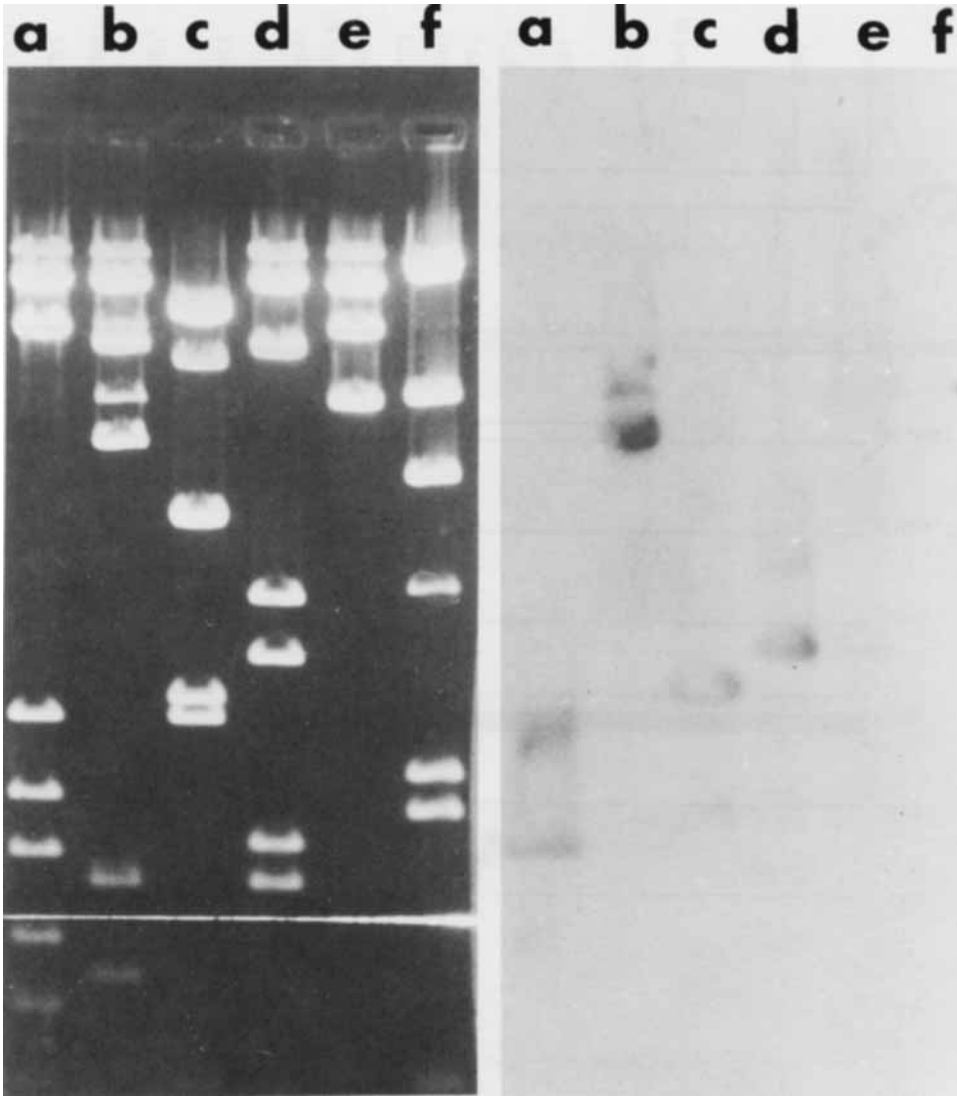

Fig. 4. Hybridization of restriction fragments of λMC29-1 DNA to a *gag* probe. The ethidium bromide stain patterns are shown in the left panel and the Southern blot is shown in the right panel. The enzymes used were: a, *Sst*I-*Eco*RI; b, *Sal*I-*Eco*RI; c, *Bam*HI-*Eco*RI; d, *Xho*I-*Eco*RI; and e, *Eco*RI. A *Hind*III digest of wild type λ DNA was run in lane f. The hybridization probe consisted of a nick-translated 1.5 kb *Bam*HI fragment from AMV proviral DNA clone λ11A1-1 [21] located between 0.9 and 2.4 kb form the cell-virus junction of the 5′ LTR.

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 (Fig. 4.). The restriction fragments that hybridized to this probe are indicated with solid bars in Figure 3. Since 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 [22, 23]. Since 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. Since the AMV-specific sequences present in this probe do not hybridize to MC29-specific sequences [24], this indicates that the proviral end of the cloned insert DNA contains helper related sequences. Since 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 *Eco*RI site separated by the same distance have been mapped within the *env* gene of several nondefective avian retroviruses [22, 23].

R-Loop Analysis of Cloned Proviral DNA

MC29 (RAV60) RNA was annealed to the cloned proviral DNA in a formamide concentration high enough to favor RNA-DNA over DNA-DNA hybridization [25]. The resulting hybrid molecules were then visualized by electron microscopy. Numerous forked structures were observed (Fig. 5), 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 size of the cloned DNA insert and that of the proviral sequences are 9.1 and 4.0 kilobases, 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 [4, 7]. We therefore annealed 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. 6). The displaced sequences ranged from 0.22 to 0.43 fractional contour units from the nearest end. This indicates the size of the hybrid region is about 1,500 bp. The apparent virus cell junction using helper RNA (0.429 ± 0.017 fraction contour length) was at a position similar to that found using MC29 RNA (0.429 ± 0.017 fractional contour length).

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 lambda vector arms. This mixture was phenol-extracted, precipitated with ethanol, and then resuspended in 10 mm Tris-HCl, pH 7.5. This DNA was then used to transfect NIH 3T3 cells. Ten days after transfection, foci of

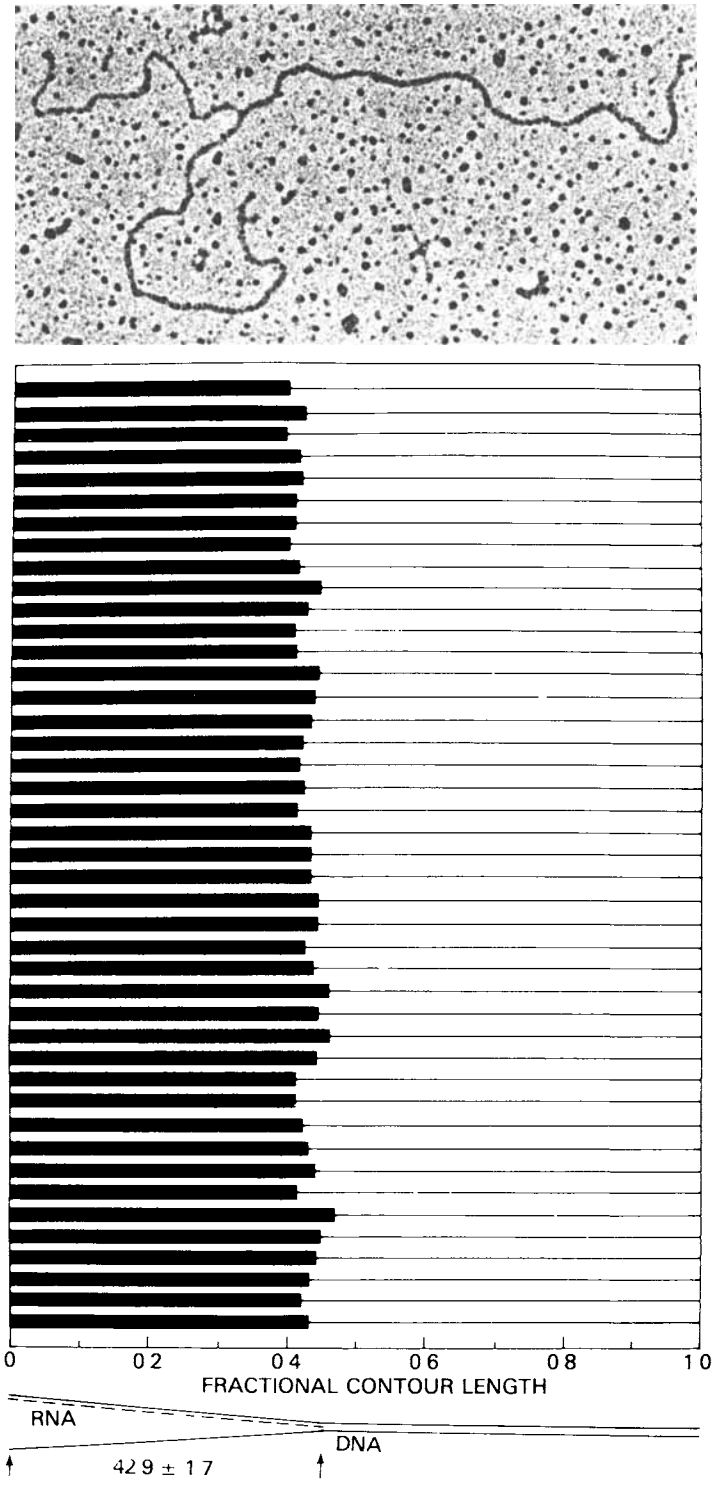

Fig 5 Electron microscopic analysis of R-loop structures formed between *Eco*RI-cleaved λMC29-1 DNA and MC29(RAV60) RNA *Eco*RI-cleaved λMC29-1 (8 μg/ml) DNA and MC29(RAV60) viral RNA (300 μg/ml) were incubated at 42° for 20 hr in a solution containing 70% formamide, 0 1 M Tricine buffer pH 8 0, 0 25 M NaCl, and 0 01 M EDTA Samples were mounted for electron microscopy and visualized as previously described [26]

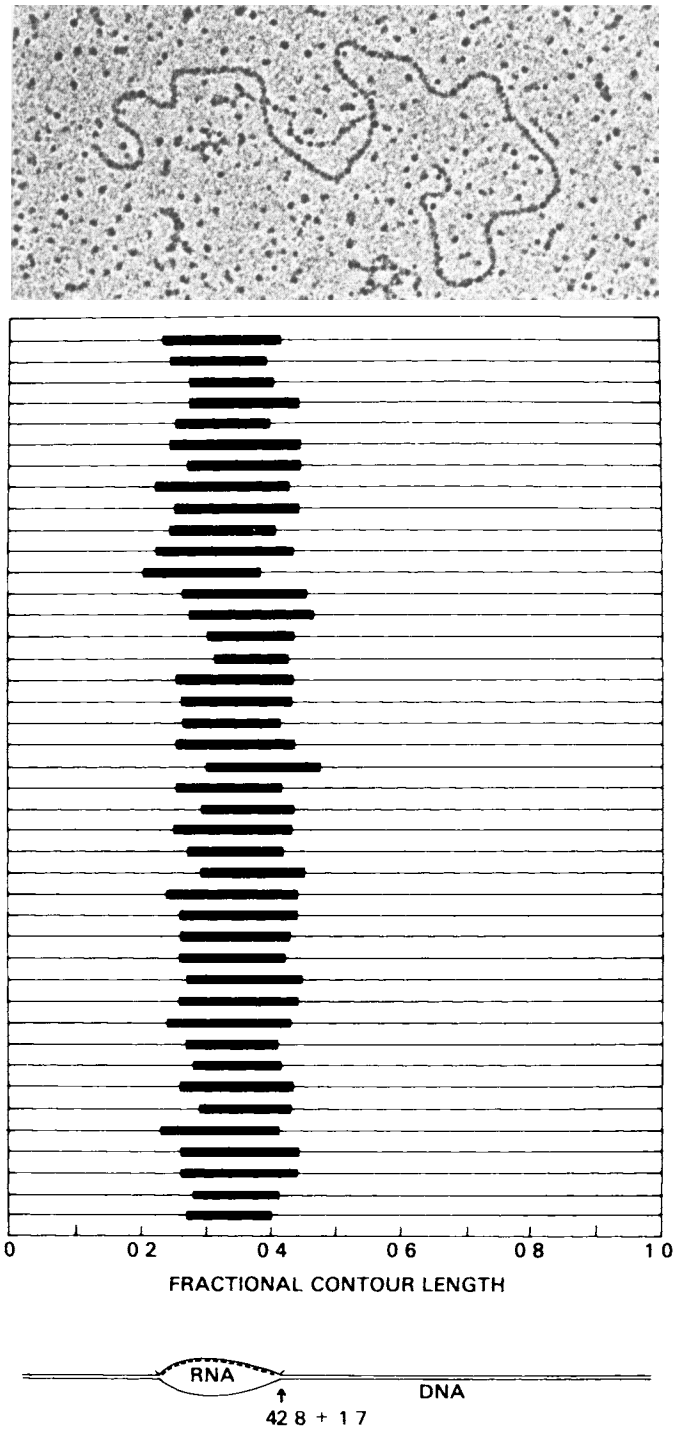

Fig 6 Electron microscopic analysis of R loop structures formed between *Eco*RI cleaved λMC29 1 DNA and MAV RNA *Eco*RI cleaved λMC29 1 DNA and MAV RNA were incubated under R loop forming conditions, mounted for electron microscopy, and visualized as described in the legend to Figure 5

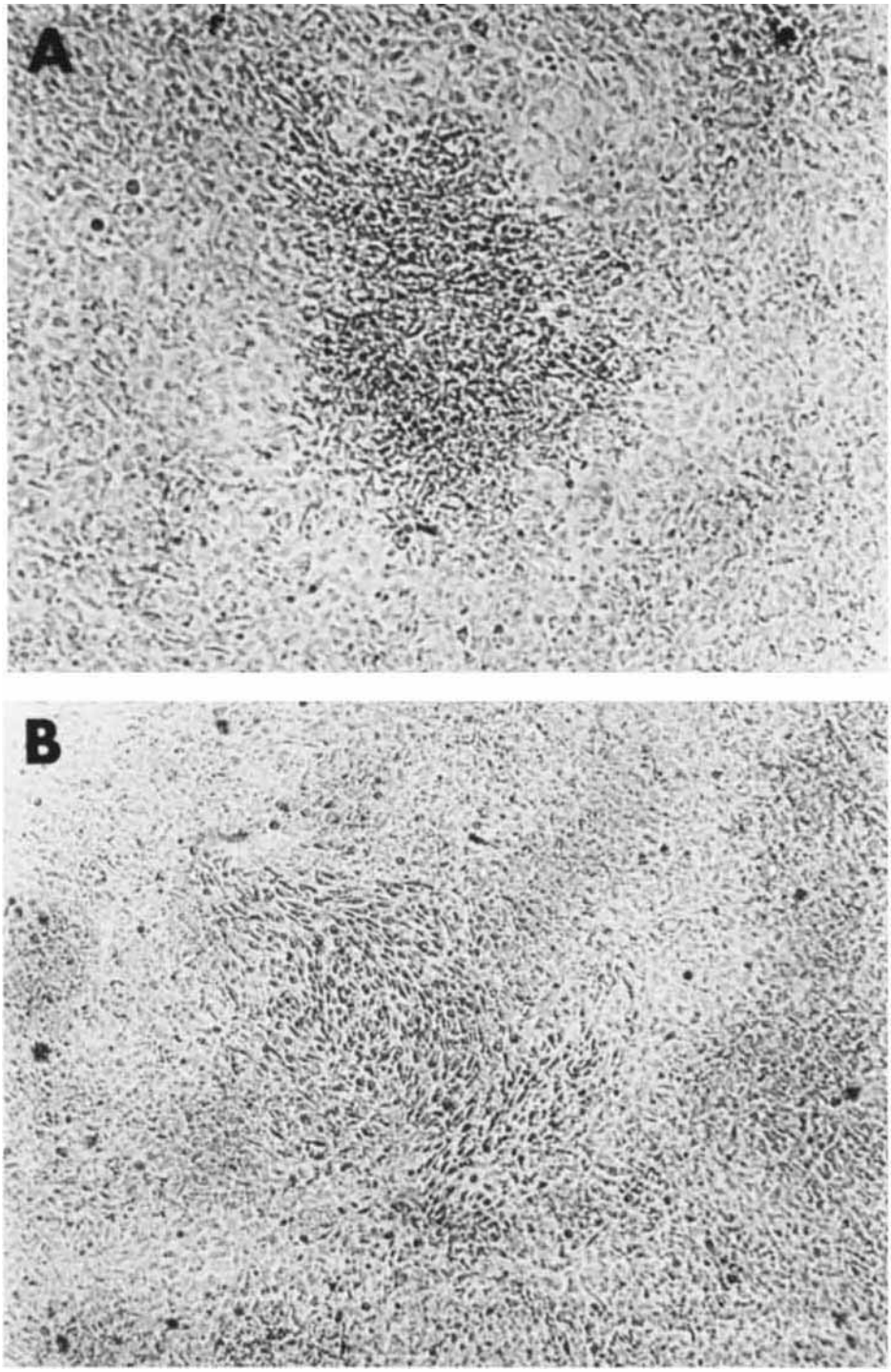

Fig 7 Foci of transformed NIH 3T3 cells induced by the DNAs of λMC29-1 (A) or the Harvey sarcoma virus clone H-1 (B)

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 that was used as a positive control in the same experiment. The MC29-induced foci consist of compact accumulations of round cells, and they have a morphology distinctly different from that of the foci induced by the cloned Harvey sarcoma virus DNA (Fig. 7).

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 [27], 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 5,350 bp in Figure 3 is located at or near the junction between the 5′ helper-related sequences and the LTR. A comparison of the restriction maps of the integrated MC29 proviral DNA and ASV DNA [23, 24] 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, whereas the other sites occur in ASV 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 exogenous avian retroviruses, is absent from MC29. This is in agreement with oligonucleotide fingerprinting data that shows distinct differences in the U_3 region between MC29 and other exogenous avian retroviruses [28].

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. 6) 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. Since 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 [29] have 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 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 [30]. 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 [28, 31]. The MC29 transforming gene can conceivably 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, 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? In addition, why would leukosis viruses act essentially only by activation of this gene in order to induce disease? The answers to these questions are not clear from the existing information, and further studies are required for them to be answered.

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