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Subgenomic mRNA in OK10 Defective Leukemia Virus-Transformed Cells

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OK10, a defective leukemia virus, is produced as a defective particle by so-called nonproducer transformed quail fibroblasts. OK10 defective viral particles contain an 8-kilobases (kb)-long genomic RNA, lack any detectable reverse transcriptase activity, and are not infectious. We studied the genetic content of OK10 RNA extracted from both virions and infected cells. As shown by RNA-cDNA hybridizations in stringent conditions, about 77% (6.4 kb) of the OK10 8.0-kb RNA was related to avian leukosis viruses in the three structural genes *gag*, *pol*, and *env*, as well as in the *c* region. The remainder of the OK10 genome-encoding capacity (≤1.6 kb) was homologous to the MC29-specific transforming sequence *myc*(m) and therefore has been named *myc*(o). *Eco*RI restriction analysis of the OK10 integrated proviral DNA with different probes indicated the presence of only one provirus in the OK10 QB5 clone, which agreed with the gene order: 5′-*gag*-Δ*pol*-*myc*(o)-Δ*env*-*c*-3′. Heteroduplex molecules formed between the viral OK10 8.0-kb RNA and the 6.8-kb *Sac*I DNA fragment of the Prague A strain of Rous sarcoma virus confirmed that structure and indicated that the *myc*(o) sequence formed a continuous RNA stretch of 1.4 to 1.6 kb long between Δ*pol* and Δ*env*. We also examined the *myc*(o)-containing mRNA's transcribed in OK10-transformed cells. OK10-transformed quail fibroblasts (OK10 QB5) transcribed two mRNA species of 8.0 and 3.6 kb containing the *myc*(o) sequence. The genetic content of the 3.6-kb species made it a possible maturation product of the genome size 8-kb species by splicing out the *gag* and *pol* sequences. In OK10-transformed bone marrow cells (OK10 BM), a stable bone marrow-derived cell line producing OK10, the *myc*(o) sequence was found in four RNA species of 11.0, 8.0, 7.0, and 3.6 kb. Again, the genetic content of these mRNA's indicated that (i) the 3.6-kb species could be spliced out of the 8.0-kb-genome size mRNA and (ii) the 11.0-kb-long mRNA could represent a read-through of the OK10 provirus, the corresponding maturation product being, then, a 7.0-kb mRNA. The 7.0- and 3.6-kb mRNA's both contained the *myc*(o) sequence, but no sequences related to the *gag* or *pol* gene. In conclusion, whereas the *myc* sequences have been generally thought to be expressed through a *gag-onc* fusion protein, as for MC29 and CMII viruses, our experiments indicate that they could also be expressed as a non-*gag*-related product made from a subgenomic mRNA in the OK10-transformed cells.

OK10 has been classified as a defective leukemia virus of the MC29 subgroup based on the differentiation phenotype of the hematopoietic cells it transforms in vitro (9). Bone marrow cells infected by these viruses (MC29, MH2, CMII, and OK10) resemble transformed macrophages and express differentiation markers of the myeloid lineage (2). These findings have been confirmed by biochemical studies. The transforming potential of MC29-type viruses is associated with the presence in their genome of a specific nucleotide sequence called *myc* (22, 26). The OK10 allele of the *myc* sequence has been called *myc*(o). The *myc* sequence has no homology with the *src* gene of Rous sarcoma viruses (RSVs) or with the structural genes (*gag*, *pol*, and *env*) of avian leukosis viruses (ALVs) (22). The *myc* sequence has been proposed, therefore, to be an oncogene (26). As for the *src* gene, *myc* is closely related to conserved nucleotide sequences (*c-myc*) found in the DNA of uninfected vertebrate cells (22).

The genetic structure of all defective leukemia viruses characterized so far conforms to the same basic model. They all contain ALV-related sequences and a unique nucleotide sequence of cellular origin (30). However, they are all deleted in ALV-related genes, which are essential

for replication, and therefore need a helper virus (ALV) for their propagation in cultured cells (9). Cells transformed by MC29 contain a novel 110,000-dalton (110K) fusion protein comprising part of the *gag* polyprotein linked to the translation product of the *myc* sequence (4). Such a product is made in the cell from a genome-size viral mRNA. A similar structure has been proposed for OK10 (5), with a 200,000-dalton (200K) fusion protein (20). Our results suggest the alternate possibility that in OK10 virus the transforming gene product could also be a non-*gag* protein translated from a subgenomic viral mRNA.

MATERIALS AND METHODS

Cells and viruses. Defective leukemia virus nonproducer quail fibroblast clones (OK10 QB5 [10, 20], MC29 Q8 [4]) were obtained through the courtesy of T. Graf and K. Bister. OK10-transformed bone marrow cells (OK10 BM) (19) were kindly provided by L. Hortling. The transformation-defective Prague C strain of RSV (PR-RSV-C) was obtained originally from R. Junghans. All viruses were grown on C/E chicken fibroblasts as described elsewhere (29).

Preparation of virus-specific cDNA's. Radiolabeled cDNA transcripts were synthesized in exogenous reactions, using 50 to 70S viral RNA templates, calf thymus primer, and purified avian myeloblastosis virus polymerase, as previously described (23, 32). The steps for selection of cDNArep (representing the transformation-defective PR-RSV-C genome), cDNAsrc, cDNAgag, cDNApol, cDNAenv, and cDNAc are outlined in Fig. 1A. cDNArep, cDNAsrc, and cDNAenv were selected as described previously (29, 31). cDNAc was prepared by hybridizing the remaining cDNA containing the *gag-pol-c* sequences to plateau value to 10S (ca. 800 nucleotides) polyadenylated [poly(A^+)] RNA of PR-RSV-C and then recovered after selection (29, 31). The 10S RNA was prepared from spontaneously nicked 70S RNA (100 μg) fractionated by sucrose gradient centrifugation followed by polyadenylate [poly(A)] selection over polydeoxythymidylate-cellulose (these two steps being repeated sequentially twice). Such 10S RNA should contain the entire *c* sequences and may contain some *src* or *env* sequences (already eliminated from the cDNA by the previous steps) but no *pol* or *gag* sequences. cDNAgag was finally separated from the remaining cDNA by annealing to MC29 Q8 cellular poly(A^+) RNA at a plateau C_rt value of 2,000 mol·s liter^{-1}. That RNA, extracted from a cell containing the MC29 genome without helper sequences, was used, since MC29 virus has been reported to have most of the *gag* sequences but no *pol* sequences (27). Specificities of these cDNA's are validated in Table 1. cDNA-myc complementary to the specific region of MC29 virus [*myc*(m)] was made as described previously (26).

cDNA5′ was prepared according to the published procedure (8). Alternatively, probes for *gag*, *pol*, *env*5′, or *env*3′ were prepared by nick translation (21) of DNA fragments B, A, C, and D (Fig. 1B) originating from a λ phage clone containing the *Sac*I 6.8-kilobase (kb) fragment of PR-RSV-A DNA, a gift of J. Taylor. The fragments were purified twice by electrophoresis

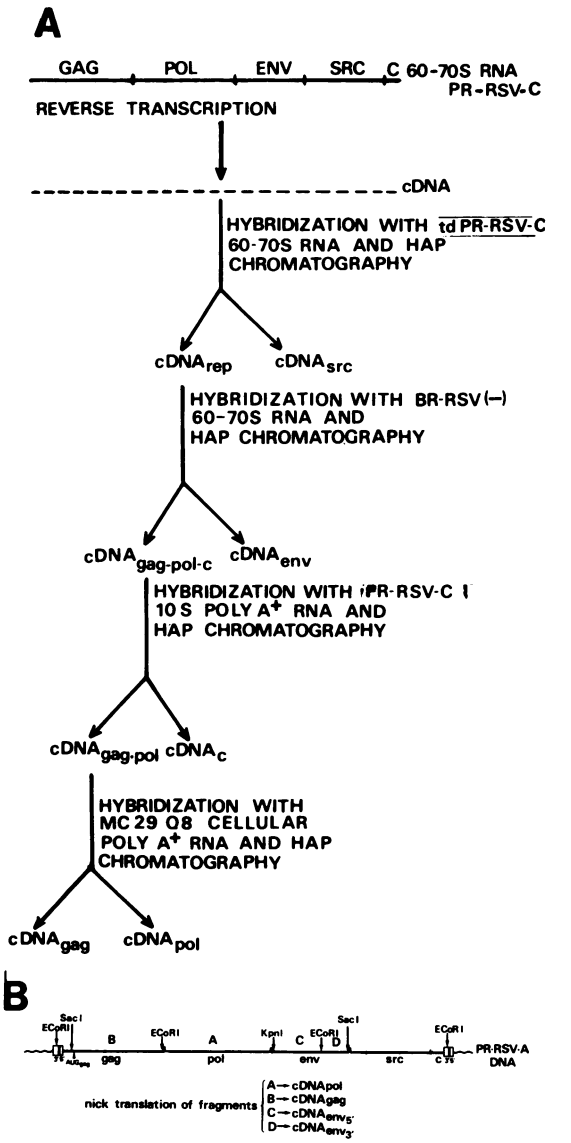
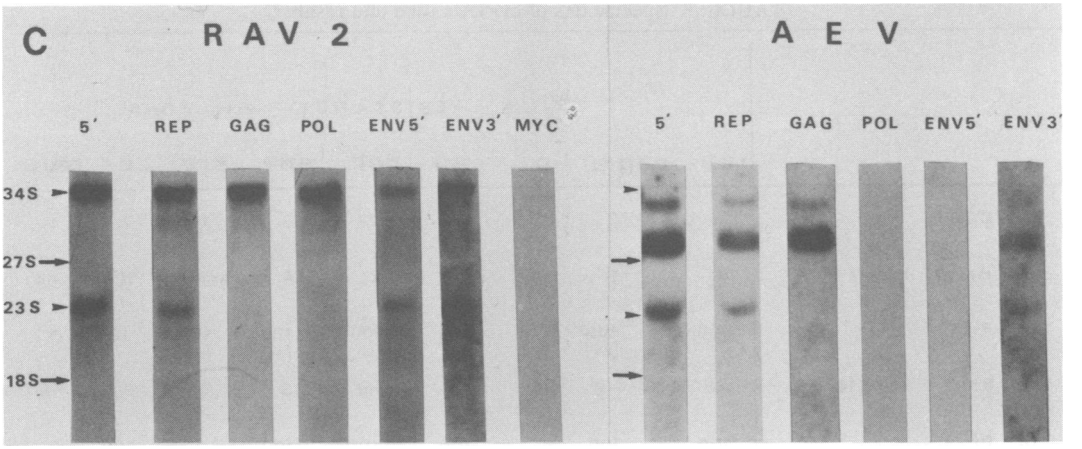


FIG. 1. Preparation of cDNA's. (A) cDNArep, cDNAgag-pol-c, cDNAc, and cDNAgag were recovered as RNA-cDNA hybrids after hydroxyapatite (HAP) chromatography. The hybrids were alkali treated, neutralized, and ethanol precipitated before the next step of selection. (B) The *Sac*I DNA fragment of PR-RSV-A was in phage Charon 16A. The insert was excised by endo·R-*Sst*I, which is an isozyme of endo·R-*Sac*I. Nick-translated cDNA's were prepared from fragments A, B, C, and D isolated by agarose gel electrophoresis after the suitable restriction cleavages. (C) Hybridization of cellular RAV-2- and AEV (nonproducer erythroblasts, clone HD3)-infected cells with nick-translated ^{32}P-labeled DNA. Total poly(A^+) RNAs were denatured by glyoxal treatment, separated on 1% agarose gels, transferred to diazobenzyloxymethyl paper, and hybridized with ^{32}P nick-translated fragments A, B, C, D, and cDNAmyc. The RNA content of HD3 cells was previously described (23). rRNA's from chicken fibroblasts were used as standard length markers.



in 0.8% agarose gels before the nick translation reaction, which was performed by using a New England Nuclear nick translation kit.

Reverse transcriptase assay. OK10 defective viral particles were purified from 1 liter of OK10 QB5 cell culture medium as described previously (15). The exogenous reverse transcriptase assay was then carried out as follows: purified virions were incubated at 38°C for 2 h with various Nonidet P-40 concentrations in 0.1 M Tris-hydrochloride (pH 8.1)–8 × 10^{-3} M $MgCl_2$–1% β-mercaptoethanol–5 × 10^{-5} M each dATP, dGTP, and dCTP–10^{-5} M dTTP–0.02 mCi of [^{3}H]dTTP.

Nucleic acid hybridization. Viral and cellular RNAs for liquid hybridization studies were extracted and purified as described previously (23). Standard liquid hybridization reaction mixtures contained 0.6 M NaCl, 2 × 10^{-3} M EDTA, 2 × 10^{-2} M Tris-hydrochloride (pH 7.4), 500 μg of calf thymus DNA per ml as carrier, 2,000 cpm (0.04) ng) of ^{3}H-labeled cDNA's, and appropriate RNAs in large excess. Hybridizations were conducted in glass capillaries at 68°C, and the extent of annealing was monitored by digestion with S1 nuclease.

Agarose gel electrophoresis of poly(A)-containing cellular and viral RNAs. Poly(A)-containing cellular and viral RNAs were prepared as described elsewhere (34). RNA samples were denatured by glyoxal treatment (18) and submitted to electrophoresis in 1% agarose horizontal gels.

Transfer hybridization of RNAs. Size-separated RNAs were transferred to diazobenzyloxymethyl paper prepared as described previously (1). The northern blots were hybridized with ^{32}P-labeled cDNA's as described previously (17), except the washing procedure which was performed as follows. After a 10-min wash in 1× Denhardt solution–2× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate), the blots were submitted to mild sonication in 0.1× SSC–0.1% sodium dodecyl sulfate for 1 to 3 min at room temperature and dried (Pons sonicator, 3 liters, 100 W, 40 kHz; Bioblock, Strasbourg, France). The blots were then subjected to autoradiography (Kodak X-ray films, Dupont Lightning Plus X-ray intensifying screens, −70°C).

Heteroduplex mapping. The 6.8-kb *Sac*I DNA fragment of PR-RSV-A cloned in lambda (Charon 16A) was excised by *Sst*I, an isozyme of *Sac*I, and purified by agarose gel electrophoresis. The gene content of this DNA fragment is shown in Fig. 1B (25). The OK10 QB5 viral 8.0-kb RNA purified by sucrose gradient centrifugation (16) was hybridized to the *Sst*I PR-RSV-A fragment by using the high formamide conditions developed by Casey and Davidson (6). The heteroduplexes were dialyzed for 1 h at 37°C against 1 M glyoxal and then dialyzed for 4 h at 4°C against 0.01 M Tris (pH 7.4)–0.001 M EDTA.

The heteroduplexes were spread from a 41% formamide hyperphase containing 0.1 M Tris (pH 8.5)–0.01 M EDTA–50 μg of cytochrome *c* per ml onto a hypophase of water. pMB9 plasmid DNA was included as an internal-length standard. Molecules were picked up on 300-mesh copper grids covered with a thin carbon film and were colored with uranyl acetate. Dried molecules were shadowed with a platinum-carbon mixture (95:5%, wt/wt), using a Balzer electron bombardment gun and a quartz crystal film thickness monitor. A 7° shadow angle and a mean shadow thickness of 0.3 mm, with a specimen rotation speed of ca. 80 rpm, were used for low rotary shadowing.

High-molecular-weight DNA isolation. Cells were gently resuspended (10^7 cells per ml) in an extraction buffer containing 0.05 M Tris-hydrochloride (pH 7.4)–0.1 M NaCl–0.01 M EDTA–100 μg of proteinase K per ml and lysed by adding slowly, under gentle rocking, Sarkosyl to a 1% final concentration. After overnight digestion, CsCl was added (2.54 g per 2 ml of lysate; ρ = 1.7 g/ml), and the DNA was centrifuged to equilibrium. The band of viscous DNA was collected through a large hole made in the bottom of the tube and dialyzed extensively against 0.001 M Tris-hydrochloride (pH 7)–0.0001 M EDTA.

Restriction endonuclease mapping. Restriction endonucleases were obtained from Boehringer Mannheim (Mannheim, West Germany) and Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). The DNA digestions were performed according to the supplier's instructions. DNA fragments were separated by electrophoresis in 0.8% agarose horizontal gels and transferred to nitrocellulose as previously described (28).

TABLE 1. Specificities of cDNA's used and results[a]

RNA SPECIES	% S_1 RESISTANCE with cDNA							
	rep	gag-pol-c	gag	pol	env	src	c	myc
E. COLI	3	3	2	2	3	1	2	1
10S Pr C POLY A+	9	14	2	2	4	nd	100 [70]	nd
Pr C	100	100 [85]	100	100 [80]	100 [75]	100 [90]	100	1
rd Br Q16	68	nd	nd	nd	3	98	97	nd
td Pr C	100 [87]	100	100	100	100	3	100	2
MC 29 Q8	45	38	100 [65]	5	66	13	38	100 [97]
OK 10 QB5	77	87	84	96	45	13	77	90
OK 10 BM	81	nd	nd	nd	77	2	nd	90
[1] COMPLEXITIES [Kb]	8.0	nd	2.0[a]	3.0[b]	2.2	1.8[2]	0.33	1.6[3]

[a] RNA extracted from different viruses (PrC, transformation-defective PrC, replication-defective Br, OK10 BM) or cells were hybridized under stringent conditions (0.6 M NaCl, 68°C) with cDNA's (2,000 cpm per point = 0.04 ng) to plateau C_rt values (10 times the $C_rt_{1/2}$ and C_rt: 4×10^4 mol·s liter^{-1} for *E. coli*). Results obtained were standardized to the values of the homologous reaction (numbers in brackets). The cDNA's were prepared as described in the legend to Fig. 1A. cDNAmyc was prepared as described by Sheiness et al. (26). [1] The complexity of the cDNAs were calculated according to Saule et al. (23); [2] according to Sheiness et al. (26); [3] according to Stehelin et al. (29). [a] cDNAgag contains only the *gag* sequences conserved in MC29; [b] cDNApol contains, in addition to the *pol* sequence of PR-RSV-C, the *gag* sequences deleted in MC29. ND, Not done.

The Southern blots were hybridized to specific ^{32}P-labeled cDNA's, washed, dried, and submitted to autoradiography as previously described (24).

Biological and physical containment. All experiments performed with the EK2-certified phage Charon A16 containing the *Sac*I DNA fragment of PR-RSV-A and its certified host *Escherichia coli* DP50 *supF* were carried out according to L2 B2 conditions in the nomenclature adopted by the French Committee (7) (equivalent to P2 EK2 in the National Institutes of Health nomenclature).

RESULTS

OK10 RNA of a nonproductively transformed quail fibroblast clone. The OK10 QB5 quail fibroblast clone has been reported to be nonproducing (10, 20, 30). It does, in fact, produce particles that accumulate in the culture fluid, but these particles are noninfectious (20, 30), suggesting the absence of a competent helper virus in these cells. The total RNA of these cells was analyzed to study the structure of the 8- to 8.6-kb OK10 RNA (5, 30). We used labeled cDNA probes related to all or parts of an ALV genome prepared as described in the legend to Fig. 1A, as well as cDNA probes corresponding to the *myc*(m) sequences of MC29 (cDNAmyc) and to the *src* gene (cDNAsrc) of PR-RSV-C. These probes were characterized by plateau hybridization experiments (Table 1). As shown, all probes exhibited the expected specificities. We thus used these probes to analyze the viral RNA of OK10 virus (Fig. 2; Table 1). Table 1 showed that the viral RNA of QB5 cells was closely related (77%) to an ALV cDNArep probe and contained homology in the *gag*, *pol*, *env*, and *c* regions. It hybridized extensively with cDNAmyc and did not contain sequences related to cDNAsrc. The following observations also resulted from Table 1 data: (i) the 84% hybridization with cDNAgag probably reflects some divergence in this gene, since it has been shown that the entire Pr76 *gag* precursor polypeptide

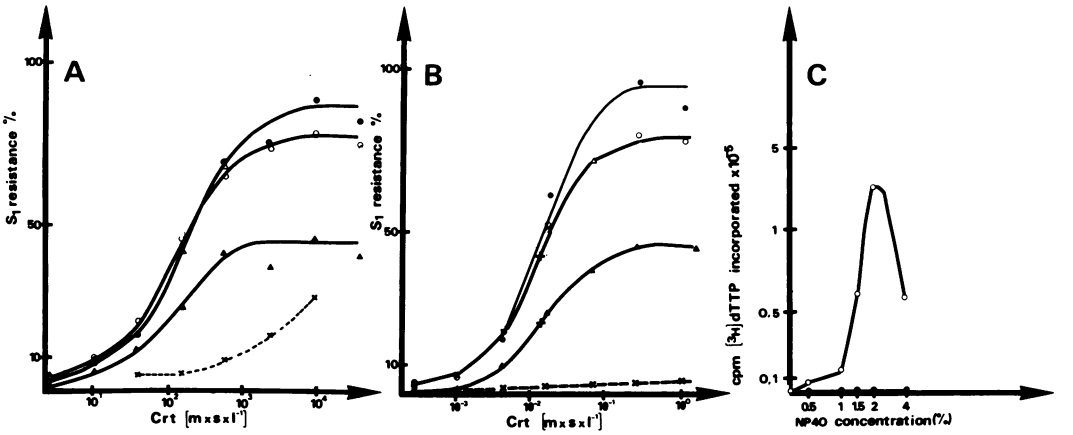


FIG. 2. Hybridization of (A) total cellular OK10 QB5 RNA and (B) 50 to 70S RNA from OK10 QB5-defective viral particles to cDNA's. (A) Hybridizations were performed in 22 μl with 0.3 mg of RNA at the highest C_rt and different ^{3}H-labeled cDNA's at the C_rt values indicated. ^{32}P-labeled cDNArep was included as a standard. Symbols: cDNAsrc, ×; cDNArep, ○, △; cDNAenv, ▲; cDNAmyc, ●. (B) Hybridizations were performed as in (A) with ^{3}H-labeled cDNA's and 20 ng of viral RNA at the highest C_rt. ^{32}P-labeled cDNArep was included as a standard. Symbols: cDNAsrc, ×; cDNArep, ○, △; cDNAenv, ▲; cDNAmyc, ●. $C_rt_{1/2}$ values on curves for *env* and *rep* have been marked by (+) symbols to indicate their similar experimental value. (C) Endogenous reverse transcriptase activity of OK10 QB5-defective particles. Virions from 1 liter each of OK10 QB5 and SR-RSV-E culture supernatants were purified separately as described in reference 15. Each point contained 0.053 μg of OK10 QB5 and 0.017 μg of SR-RSV-E virion proteins. Incubation were performed for 2 h at 37°C in the presence of increasing concentrations of Nonidet P-40. Symbols: SR-RSV-E, ○; OK10 QB5, ●.

was made in these cells (20); (ii) the 96% annealing with cDNApol indicated the presence of most or all of the *pol* gene, although this number may be an overestimation due to some *gag* sequences being present in this probe (see Table 1); (iii) cDNAenv annealed only to 45%, indicating that the *env* gene of OK10 was partially missing, or quite diverged (12), from its ALV homolog; (iv) the homology of OK10 RNA in the *c* region was much higher than for MC29, (and also for MH2; unpublished data), 35% as tested by this method.

We next tested the amount of OK10 RNA produced within the OK10 QB5 cells as well as in the culture fluid harvested from them. Figure 2A indicated about 330 copies of intracellular viral RNA ($C_rt_{1/2}$, 120 mol·s liter^{-1}), and the colinearity of the curves obtained with cDNAmyc and cDNAenv showed that these sequences were present in stoichiometric amounts. Figure 2B showed a similar pattern for the virus produced in the culture fluid of these cells. From the yield of RNA, we deduced a production of ca. 5×10^7 virions per ml. As reported previously (20), this virus was noninfectious and had no polymerase activity (Fig. 2C).

Proviral DNA of OK10 in the QB5 cells. According to the ALV relatedness determined above for OK10 viral RNA, we made the assumption that some of the *Eco*RI sites present in the DNA of several strains of RSVs and ALVs (25) (Fig. 1B) could be conserved in OK10. Our assumption was correct (Fig. 3). The *Eco*RI pattern of digestion of OK10 QB5 cellular DNA yielded three fragments of 2.7, 1.6, and 0.8 megadaltons (Md) hybridizing with cDNArep. These fragments were further analyzed by using DNA probes to the *gag*, *pol*, and *env* (3′ or 5′ part) genes, prepared as described in the legend to Fig. 1B, as well as cDNA5′ and cDNAmyc, prepared as described by Friedrich et al. (8) and Sheiness et al. (26), respectively. These probes exhibited the expected specificities as documented by their hybridization with northern blots containing polyadenylated RNA extracted from chicken fibroblasts infected by Rous-associated virus-2 (RAV-2) and chicken erythroblasts (HD3) transformed by avian erythroblastosis virus (AEV) (Fig. 1C; 23). The *gag* probe did not hybridize to the RAV-2 or AEV subgenomic RNAs. The *pol* probe contained probably about 160 bases related to *gag* (sequence data from D. Schwartz, R. Tizard, and W. Gilbert [personal communication], referred to throughout this paper), but did not hybridize with AEV RNA, which lacks these sequences (16, 27, 33), or to viral *env* sequences. The *env* 5′ probe contained about 190 bases into the *pol* gene (sequence data cited) and did not hybridize with AEV RNA, unlike the *env*3′ probe, which scored a different set of sequences. Finally,

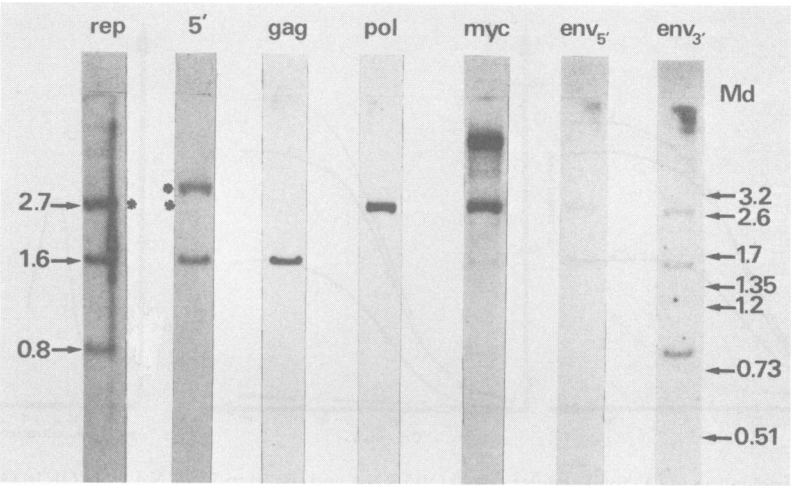


FIG. 3. *Eco*RI mapping of OK10 QB5 proviral DNA. DNA from OK10 QB5 cells was isolated and digested by *Eco*RI as described in the text. DNA fragments separated by electrophoresis in a 0.8% horizontal agarose gel were hybridized to different ^{32}P probes. The DNA fragments used as size markers were obtained by digestion with *Kpn*I, *Sst*I, and *Eco*RI of the clone containing the *Sac*I DNA fragment of PR-RSV-A. The probes used are listed across the top. Note that the band at 2.7 Md with the rep probe is different from the one seen at 3.2 Md with the 5′ probe (*).

cDNAmyc did not hybridize with the RNA of RAV-2-infected fibroblasts (Fig. 1C).

Using these probes with Southern blots containing *Eco*RI digests of OK10 QB5 cell DNA, we reached the following conclusions (Fig. 3). (i) As expected, the 1.6-Md fragment hybridized with cDNA5′ and with the *gag* probe only, thus representing probably the 5′ part of the OK10 genome. (ii) The 2.7-Md DNA fragment hybridized with the *pol* probe and DNAmyc but not with cDNA5′ or to the *gag* or the two *env* probes. This fragment thus appeared to represent the internal part of the OK10 genome. This indicated that the *myc*(o) sequences, if contiguous, had to be located next to the 3′ part of *pol* or within *pol*. Furthermore, that this fragment showed no hybridization with the two *env* probes indicated that it did not contain a significant part of the *env* sequences at the left of the *Eco*RI site in the *env* gene of ALV. This is compatible with the liquid hybridization value of 45% observed with OK10 RNA and ^{3}H-labeled cDNAenv (Fig. 2A and B; Table 1) (unless these sequences were so diverged that they were no longer detected under Southern blot conditions). (iii) The 0.8-Md DNA fragment hybridized only with the *env*3′ probe and thus represented the 3′ part of the viral DNA. The size of this fragment coincided with the 3′ *Eco*RI fragment observed for an ALV (25) and indicated that this *Eco*RI site probably also was conserved in OK10 (unless by coincidence the site was lost and a new site appeared in its vicinity). We concluded from these experiments that the usual *Eco*RI sites of RSVs and ALVs (i.e., Pr-C or Schmidt-Ruppin strain [SR-A]) were conserved in OK10, including the one in the large terminal repeats (LTR). Of the two bands seen with cDNA5′, one has been shown to represent the 5′-*gag* fragment of 1.6 Md. The other band at 3.2 Md, larger than 2.7 Md, did not hybridize to any other viral probes and thus represented the 3′ LTR fragment extending into the cellular DNA. This indicated that the QB5 cells contained only one provirus. The *myc*-containing band at high molecular weights represented the cellular equivalent (*c-myc*) DNA since it did not hybridize to any of the viral probes.

Heteroduplex mapping of OK10 RNA. The observations described above favored the following structure of OK10 viral RNA: 5′-*gag*-(Δ*pol-myc*(o))-*env*3′-*c*-3′. Further investigations were undertaken. We built heteroduplexes between the OK10 viral 8.0-kb RNA and the 6.8-kb *Sst*I DNA fragment of PR-RSV-A and analyzed the hybrids by electron microscopy. From sequence data, the *Sst*I (or *Sac*I, isoenzyme) DNA fragment of PR-RSV-A lacks ca. 250 bases at the 5′ end of the genome, left of the *gag* gene, and contains the whole *pol* and *env* genes, but the *c* region is totally missing, as are the 5′ and 3′ large terminal repeats (Fig. 1B). We detected in the electron microscope only one type of heteroduplex molecule. All hybrids contained reproducibly a single large substitution loop flanked by two double-stranded regions of 4.33 ± 0.065

and 0.98 ± 0.06 kb (Fig. 4). The two strands of the loop were of similar sizes, 1.43 ± 0.11 and 1.55 ± 0.07 kb, indicating that the inserted sequences were comparable in length to the deletion of ALV-related sequences (we could not determine which of the strands was RNA). These observations agreed with the results of the Southern blots and with the size of OK10 RNA as well as with the 90% annealing of OK10 mRNA with cDNAmyc, representing a com-

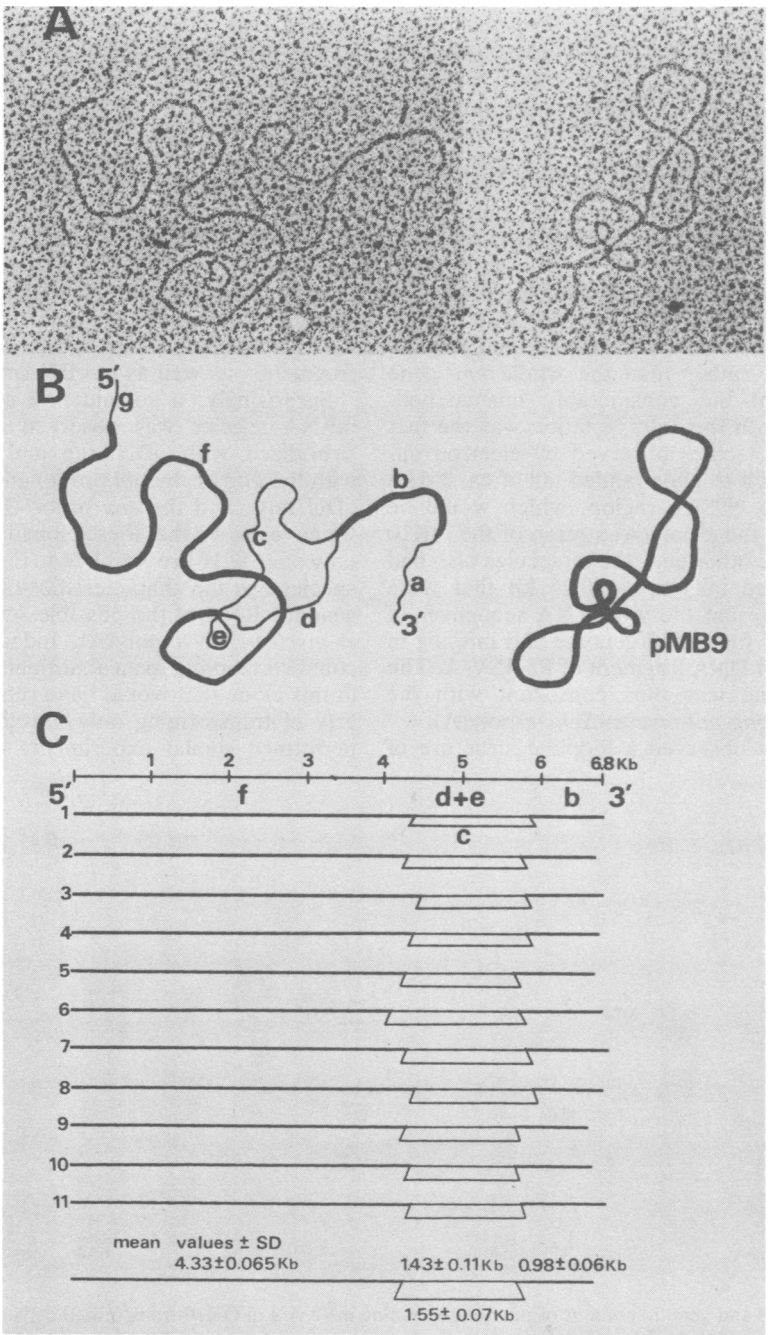


FIG. 4. Electron micrograph of OK10 RNA PR-RSV-A DNA heteroduplexes. One typical hybrid molecule (A) observed in the electron microscope was drawn on a transparency (B). In (C), we present the measurements made on 11 molecules (*n*), using pMB9 as a length standard. Standard deviation = Σ (variations from mean value)/*n*.

plexity of 1.6 kb (26). Thus, the *myc*(o) sequence in OK10 appeared to be very similar to the corresponding *myc*(m) sequence in MC29 (13). We next had to order the two double-stranded stretches of 4.33 and 0.98 kb, deciding between the structures *gag*-Δ*pol*-*myc*(o)-Δ*env* and Δ*gag*-*myc*(o)-Δ*pol*-Δ*env*. Since OK10 virus makes the Pr76 polyprotein (20), its *gag* gene must be complete and is expected from sequence data of RSV to amount to 2.1 kb (sequence data cited above) minus the ca. 0.25 kb of missing sequences of the *Sac*I fragment in the *gag* region, a total of ca. 1.85 kb. Such a size is incompatible with the size of the small fragment. Thus, it had to be included with the *pol* sequences in the 4.3-kb double-stranded stretch. It followed that the 0.98-kb segment had to correspond to the *env* sequences present in OK10, confirming the hybridization data that the 5′ half of the *env* gene was missing, rather than the whole *env* gene being present but considerably mismatched. Consistent with this interpretation was the fact that most molecules observed by electron microscopy had a single-stranded tail of ca. 0.6 kb following the 0.98-kb region, which would be expected for the *c*-poly(A) stretch of the OK10 RNA. On the other end, the molecules also had single-stranded tails up to 0.25 kb that were likely to represent the viral RNA sequences at the 5′ end of the OK10 genome and missing in the 6.8-kb *Sst* DNA fragment of Pr-RSV-A. The OK10 genome was thus consistent with the structure 5′-*gag*-*pol*-*myc*(o)-Δ*env*-*c*-poly(A).

We always observed a looplike structure of 0.37 to 0.1 kb at the 5′ end of the insertion-substitution loop. This observation was not studied further, but could indicate that the OK10 genome had a small deletion somewhere at the end of the *pol* gene.

Subgenomic viral mRNA in OK10 QB5-transformed cells. Having determined the organization of the OK10 genome, we next analyzed the strategy of viral expression in OK10-transformed cells.

Poly(A$^+$) QB5 cell RNA was fractionated by agarose gel electrophoresis and transferred to activated diazobenzyloxymethyl paper. The blots were then hybridized with different labeled probes. Two viral RNA species accumulated in these cells (Fig. 5A). The genomic OK10 RNA of the expected 8.0-kb size (30) hybridized to the different ^{32}P-labeled probes tested, cDNArep, cDNA5′, and the *gag* and *pol* nick-translated fragments, as well as to cDNAmyc.

Surprisingly, a second *myc*-containing viral mRNA species was observed at 3.6 kb that hybridized with cDNArep and cDNA5′ (not with the *gag* or the *pol* probe) and strongly with cDNAmyc and the *env* probe (Fig. 5A). Since we have shown that these cloned cells contained only one OK10 proviral DNA (Fig. 3), the 3.6-kb species had the characteristics of a spliced subgenomic RNA of the possible structure 5′ leader-*myc*(o)-*env*3′-*c*-poly(A). Indeed, our finding could correspond to an abnormal OK10 provirus in this clone that would have retained the property of transforming only fibroblasts. We thus performed similar experiments with a chicken

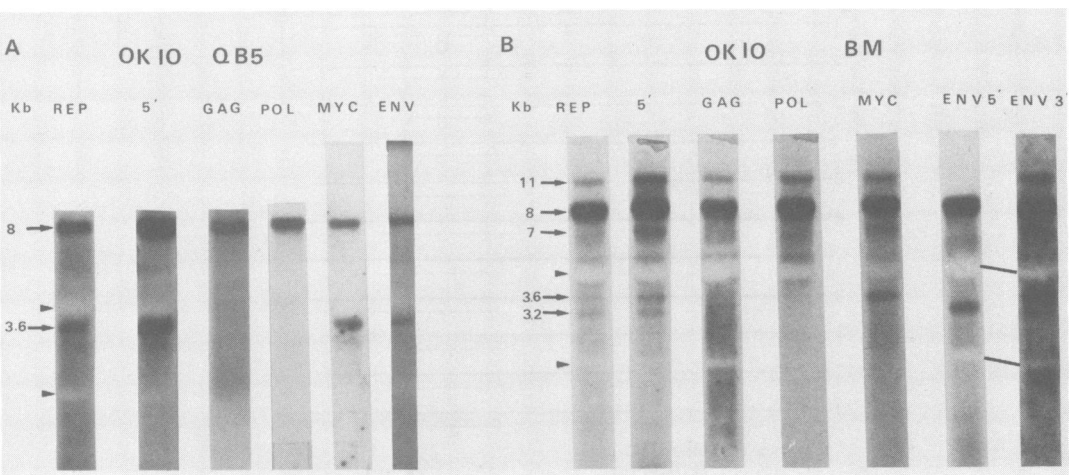


FIG. 5. Size and genetic content of *myc*(o)-containing mRNA's in OK10-transformed cells. Poly(A$^+$) RNAs were denatured by glyoxal treatment, separated on agarose gels, transferred to diazobenzyloxymethyl paper, and hybridized with the ^{32}P probes DNAgag, DNApol, DNAenv3′, and DNAenv5′ were nick translated and are described in the legend to Fig. 1. rRNA's from chicken fibroblasts and from *E. coli* were used as standard length markers. (A) OK10 QB5 intracellular poly(A$^+$) RNA. Probes are listed across the top. (B) OK10 BM intracellular poly(A$^+$) RNA.

bone marrow cell line (OK10 BM) obtained by in vivo tranformation with the original OK10 virus (kindly provided by N. Oker Blom and L. Hortling). Although this cell line produced infectious virus (19), we observed again, with the 8.0-kb band and among other species that will be analyzed further below, the other OK10-derived mRNA species of 3.6 kb (Fig. 5B) that hybridized as expected to the different probes used: to cDNArep and cDNA5′, not to the *gag* or the *pol* probe, to cDNAmyc, not to *env*5′, and to *env*3′.

Supergenomic mRNA in OK10 BM cells. Although quite complicated, the pattern of viral transcripts (Fig. 5B) present in the OK10 BM cells presented other interesting features besides the 8.0- and 3.6-RNAs just described.

Two mRNA's indicated the presence of a helper virus in these cells. One species at 8.0 kb coincided with the OK10 genomic RNA and could be shown with the ^{32}P-labeled *env*5′ probe, although these sequences are absent from the OK10 genome. This helper produced, as expected, a subgenomic mRNA at 3.2 kb that reacted with cDNArep, cDNA5′, and not with the probes to the *gag*, *pol*, and *myc* sequences, but again with *env*5′ and *env*3′, thus representing probably the spliced *env*-mRNA (17).

Two other viral species that contained *myc*(o)-related sequences were also observed in these cells. An 11.0-kb band reacted to the same probes as the 8.0-kb OK10 RNA. Similarly, a 7.0-kb species reacted with the same probes as the OK10 subgenomic 3.6-kb mRNA. Thus, these two bands were OK10 related but of abnormally large size. We thus hypothesized that they represented species that extend into the cellular DNA where they were modulated (at termination or initiation or both) by cellular signals. Despite this, the 7.0-kb species was apparently spliced, possibly from the 11.0-kb mRNA. These data and hypotheses are summarized in Fig. 6. Alternatively, these cells could contain an additional OK10-like provirus of larger size.

DISCUSSION

Genetic structure of OK10. Although OK10 does not contain nucleotide sequences related to the *src* gene of RSVs (22), this virus, like all known MC29-type viruses, can transform specific hematopoietic target cells in vitro (2) as well as avian fibroblasts (9). Its ability to produce a transformed phenotype in these cells has been associated with the presence in its genome of a unique nucleotide sequence termed *myc*(o) (22). This sequence has its counterpart in normal

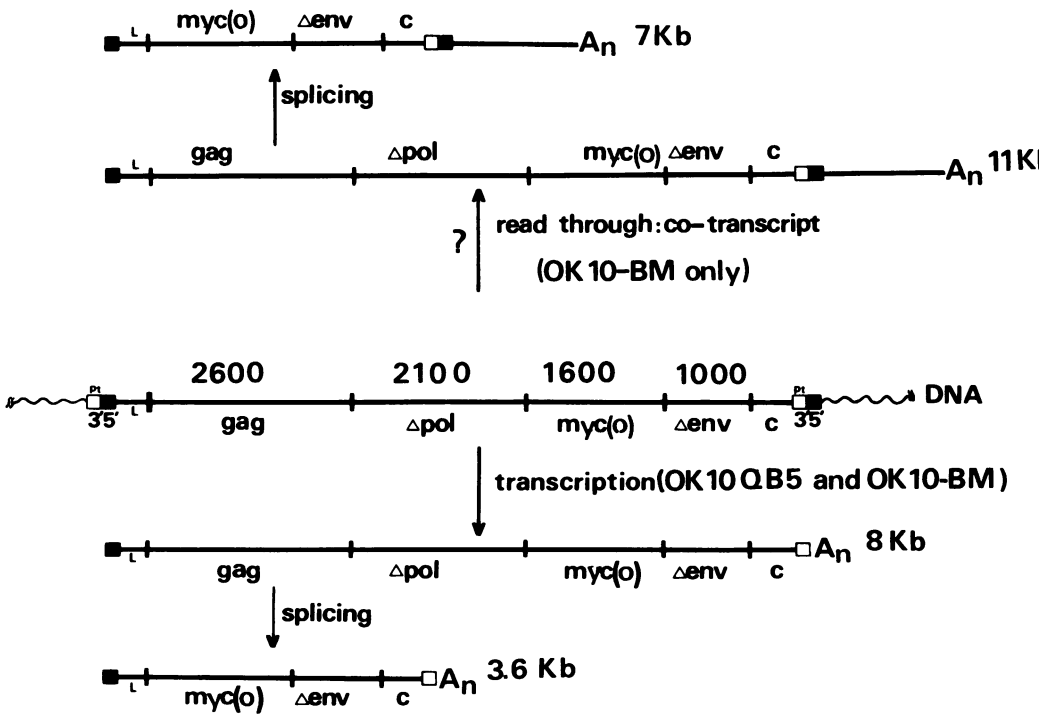


FIG. 6. Transcription and processing of OK10 mRNA's. This model has been built to explain the results obtained and presented in Fig. 5, favoring the 3′ cellular-extended 11.0-kb RNA species in OK10 cells. Pt, Putative transcription promoter and termination signals. The 11-kb supertranscript could as well extend upstream or at both ends of the OK10 provirus.

cell DNA (22) and is unrelated to any known RSV/ALV genes (30). Our studies showed that the OK10 *myc*(o) allele of the putative *myc*-transforming gene formed a continuous stretch of 1.43 to 1.55 ± 0.07 kb in the 8.0-kb-long OK10 genomic RNA extracted from defective viral particles produced by OK10 QB5 cells. The *myc*(o) sequence is flanked by sequences that are isogenic to part or all of the *gag*, *pol*, and *env* genes of ALVs.

The *gag* gene is fully represented in OK10 genomic RNA. This finding fits with the fact that OK10-defective viral particles containing the *gag* gene products p27, p19, and p15/p12 (20) are produced by OK10 QB5 cells. The *pol* gene is deleted by about 0.5 kb at the 3′ end, and that could explain why no polymerase activity could be detected in OK10 QB5-defective viral particles (Fig. 2C). The *env* gene is deleted by ca. 0.9 kb at the 5′ end, and again this finding has been corroborated by the absence of any detectable *env* gene-related proteins in OK10 QB5-defective particles (20). Moreover, in OK10 QB5 cells, two proteins were identified, the OK10 76K, antigenically related to the *gag* gene, and the OK10 200K, antigenically related to the *gag* and *pol* genes but not to the *env* gene product (20). Tryptic peptide mapping showed that the OK10 76K protein was almost indistinguishable from the *gag* gene product $Pr76^{gag}$ of an ALV and that the OK10 200K polyprotein contained all but one methionine tryptic peptide of $Pr180^{gag\text{-}pol}$, the missing peptide being a *pol* peptide (20). In short, the genetic structure of OK10 as probed by hybridization and heteroduplex mapping, i.e, 5′-*gag*-Δ*pol*-*myc*(o)-Δ*env*-*c*-poly (A), is compatible with determinations by T1 fingerprinting (5) and with immunological analyses of the OK10-coded proteins present in OK10 QB5 cells and the OK10 QB5-defective particles. We have no clear explanation for the presence of the 0.37-kb looplike structure at the 5′ end of the deletion-substitution loop observed reproducibly in the heteroduplexes studied; it could represent an additional deletion in the OK10 genome, probably near the end of the *pol* gene. The OK10 200K polyprotein covers the total OK10 *gag*-Δ*pol*-*myc*(o) coding capacity (ca. 6.2 kb) and has been described as a fusion product between the OK10 *gag* and *pol* gene-coded polypeptides and the *myc*(o) cellular information. Similarly nonproducer quail fibroblasts transformed by the other MC29-related defective leukemia viruses MH2 and CMII also contain *gag*-related polyproteins which were shown to be fusion products of the *gag* gene-coded polypeptides and possibly the *myc* cellular derived information (4, 11, 14). Therefore, the oncogenic potential of MC29-related viruses might be expressed through *gag-myc* or *gag*-Δ*pol-myc* proteins translated from genome-size mRNA's.

Subgenomic mRNA of OK10. It must be pointed out that, in the case of OK10, the amount of 200K polyprotein observed is very low and similar to the precursor of the polymerase $180^{gag\ pol}$ polyprotein observed in ALV-infected cells. This is about 20 to 50 times less abundant than the *gag-onc* fusion protein in MC29- or CMII-infected cells, although the OK10 virus is, like MC29 or CMII, acutely transforming and similar to that of MC29. We raise here the possibility that the OK10-transforming protein could be made in another way. The 3.6-kb subgenomic mRNA observed in abundance in the OK10-transformed cells studied represents a candidate of choice for such a function with its structure 5′-*myc*(o)-Δ*env*-*c*-poly(A) where the ALV-related sequences represent, at most, ca. 2.2 kb (see Results). Thus, this RNA is likely to contain 3.6 minus 2.2 = 1.4 kb of *myc*(o), which represents most or all of the *myc*(o) of OK10. The interesting implication of these experiments is that they allow us to think that a given single transforming gene information could be translated in a *gag-onc* fusion protein (in MC29) as well as in a non-*gag* protein (in OK10), the important feature for transformation being, then, the efficient translation of the active gene in the correct phase no matter by which strategy of mRNA expression. Alternatively, the 3.6-kb RNA only would code for a competent transforming protein in OK10 virus, the P200 polypeptide being inactive because of an early termination or an incorrect frame. The latter possibility is less likely, since one methionine-containing tryptic peptide related to the MC29 P110 polyprotein in the *myc* region has been found for OK10 P200 (20). The structure of the 3.6-kb spliced mRNA will have to be determined more precisely to determine how the splice occurs. If indeed the *myc*(o) sequence is next to the 5′ leader, it must contain a splice acceptor site compatible with the donor site at the 5′ end of the viral RNA. Another possibility is that the splice signal of the *env* gene is still conserved and used and that the insertion-deletion occurred all within the *env* gene. The structure of this mRNA would then be 5′ leader-5′-*env* splice acceptor site-*myc*(o)-*env*3′-*c*-poly(A). Such a possibility would then also explain the small loop observed at the *pol*-*env* junction in the heteroduplex shown in Fig. 4. The loop would represent the DNA corresponding to the 3′-end *pol* deletion of OK10 and would be followed by a small double-stranded stretch corresponding to the 5′ end of *env*, presumably then conserved in OK10 and located at the 5′ side of the *myc* insertion but too small to anneal with our *env*5′ probe. This hypothesis remains to be documented further.

In conclusion, OK10 virus presents at least two interesting features compared with MC29. (i) It is produced as a noninfectious form without helper in the culture fluid of nonproducer cells. (ii) It produces in fibroblasts, but also in in vivo transformed myeloid cells (19), a subgenomic spliced mRNA containing most or all of the *myc*(o) sequences. Its putative transforming protein could thus be a non-*gag* polypeptide rather than a *gag-myc* fusion protein as in MC29. Preliminary findings (S. Saule, M. B. Raes, C. Lagrou, and D. Stehelin, unpublished data) indicate that MH2-transformed cells also contain a subgenomic mRNA species that hybridizes with cDNAmyc.

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