Deletions Within the Transformation-Specific RNA Sequences of Acute Leukemia Virus MC29 Give Rise to Partially Transformation-Defective Mutants

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The viral RNAs of three nonconditional mutants of avian myelocytomatosis virus MC29 were analyzed. These mutants, which were originally isolated from the quail producer line Q10 and were designated 10A, 10C, and 10H, have lost most of the ability to transform hematopoietic cells in vitro and to induce tumors in vivo, but they still transform cultured fibroblasts with the same efficiency as wild-type (*wt*) MC29. Electrophoretic analyses showed that the mutant genomic RNAs were smaller than the 5.7-kilobase genome of *wt* MC29; the genomes of mutants 10A, 10C, and 10H were about 5.5, 5.3, and 5.1 kilobases long, respectively. Analyses of the transformation-specific sequences of these mutant RNAs by a combination of T₁ oligonucleotide fingerprinting and hybridization with cDNA from the transformation-specific sequences *myc* of *wt* MC29 or competition hybridization including *wt* MC29 RNA revealed that deletions of *myc*-specific sequences had occurred. The deletions in all three mutants overlapped, since they all had lost one particular *myc*-specific oligonucleotide. In agreement with the size of the genomic RNAs, mutants 10C and 10H had lost two additional *myc* oligonucleotides, and mutant 10A contained a modified *myc* oligonucleotide. The locations of the deletions were deduced from comparisons with previously established oligonucleotide maps of several members of the MC29 subgroup of acute leukemia viruses and by hybridization of *wt* and mutant RNAs to molecularly cloned subgenomic fragments of *wt* MC29 proviral DNA, representing the 5' and 3' domains of the *myc* sequence. We found that the deleted sequences represented overlapping internal segments of the *myc* sequence and that the borders of *myc* with the partial complements of the virion genes *gag* and *env* appeared to be conserved in mutant and *wt* MC29 RNAs. The correlation between the altered transforming potential for hematopoietic cells and the partial deletion of *myc* in the mutant RNAs provided direct genetic evidence for the involvement of *myc* in oncogenesis. However, the unaffected efficiency of these mutants in fibroblast transformation suggested that the deleted sequences are not essential for the fibroblast-transforming potential of the *onc* gene of MC29.

Avian acute leukemia virus MC29 was isolated in Bulgaria in 1960 (17) and induces a broad spectrum of malignant diseases in fowl, including myelocytomatosis, renal and liver tumors, and, less typically, sarcomas and even erythroblastosis (1, 23). The oncogenic agent in MC29 stocks is defective in all three genes essential for retrovirus replication (i.e., *gag*, *pol*, and *env*) (4, 8, 16) and requires a nondefective helper virus (MC29-associated virus [MCAV]) for replication. Analyses of the viral genome and its protein product led to the definition of the trans-

forming (*onc*) gene of MC29; the viral RNA contains a specific sequence (*myc*) which is not related to known virion genes or to the *src* gene of Rous sarcoma virus (RSV) and is flanked by partial (Δ) sequence elements of *gag* and *env* (3, 11, 22). (Because of a recent proposal for a uniform nomenclature of transformation-specific RNA sequences of retroviruses [9a], the term *myc* is used throughout this paper and the accompanying paper [26]; this term is synonymous with *mcv* and *mac*, which we used previously [7, 24].) The only viral protein product which is found in cells transformed by MC29 in the absence of helper virus (nonproducer cells) is a 110-kilodalton phosphorylated polyprotein

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(p110) that is antigenically related to the *gag* gene (4, 5). In vitro translation and peptide mapping have shown that this protein is coded for by Δgag and the MC29-specific RNA sequence (19, 22, 27). Hence, it appeared that the genetic unit Δgag -*myc* is the *onc* gene of MC29 and that p110 is the transforming protein.

Despite the biochemical evidence, a strict genetic definition of the *onc* gene of MC29 was missing due to the absence of conditional or nonconditional mutants for transforming function. However, strong genetic support for the biochemically defined *onc* gene of MC29 was first achieved by comparing MC29 with three other acute leukemia viruses (CMII, MH2, OK10), which represented totally independent isolates but shared a closely related oncogenic spectrum (1, 14). It has been shown that the viral RNAs of all of these MC29 subgroup viruses contain conserved Δgag and *myc* sequences and code for *gag*-related phosphoproteins (3, 5-7, 10, 12, 15, 25, 28, 29).

The recent isolation of three nonconditional mutants of MC29, designated 10A, 10C, and 10H, which have lost most of their potential to transform hematopoietic cells in vitro and to induce tumors in vivo (24; G. Ramsay and L. N. Payne, unpublished data) but are still capable of transforming fibroblasts, enabled us to aim for direct genetic definition of essential *onc* gene elements of the MC29 genome. The partially transformation-defective (*td*) mutants 10A, 10C, and 10H encode smaller *gag*-related polypeptides than wild-type (*wt*) MC29 (100, 95, and 90 kilodaltons, respectively [24]). In this paper, we describe an analysis of the genomic RNAs of all three mutants and provide evidence that they were generated by deletions within the *myc* sequence.

MATERIALS AND METHODS

Cells and viruses. Nonproducer cultures of quail embryo fibroblasts transformed by the MC29 mutant viruses 10A, 10C, and 10H were isolated and maintained as described previously (24). MC29-Q10(MCAV-Q10) released from the producer line Q10 of MC29-transformed quail fibroblasts (4), MC29-I (MCAV-I) released by a chicken cell line of epithelial morphology (10), and MC29 (ring-necked pheasant virus [RPV]) released from the quail nonproducer line Q8 (4) superinfected with nontransforming helper virus RPV were used as sources of *wt* MC29 virus. Rescue of the defective mutant viruses from nonproducer cultures was carried out by superinfection with RPV.

Preparation and analysis of viral RNAs. Labeling of virus with $H_3^{32}PO_4$ or $[^3H]$ uridine, virus purification, RNA extraction and sedimentation, RNA gel electrophoresis, and fingerprinting of T_1 -resistant oligonucleotides by two-dimensional electrophoresis and homochromatography were carried out essentially as described previously (3, 6, 7, 11).

Preparation of cDNA specific for *myc*. Viral cDNA was synthesized by using the following modification of previously described procedures (11). A total of 12.5 units of absorbance at 260 nm (measured in NTE [0.1 M NaCl, 0.01 M Tris, pH 7.4, 1 mM EDTA] containing 0.2% sodium dodecyl sulfate) of purified MC29-I (MCAV-I) was incubated in 5 ml of a solution containing 60 mM KCl, 10 mM dithiothreitol, 20 mM Tris (pH 8.0), 0.025% Nonidet P-40, 8 mM magnesium acetate, 18 μ g of actinomycin D per ml, 0.1 mM EDTA, 7.5% glycerol, 2 mM dATP, 2 mM dGTP, 2 mM dCTP, and 2 mM dTTP supplemented with 250 μ Ci of $[^3H]$ dTTP; this incubation yielded cDNA at 40 dpm/ng. After 15 h at 40°C, 2 ml of NTE and 0.5 ml of 0.5 M EDTA were added, and the mixture was extracted with an equal volume of phenol-chloroform (1:1). The organic phase was reextracted with 5 ml of NTE, and the combined aqueous phases were extracted once more with phenol-chloroform. After ethanol precipitation, nucleic acids were dissolved in 300 μ l of 0.3 M NaOH and incubated at 40°C for 75 min and then at 20°C overnight to degrade the viral RNA. The mixture was neutralized with 100 μ l of 1 N acetic acid and then applied to a Bio-Gel p100 (Bio-Rad Laboratories) column (15 by 1 cm). The cDNA was eluted in the void volume with a solution containing 10 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, and 0.1% sodium dodecyl sulfate. The pooled fractions (2 ml) were diluted with 3 ml of NTE, 30 μ g of yeast RNA was added, and the mixture was extracted twice with phenol-chloroform. After ethanol precipitation, the cDNA was dissolved in 3 ml of 10 mM NaCl-10 mM Tris (pH 7.4)-1 mM EDTA. The yield was 100 μ g of MC29-I(MCAV-I) cDNA. *myc*-specific cDNA was selected from this preparation by hybridization (see below) with excess viral RNA from the Prague strain of RSV (PRB-RSV) or from RPV or from both of these viruses.

Cloned DNAs representing 5' and 3' domains of *myc* or the chicken locus homologous to *myc*. A subclone of MC29 proviral DNA in pBR322 containing a 3-kilobase (kb) *Bam*HI-resistant fragment encompassing most of Δgag , all of *myc*, and part of Δenv was kindly provided by T. Papas (National Cancer Institute, Bethesda, Md.); this clone was derived from the molecularly cloned provirus (20) of the Q5 nonproducer line (4). Subclones containing the 5' domain of *myc* and part of Δgag or the 3' domain of *myc* and part of Δenv were constructed as follows. The plasmid DNA of pBR322 containing the *Bam*HI fragment of MC29 DNA was cut with restriction enzymes *Bam*HI and *Sal*I at the *Bam*HI insertion sites and at the *Sal*I sites of pBR322 and *myc*; 0.5 μ g of the DNA of this digest was ligated in 20 μ l of ligation buffer (66 mM Tris, pH 7.4, 33 mM NaCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM ATP) containing 0.1 U of T4 DNA ligase. Incubation was at 15°C for 20 min and then, after dilution with 500 μ l of ligation buffer, at 4°C for 48 h (18). A 10-ng portion of ligated DNA was used to transform *Escherichia coli* strain HB101 (21). Bacterial colonies were picked from ampicillin-containing plates and then grown in 1-ml cultures to screen for recombinant plasmids. Plasmid DNA was isolated (2) and analyzed by digestion with *Bam*HI and *Sal*I. Two clones containing the 5' or 3' *Bam*HI-*Sal*I fragments of MC29 DNA were used in this study (see Fig. 5).

Recently, the chicken locus *c-myc*, which is related

to the viral *myc* sequence, was isolated from a chicken DNA recombinant library (27a). In this study we used a subclone of a 10-kb *Bam*HI fragment of chicken DNA containing the complete *c-myc* sequence in a modified pBR322 vector that also contained the chicken thymidine kinase gene.

Work on recombinant DNA was done in accordance with the National Institutes of Health guidelines.

Nucleic acid hybridizations. Hybridizations of viral RNAs with cDNA or cloned DNA were carried out in 10 to 20 μ l of a solution containing 70% formamide, 0.3 M NaCl, 0.03 M sodium citrate, and 0.01 M sodium phosphate (pH 7.0) or a solution containing 50% formamide, 0.45 M NaCl, 0.045 M sodium citrate, and 0.01 M sodium phosphate (pH 7.0) at 40°C for 2 to 18 h. Digestion of unhybridized RNA, isolation of the RNA-DNA hybrids by exclusion chromatography, melting, and fingerprinting of the hybridized RNA were done essentially as described previously (3, 7, 22).

Plasmid DNA to be used in hybridization reactions was fragmented by alkaline treatment (9) as follows. A 30- μ g portion of DNA in 200 μ l of TE (10 mM Tris, pH 8.0, 1 mM EDTA) was mixed with 100 μ l of 1 M NaOH, and the mixture was heated at 100°C for 10 min; then the mixture was chilled on ice, and the pH was adjusted to 5 to 6 with 125 μ l of 1 N acetic acid. The denatured DNA was precipitated with 1.3 ml of ethanol and redissolved in 300 μ l of TE. This treatment yielded fragmented DNA in a size range from 500 to 1,500 bases.

RESULTS

Electrophoretic comparison of viral RNAs from wt MC29 and *td* mutants 10A, 10C, and 10H. Quail embryo fibroblast cultures infected with 10A(RPV), 10C(RPV), 10H(RPV), and the Q10

producer line releasing wt MC29-Q10(MCAV-Q10) were labeled with [³H]uridine, and viral RNA was prepared as described previously. Since all three mutants were isolated from virus stocks that had been harvested from the Q10 line at later passages (24), it should be noted that the Q10 cells used here for comparison had been passaged only 17 times since their original isolation in 1976 (4), with intermediate periods of storage at -70°C. An electrophoretic comparison of the heat-denatured 70S [³H]-labeled RNAs of these viruses with [¹⁴C]uridine-labeled viral RNA of wt MC29-I(MCAV-I) showed that all of the RNA preparations contained helper viral RNAs of similar size (about 8.5 kb) and that the MC29-specific RNA components of wt MC29-I and wt MC29-Q10 comigrated exactly (Fig. 1). However, the defective viral RNAs of MC29 mutants 10A, 10C, and 10H migrated one, two, or three gel slices ahead of the wt MC29 RNA (5.7 kb) (3, 11) (Fig. 1). This corresponded to size estimates of 5.5 kb for 10A RNA, 5.3 kb for 10C RNA, and 5.1 kb for 10H RNA. This result provided the first evidence suggesting that the altered oncogenic properties of these mutants might be due to deletions of viral RNA sequences.

Deletions in the *myc* sequences of *td* mutants 10A, 10C, and 10H. First, we wanted to obtain information about the genetic composition of the wt MC29 RNA species released as a predominant component of virus from the Q10 cell line (Fig. 1A) (4, 11). The ratio of MC29 RNA to MCAV RNA was favorably high in MC29-Q10(MCAV-Q10) virus preparations (Fig. 1A).

FIG. 1. Electrophoresis in 2.1% polyacrylamide gels (6, 7, 11) of the [³H]RNA monomers of wt MC29-Q10(MCAV-Q10) (A), *td* MC29-10A(RPV) (B), *td* MC29-10C(RPV) (C), and *td* MC29-10H(RPV) (D) in the presence of [¹⁴C]RNA from wt MC29-I (MCAV-I). Symbols: Δ , [³H]RNA; $\circ$, [¹⁴C]RNA. Gels were cut into 1-mm slices and radioactivity was determined after the gel slices were incubated for 2 h at 60°C in a toluene-based scintillation fluid containing 10% NCS (Amersham Corp.) and 1% water.

Therefore, $H_3^{32}PO_4$ -labeled RNA from the trailing half of the 60 to 70S peak of viral RNA sedimented through a glycerol gradient as described previously (6, 7, 11) was used directly for a fingerprint analysis of the T_1 oligonucleotides. Figure 2A shows that all of the oligonucleotides were obtained in nearly equimolar amounts and hence were integral components of MC29-Q10 RNA. The oligonucleotides identical to the oligonucleotides previously identified in other MC29 subgroup viral RNAs were numbered accordingly (3, 6, 7, 10, 22), and the MC29-specific (*myc*) oligonucleotides are indicated on Fig. 2. The compositions of these oligonucleotides in terms of RNase A-resistant fragments are shown in Table 1.

In general, the oligonucleotide composition of MC29-Q10 RNA was very similar to the compositions described previously for other *wt* MC29 viruses (3, 10, 22). In particular, 11 of 12 *myc*-specific oligonucleotides identified previously in the RNAs of all members of the MC29 subgroup

(3, 7) were present. MC29-specific oligonucleotide 3 was missing in MC29-Q10 RNA, although it was found previously in all other *wt* MC29 RNAs; however, it has not been found in CMII, MH2, or OK10 RNA. Instead, MC29-Q10 contained a novel oligonucleotide, oligonucleotide 3a, which appeared to be closely related to *wt* oligonucleotide 3 (Table 1). That oligonucleotide 3a might indeed be a variant of oligonucleotide 3 was supported by the fact that it appeared in a hybrid of MC29-Q10 RNA with *myc* cDNA (Fig. 2B). The fingerprint in Fig. 2B contains all of the *myc*-specific oligonucleotides and, in addition, some *env*-related oligonucleotides (3, 10), since the *myc* cDNA was prepared by preselection with RPV RNA, which belongs to envelope subgroup F (13) and appears to be related only distantly to the Δenv region of MC29-I RNA that was used for the preparation of cDNA (Fig. 2B).

Direct fingerprint analyses of electrophoretically (Fig. 1) prepared ^{32}P -labeled RNAs from 10A, 10C, and 10H initially demonstrated that

FIG. 2. Fingerprint analysis of *wt* MC29-Q10 RNA. (A) $[^{32}P]$ RNA of MC29-Q10(MCAV-Q10) was sedimented through a glycerol gradient, as described previously (7, 11). The trailing half of the 60 to 70S peak of viral RNA containing practically pure MC29-Q10 RNA (see text) was used for two-dimensional electrophoresis and homochromatography (fingerprinting) (3, 11) of RNase T_1 -resistant oligonucleotides. (B) cDNA (1 μ g) prepared from MC29-I(MCAV-I)RNA was first hybridized with 6 μ g of RPV RNA in 10 μ l of a solution containing 70% formamide, 0.3 M NaCl, 0.03 M sodium citrate, and 0.01 M sodium phosphate (pH 7.0) at 40°C for 16 h. This prehybridized cDNA was then incubated in 20 μ l (total volume) of the same hybridization buffer with 0.1 μ g (4.1×10^5 cpm) of MC29-Q10 $[^{32}P]$ RNA at 40°C for 2 h. Unhybridized RNA was digested with 5 U of RNase T_1 in 200 μ l of 0.15 M NaCl–0.015 M sodium citrate, and the hybrid was isolated by exclusion chromatography on a Bio-Gel p100 (Bio-Rad) column (15 by 1 cm) equilibrated in a solution containing 0.1 M NaCl, 0.01 M Tris (pH 7.4), 1 mM EDTA, 0.1% mercaptoethanol, and 0.1% sodium dodecyl sulfate. The hybrid eluted in the void volume was phenol extracted together with 20 μ g of yeast RNA and ethanol precipitated. After heat denaturation, the RNA present in the hybrid was digested with 10 U of RNase T_1 in 200 μ l of 5 mM Tris (pH 7.4)–1 mM EDTA, and the T_1 oligonucleotides were fingerprinted. The *myc*-specific oligonucleotides are circled.

TABLE 1. Composition of T₁ oligonucleotides of *wt* and *td* MC29 RNAs

Oligonucleotide in <i>wt</i> and <i>td</i> MC29 ^a	RNase A digestion products ^b	Oligonucleotide in: ^c			
		MC29	CMII	OK10	MH2
1	2U, 3C, G, 2AC, AU, 3AAC	1	1	2	4a
1a	U, 3C, G, 2AC, AU, 2AAC				
3	15U, 2C, G, 2AAC, A ₄ U	3			
3a	6U, 2C, G, 2AC, 2AAC, A ₄ U				
6	U, 6C, 3AU, AG, A ₃ U	6	8	5b	
7b	3U, 8C, AC, AAG	7b	10	9	9
8b	2U, 4C, AC, AG, AAU, A ₃ C	8b	11	10	10
15	4U, 4C, G, AC, 2AU, AAU	15	20	22	
26	4C, 2AC, AG	26 ^d	22 ^d	27	26
31a	2U, 2AU, AG, A ₄ C/U	NA	NA	31a	NA
32a	5U, 3C, G, 2AC	NA	NA	32a	NA
43 + 44	3U, 2C, 2G, AC, 2AAC, A ₃ C, A ₄ C/U	NA	NA	43 + 44	NA
45	3U, C, G, 2AC, AAC	NA	NA	NA	NA
120	U, 4C, 2AC, AAG	120	23	25	
2	6U, 6C, G, AC, 3AU, AAC, AAU	2			
7a	2U, 6C, G, 2AC, AU, AAC	7a			
8a	2U, 2C, G, 3AC, A ₃ C	8a			
9	4U, 6C, AAG, A ₃ C	9	16	5a	NA
10	3U, 2C, 2AU, AG, 2AAU	10	17a		
11	2U, 4C, G, 2AC, AU, AAC	11		11	NA
12	U, 4C, 2AC, AU, AG, AAC	12	12	12a	NA
13	U, 4C, G, 2AC, A ₄ C	13	14	13	
14a	3U, AC, AU, AG, 2AAC	14a			
18	4U, 3C, G, AU, AAU	18	24	24	
20a	6U, 9C, G, AU	14b ^d	17b	20a	NA
25	3C, AC, AG, AAC	25	NA	NA	NA
31b	4U, 4C, G, AC, AU	NA	NA	31b	NA
60	U, 3C, G, 3AC	NA	NA	NA	NA
116	5U, 3C, G, AAC, AAU	116			
117	8U, 5C, G, 2AC, AU	117			
119	U, 5C, 2AC, AG	119 ^d			
130	4U, 2C, G, 2AC, AAU	130			
200	4U, 2C, AC, A ₃ C, A ₄ G	200			
4-cap	4U, 4C, G, 3AC, 3AU, m ⁷ GpppGmC	4	4	4	

^a T₁ oligonucleotides found in *wt* MC29 or in *td* MC29 or in both (see text and Fig. 2 through 6). Oligonucleotides 1, 1a, 3, 3a, 6, 7b, 8b, 15, 26, 31a, 32a, 43, 44, 45, and 120 were *myc*-specific oligonucleotides.

^b T₁ oligonucleotides were located on fingerprints by autoradiography, and each corresponding region of the DEAE-cellulose layer was removed by suction into a 1-ml Eppendorf pipette tip plugged with a filter of porous hydrophilic polyethylene. The cellulose was washed repeatedly with ethanol, and nucleic acid was eluted three times with 100 μ l of 30% triethylamine equilibrated to pH 9 with CO₂. The eluate was lyophilized three to four times by adding 100 μ l of water each time. The oligonucleotides were digested with 10 μ l of RNase A (0.5 mg/ml; Calbiochem) in 10 mM Tris (pH 7.4)–1.5 mM EDTA for 3 h at 37°C. The resulting digests were electrophoresed on DEAE paper (Whatman no. DE81) in 5% acetic acid–0.5% pyridine (pH 3.5) at 1,500 V.

^c Homologous oligonucleotides analyzed previously in MC29, CMII, OK10, and MH2 RNAs (3, 6, 7, 10–12, 22). NA, Not analyzed, but might be present. Some oligonucleotide compositions were revised slightly.

^d *myc* oligonucleotide 26 of MC29 and *myc* oligonucleotide 22 of CMII had been assigned previously to the *gag* region, *gag* oligonucleotide 119 had been assigned to the *myc* region, and *gag* oligonucleotide 14b had been assigned to the *env* region (3, 6, 10). The composition of oligonucleotide 22 of CMII-associated virus (3, 6) was corrected to 2C, 3AC, AG.

some *myc*-specific oligonucleotides were missing (data not shown). Because of the rather unfavorable ratio of RPV RNA to mutant RNA (Fig. 1B through D), our analysis of mutant RNA fingerprints was complicated by the presence of contaminating RPV RNA. Therefore, in subsequent studies we used fingerprinting of RNA hybridized with specific cDNA or cloned DNAs. Figure 3 shows fingerprints of RNAs

from 10A (fingerprint IA), 10C (fingerprint IIA), and 10H (fingerprint IIIA) after hybridization with *myc*-specific cDNA which was prepared by prehybridizing MC29-I(MCAV-I) cDNA with PRB-RSV and RPV RNAs (3, 7, 22). Of the 12 *myc* oligonucleotides, 9 were conserved in all of the mutant RNAs. All *td* RNAs had lost oligonucleotides 26 and 1, and mutants 10C and 10H were also missing oligonucleotide 7b. MC29

FIG. 3. Fingerprint analysis of *myc*-specific sequences of *td* MC29 mutant RNAs (fingerprints IA, IIA, and IIIA) and of *wt myc*-specific sequences not competed by mutant RNA sequences in molecular hybridization (fingerprints IB, IIB, and IIIB). For fingerprints IA, IIA, and IIIA 0.1 to 0.2 μg (6×10^5 to 10×10^5 cpm) of [^{32}P]RNA from 10A(RPV), 10C(RPV), and 10H(RPV), respectively, was hybridized for 2 h at 40°C with 1 μg of *myc*-specific cDNA prehybridized with 3 μg (fingerprint IA) or 6 μg (fingerprints IIA and IIIA) of PRB-RSV RNA and 3 μg (fingerprints IIA and IIIA) or 10 μg (fingerprint IA) of RPV RNA, as described in the legend to Fig. 2. The hybridized RNA was fingerprinted as described in the text. For fingerprints IB, IIB, and IIIB 1 μg of MC29-I(MCAV-I) cDNA was first hybridized for 18 to 20 h under the conditions described in the legend to Fig. 2 with 40 μg of unlabeled 10A(RPV) RNA and 3 μg of PRB-RSV RNA (fingerprint IB), with 17 μg of 10C(RPV) RNA and 3 μg of PRB-RSV RNA (fingerprint IIB), or with 34 μg of 10H(RPV) RNA and 3 μg of PRB-RSV RNA (fingerprint IIIB). It was then hybridized for 2 h to 0.2 μg (10^6 cpm) of [^{32}P]RNA from *wt* MC29(RPV). In fingerprints IA and IB 50% formamide was used in the hybridization buffer, and in fingerprints IIA, IIB, IIIA, and IIIB 70% formamide was used (see text). The *myc* oligonucleotides are circled.

mutant 10A contained a novel oligonucleotide, oligonucleotide 1a, which appeared to be a derivative of oligonucleotide 1 (Table 1). Again, faint oligonucleotides, especially from the *env* region, were visible, probably due to partial replacement of poorly matched RSV/RPV RNA-MC29 cDNA hybrids by the challenging ^{32}P -labeled mutant RNAs. Nonequimolar ratios among the *myc* oligonucleotides (Fig. 3, fingerprints IA, IIA, and IIIA) have been observed

previously in such experiments (3, 7, 22) and are probably due to under-representation of certain *myc* sequences in the cDNA.

To determine whether the lack of these oligonucleotides was due to point mutations or to true deletions of RNA sequences, unlabeled MC29 mutant (RPV) RNAs and unlabeled RSV RNA were prehybridized with MC29-I(MCAV-I) cDNA. The remaining unhybridized *myc* cDNA was used to protect ^{32}P -labeled RNA from *wt*

MC29 from digestion with T_1 . The fingerprints of the RNAs present in these hybrids are shown in Fig. 3 (fingerprints IB, IIB, and IIIB). In each case, the *myc* oligonucleotides of *wt* RNA that were missing from the mutant RNAs were found in these hybrids. In the case of mutant 10A, oligonucleotide 7b was recovered from these hybrids, although it was present in 10A RNA (see below). These results indicated that the lack of *myc*-specific oligonucleotides in the RNAs of *td* MC29 mutants was probably due to deletions of *myc* sequences. We observed that *env* oligonucleotide 2 was also found in hybrid fingerprints IB, IIB, and IIIB shown in Fig. 3, probably because of replacement of mismatched RSV/RPV RNA-MC29 cDNA hybrids by ^{32}P -labeled RNA. A similar explanation is also likely to be true for the fact that *wt* oligonucleotide 1 effectively competed with its probable variant, oligonucleotide 1a, in mutant 10A. It is not clear whether oligonucleotide 7b of mutant 10A was also modified and, therefore, competed poorly with the *wt* homolog, since it was always recovered as a double or triple oligonucleotide (Fig. 3 and 4) and, hence, its composition was not absolutely certain.

To determine more directly the origin of variant oligonucleotide 1a and to determine whether the *myc* oligonucleotides were present in equimolar ratios, *wt* and 10A RNAs were hybridized

to DNA from the chicken *c-myc* locus, which has recently been cloned and characterized (27a). The fingerprints in Fig. 4 show that all *myc*-specific oligonucleotides were recovered at equimolar ratios from such hybrids and that no oligonucleotides from the structural genes were present. Variant oligonucleotide 1a was *myc* specific, and the absence of oligonucleotide 26 from 10A RNA was confirmed. The composition of an additional *myc* oligonucleotide (oligonucleotide 45), which was present in *wt* and 10A RNAs, was determined (Table 1).

Location of the *myc* deletions in 10A, 10C, and 10H RNAs. Previous oligonucleotide mapping had shown that the relative map order of the *myc*-specific oligonucleotides is conserved among all members of the MC29 subgroup of acute leukemia viruses (3, 6, 7, 10, 22), and it was reasonable to assume that the same was true for the mutant RNAs. This was confirmed by oligonucleotide mapping studies of the 10C and 10H RNAs (data not shown). However, the order of oligonucleotides within about 1 kb, as determined by oligonucleotide mapping, is uncertain, and precise location of the deletions was not possible. We were especially interested in determining whether the deleted sequences extended into the partial structural genes Δgag and Δenv or whether they were located internal to *myc*. To do this, we subcloned *myc*-5' and

FIG. 4. Fingerprint analysis of *wt* and *td* MC29 RNA sequences related to the cellular *myc* locus. A 1- μg portion of [^{32}P]RNA (5×10^6 cpm) from *wt* MC29(RPV) (A) or *td* 10A(RPV) (B) was hybridized with 20 μg of denatured and fragmented plasmid DNA (15 kb) containing 1.6 kb of cellular *myc* DNA in a solution containing 50% formamide, 0.45 M NaCl, 0.045 M sodium citrate, and 0.01 M sodium phosphate (pH 7.0) for 20 h at 40°C. The RNA hybridized was fingerprinted.

myc-3' domains of *wt* MC29 proviral DNA into plasmid vector pBR322, as shown in Fig. 5A and C. *myc*-5' contained most of Δgag (about 1.1 kb) and the 5' 0.8 kb of *myc*, and *myc*-3' contained the 3' 0.9 kb of *myc* and 0.2 kb of Δenv . DNAs from these subclones were used in hybridizations with ^{32}P -labeled *wt* MC29(RPV) RNA. Fingerprints of the hybridized RNAs are shown in Fig. 5B. The hybrid formed with *myc*-5' DNA (Fig. 5B, fingerprint 1) contained three *myc* oligonucleotides (oligonucleotides 120, 32a, and 6) and six *gag* oligonucleotides. Some of these were shared with RPV and, hence, were present at more than equimolar amounts. The conserved *gag* oligonucleotides (oligonucleotides 13, 20a, 60, and 25) were identified in the recently determined sequence of PRC-RSV proviral DNA (D. Schwartz, R. Tizard, and W. Gilbert, Cell, in press) and were located accordingly on the map of *wt* MC29 RNA (Fig. 5C), in good agreement with previous oligonucleotide mapping. All other *myc* oligonucleotides and three *env* oligonucleotides were present in the hybrid of *wt* MC29 RNA with subclone *myc*-3' DNA (Fig. 5B, fingerprint 2). In very good agreement with previous mapping data (10), it appeared that *env* oligonucleotides 2, 7a, and 14a mapped directly adjacent to the *myc*-*env* border. The relative order of the 3' *myc* oligonucleotides remained somewhat uncertain; however, all previous mapping data on MC29 subgroup viral RNAs have indicated that oligonucleotides 3, 8b, and 15 map closest to the *myc*-*env* border (6, 10).

Similar results were obtained when *td* MC29(RPV) RNAs were hybridized with *myc*-5' and *myc*-3' DNAs (Fig. 6). The absence of *myc* oligonucleotides 1 and 26 in all *myc*-3' hybrids and of oligonucleotide 7b in *myc*-3' hybrids with 10C and 10H RNAs was confirmed. The presence in the mutant RNAs of the three *env* oligonucleotides which were not present in RPV RNA (Fig. 2B) (11) and of some *gag* oligonucleotides (oligonucleotides 119, 20a, and 31b) that were not shared with RPV RNA could be deduced directly from the data in Fig. 6. The presence in the mutant RNAs of the other *gag* oligonucleotides shared with RPV was demonstrated by fingerprinting polyadenylic acid-containing 6-kb fractions of *td* MC29(RPV) RNAs (data not shown). It appeared that one conserved *gag* oligonucleotide (oligonucleotide 13) was missing only in the case of 10A.

It followed from the locations of deleted *myc* oligonucleotides 1, 7b, and 26 (Fig. 5C) that all three mutants (10A, 10C, and 10H) suffered overlapping deletions within the *myc* sequences of their viral RNAs. The approximate complexities, as deduced from the smaller viral genome sizes (Fig. 1), and the locations of the deletions, are shown schematically in Fig. 7. The presence

in 10A RNA of oligonucleotide 7b and of a partial complement of oligonucleotide 1 correlated well with the larger genome size of 10A compared with 10C and 10H. The loss of sequences from the viral RNAs was in good agreement with the observation that these mutant RNAs code for smaller *gag*-related proteins (Fig. 7) (24) which have lost *myc*-specific tryptic peptides (26).

DISCUSSION

Genetic structure of *td* MC29 mutants. The three nonconditional mutants of MC29 (10A, 10C, and 10H) have an approximately 100-fold reduced ability to transform hematopoietic cells in vitro (24) and are not known to cause in vivo tumors typical of infections by *wt* MC29 (G. Ramsay and L. N. Payne, unpublished data). However, these mutants do transform cultured chick and quail fibroblasts with an efficiency equal to that of *wt* MC29. Therefore, we consider these mutants to be partially *td*.

In this work we found that the altered oncogenic properties of these *td* mutants can be correlated with deletions within the transformation-specific (*myc*) sequences of their viral RNAs. The smaller genome sizes and the complete or partial loss of one to three *myc*-specific oligonucleotides are in excellent agreement with the analysis of the smaller *gag*-related polypeptides by peptide mapping techniques described in the accompanying paper (26). From these studies and from the data presented here, it clearly follows that all three of these mutants contain overlapping deletions of *myc*-specific sequences, increasing in size from mutant 10A to 10C to 10H. We suggest from the oligonucleotide mapping and hybridization data and from the overlap among the deletions in the three mutant RNAs that these deletions are probably contiguous and may have originated from the same point in the *wt myc* sequences (see below). It is almost certain that the Δgag -*myc* border is not changed in these mutant RNAs since the deletions at least originate in the 3' half of *myc*. It is also very likely that the *myc*- Δenv border is conserved since all mutant RNAs contain *env* oligonucleotides 2, 7a, and 14a and *myc* oligonucleotides 3, 8b, and 15, which all map close together. Hence, the deletions appear to have occurred internally in the *myc* sequence (Fig. 7). However, only molecular cloning and sequencing of the mutant proviral DNAs could unambiguously determine the precise locations of the deletions and whether these deletions were generated in phase and have preserved the reading frame of the 3' *myc* sequences. If this is the case, then the smaller *gag*-related proteins probably contain the same carboxy terminus as the *wt*

FIG. 5. Construction of subclones of wt MC29 proviral DNA and fingerprint analysis of wt MC29 RNA sequences related to them. (A) Electrophoretic analysis in 0.8% agarose (30) of pBR322 (lane 8), pBR322 containing the 3-kb *Bam*HI fragment of MC29 proviral DNA (lane 9), pBR322 containing the 1.9-kb *Bam*HI-*Sal*I fragment (lane 10), and pBR322 containing the 1.1-kb *Bam*HI-*Sal*I fragment (*myc*-3') (lane 11). Lanes 1 through 4 contained *Bam*HI digests of the DNAs from lanes 8 through 11, and lanes 5 through 7 contained *Bam*HI-*Sal*I digests of the DNAs from lanes 9 through 11. The *Sal*I digestion in lane 5 was incomplete. Lane M contained the *Hind*III digest of phage λ cl857 S₇ DNA as a molecular weight marker. (B) A 0.2- μ g portion (10⁶ cpm) of [³²P]RNA from wt MC29(RPV) was hybridized with 10 μ g of pBR322 *myc*-5' DNA (fingerprint 1) or with 10 μ g of pBR322 *myc*-3' DNA (fingerprint 2) for 12 h at 40°C in a hybridization mixture containing 70% formamide. The hybridized RNA was fingerprinted, and the *myc* oligonucleotides are circled. (C) Ordering of *gag*, *myc*, and *env* oligonucleotides on the map of MC29 genomic RNA according to the results in (B) and to previous oligonucleotide mapping (3, 6, 7, 10, 22) and *gag* gene sequencing data (Schwartz et al., in press). The absolute order of *myc* oligonucleotides within the 5' and 3' segments of *myc* is uncertain.

FIG. 6. Fingerprint analysis of [32 P]RNAs from 10A(RPV) (fingerprints IA and IB), 10C(RPV) (fingerprints IIA and IIB), and 10H(RPV) (fingerprints IIIA and IIIB) hybridized with subcloned *myc*-5' DNA (fingerprints IA, IIA, and IIIA) or *myc*-3' DNA (fingerprints IB, IIB, and IIIB). A 0.4- μ g portion (2×10^6 cpm) of each *td* MC29(RPV) RNA was hybridized with 10 μ g of DNA under the conditions described in the legend to Fig. 5. The *myc* oligonucleotides are circled.

FIG. 7. Schematic diagram of the genomic RNAs and protein products of MC29 mutants 10A, 10C, and 10H. Δ indicates the presence of partial complements of *gag*, *myc*, and *env*. The dotted lines indicate that the precise locations of the *gag-myc* borders and the absolute map positions of the deletions within the *myc* sequences are uncertain.

p110 protein. Alternatively, if the deletions map closer to the 3' end of *myc*, then the smaller size of the proteins could be explained by termination of translation at the point of the deletion due to disturbance of the translational reading frame. The proteins would then lack the deleted sequences, as well as the 3' sequences of *myc* at the *myc- Δenv* border.

Origin and genesis of *td* MC29 mutants. The origin of these mutants was a quail fibroblast line (Q10) that was transformed by MC29 and released infectious virus. This line was isolated from a single focus of MC29-transformed cells and shortly after isolation contained only the p110 protein of *wt* MC29, together with helper viral proteins (4). However, after only 15 to 18 passages in cell culture, smaller *gag*-related proteins were observed as minor components in addition to the *wt* p110 protein, and this led later to the isolation of the three *td* mutants described here and previously (24). It is very unlikely that the original cell that gave rise to the Q10 focus was infected with the MCAV helper virus and several variants of MC29 present in the MC29 stock used in the focus assay; no variant MC29 virus was isolated directly in this assay, and all other MC29-transformed cell lines isolated at the same time contained only *wt* MC29 (4), as did all subclones derived from cell lines other than Q10 (26; Bister, unpublished data). Hence, the deletion mutants apparently arose by genetic changes of the original *wt* RNA or proviral DNA. It is a surprising distinction of the *wt* MC29-Q10 virus that it spontaneously and at a rather high frequency segregates deletion mutants that are partially defective for transformation. In contrast to this, a previous study of *wt* MC29 virus stocks from several laboratories and with greatly varying passage histories demon-

strated a remarkable genetic stability of the *gag-myc* sequences and of the p110 protein product (10). A possible indication for a unique mutation of the *wt* Q10 virus which conceivably could give rise to the spontaneous deletions is the presence of the novel *myc*-specific oligonucleotide 3a. Since this oligonucleotide maps close to the sequences deleted from the mutant RNAs which contain the *wt* form of oligonucleotide 3, it could be hypothesized that this unique mutation in Q10-MC29 RNA is involved in the initiation of the deletions. It has been noted that oligonucleotide 3 is missing from the related viruses CMII, OK10, and MH2 (6, 7, 12). Further analysis of *wt* Q10-MC29 provirus and its integration site might reveal more definitive clues concerning the ability of the Q10 cell line to generate a variety of deletion mutants of MC29.

Effect of partial deletions in *myc* on the oncogenicity of MC29. Unlike most *src* deletion mutants of RSV, which generally have completely lost the ability to transform fibroblasts in cell culture or to induce sarcomas in animals (31), the *myc* deletion mutants of MC29 described here have lost only part of their oncogenic potential. They transform cultured fibroblasts with unaltered efficiency, but have decreased ability to induce tumors *in vivo* or to transform efficiently hematopoietic cells *in vitro*. However, even in macrophage and bone marrow assays, the reduction in focus or colony titer is a quantitative phenomenon (100-fold reduction [24]) and not an absolute loss of transforming activity. These considerations might be important for understanding the multioncogenic potential of the MC29 *onc* gene. For instance, if fibroblast and hematopoietic cell transformation were encoded by two separate and independent

domains of the MC29 *onc* gene and the deletion mutants had lost the domain essential for blood cell transformation, then a total loss of that activity would have to be expected, as for the nonleaky *src* deletion mutants of RSV. On the other hand, if the sarcomagenic potential and the leukemogenic potential of MC29 are mediated basically by the same activity of the *onc* gene product, then the biological data obtained with the partial *myc* deletion mutants would argue that the deletions resulted in a quantitative change in the activity of the *onc* gene product which is now sufficient to transform fibroblasts, but not blood cells, efficiently. To distinguish between these possibilities, it would be necessary to isolate and analyze mutants which have lost fibroblast-transforming activity and, if they exist, have retained the potential to transform blood cells.

The p110 transforming protein of *wt* MC29 is a phosphoprotein (5), and we have shown recently that the *gag*-related proteins of the three *td* mutants have all lost a *myc*-specific phosphorylation site (G. Ramsay, M. J. Hayman, and K. Bister, manuscript in preparation). The correlation of deletions in *myc*-specific RNA and protein sequences resulting in the loss of possibly important chemical modification sites on the transforming proteins with the altered oncogenic properties of the mutant viruses provides direct genetic evidence for the involvement of *myc* in oncogenesis.

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ADDENDUM IN PROOF

Recent DNA sequence analysis of the *myc*-3' subclone of *wt* MC29 proviral DNA demonstrated that *myc* oligonucleotide 3 and *env* oligonucleotide 7a map adjacent to each other, separated by a segment of 33 nucleotides (K. Bister and P. Bullock, unpublished data). This directly supports the observation that the junction of *myc* and *env* sequences appears to be conserved in *td* and *wt* MC29 RNAs.

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