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Mutations within the 5′ Half of the Avian Retrovirus MC29 v-*myc* Gene Alter or Abolish Transformation of Chicken Embryo Fibroblasts and Macrophages

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Avian myelocytomatosis virus MC29 induces a wide variety of neoplastic diseases in infected birds and transforms cells of the macrophage lineage as well as fibroblasts and epithelial cells. A biological and biochemical analysis, carried out on a series of in-frame insertion and deletion mutations within the *gag-myc* gene of MC29, revealed several mutations within the 5′ portion of the v-*myc* gene that encode proteins either completely defective for transformation or compromised in their ability to transform chicken embryo fibroblasts but not macrophages. Mutations within the 3′ end of the v-*myc* gene which disrupt sequences encoding the basic/helix-loop-helix region were defective for transformation of both fibroblasts and macrophages. Eight variants were cloned into the replication-competent avian expression vector RCAS. Analysis of cells infected with transformation-defective, replication-competent viruses confirmed the expression of functionally defective Myc proteins. Further, expression of the transformation defective variant *dl*91-137 in chicken fibroblasts inhibited subsequent transformation by wild-type MC29. The results reported herein support the hypothesis that Myc proteins function as regulators of transcription in a variety of cell types and clearly point out the necessity of putative regulatory domains within the amino-terminal half of the Myc protein.

Avian myelocytomatosis virus MC29 (8, 48) induces a wide variety of neoplastic diseases in infected birds, including myelocytoma, carcinomas, and sarcomas (4, 37). In cell culture, the effects of MC29 parallel those observed in animals; MC29 transforms myeloid cells of the macrophage lineage as well as fibroblasts and epithelial cells (18, 20, 21). The myeloid targets of MC29 transformation resemble mature macrophages by a number of morphological and biochemical criteria (5, 21, 22, 30).

The v-Myc protein encoded by MC29 and that encoded by the cellular homolog c-*myc* are localized in the nucleus (1, 3, 16, 24, 43). Both v-*myc* and c-*myc*-encoded proteins are phosphorylated on serine and threonine residues (24, 49) and have a short half-life of 20 to 30 min (25). Comparison of the deduced amino acid sequences of chicken, mouse, and human c-Myc have revealed several interesting structural motifs (Fig. 1A). The amino-terminal half of the protein has three regions, referred to as Myc boxes I, II, and III, which are highly conserved among c-*myc* genes of different species and are present in all Myc family members (13). In addition to the Myc boxes, the amino-terminal half of the v-Myc and c-Myc proteins also contain a region rich in glutamine residues and two short stretches which exhibit an unusually high content of proline residues (Fig. 1A). The interior of the protein is rich in acidic residues and contains limited sequence similarity to regions of the Myb and adenovirus E1A proteins (47). Glutamine, proline, and acidic regions have been described in several proteins which function as *trans*-acting regulators of transcription (36). The carboxyl-terminal one-third of the Myc protein is highly conserved among Myc family members and is enriched in basic residues. The carboxyl-terminal portion of the Myc protein contains a basic region and a helix-loop-helix motif (29, 38, 40), which are followed by a leucine zipper motif (32) (Fig. 1A). These sequence motifs are characteristic of a number of proteins implicated in regulation of gene expression. Their presence in c-Myc and v-Myc proteins contributes to the notion that Myc proteins are intimately involved in gene regulation (for further discussion, see reference 33).

The MC29 viral genome encodes a 110-kDa protein, $P110^{gag\text{-}myc}$. $P110^{gag\text{-}myc}$ is composed of 452 amino-terminal residues encoded by viral *gag* sequences, linked to 7 amino acid residues from the c-*myc* 5′ untranslated region, followed by 416 amino acids encoded by exons 2 and 3 of the c-*myc* locus (2, 50). Natural variants of MC29 which have deletions in the *myc* coding region resulting in altered transforming properties (8, 48) have been isolated (7). The variant MC29-10-A lacks residues 224 through 280 (a deletion of the acidic domain) and is defective for macrophage transformation but retains the ability to transform fibroblasts (9). Heaney et al. (26) described site-directed mutations in the MC29 v-*myc* gene that encode proteins with deletions of 11 to 84 amino acids near and including the acidic region in the middle of the Myc protein. Each of these variants was able to transform chicken fibroblasts but was defective for the transformation of chicken macrophages. These variants also exhibited an interesting species-dependent phenotype, in that viruses unable to transform chicken macrophages (or induce tumors in chickens) were able to induce transformation of quail macrophages and induce tumors in newborn quail (6).

In this report, we describe additional regions of the v-Myc protein that are functionally important for the transforming activity of $P110^{gag\text{-}myc}$. A biological and biochemical analysis carried out on a series of in-frame insertion and deletion

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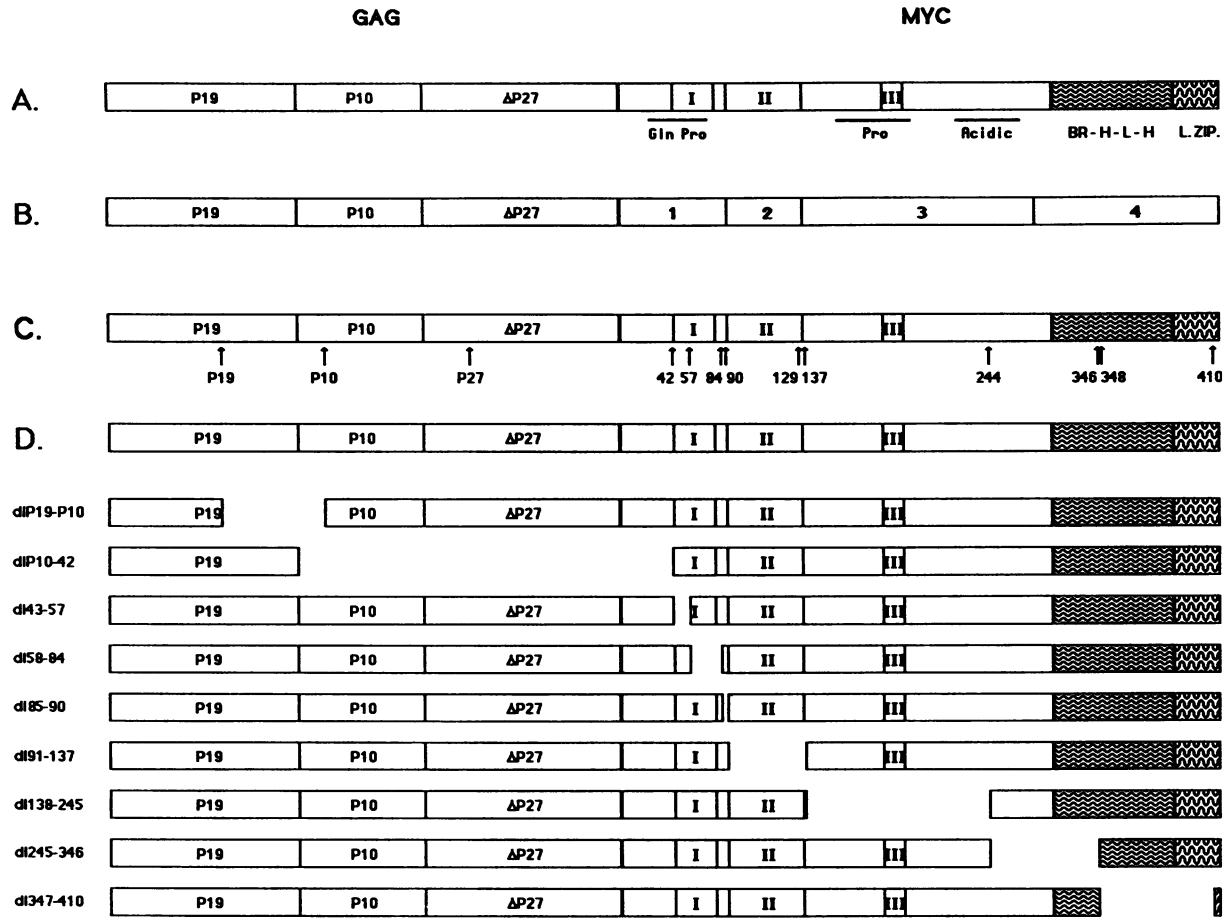

FIG. 1. Schematic illustration of the structural motifs and functional regions of Myc proteins and the positions of insertion and deletion mutations. (A) Positions of the Myc boxes and other structural motifs predicted from the amino acid sequences of Myc proteins. (B) Functional regions of Myc proteins defined by Stone et al. (53). (C) Positions of the linker insertion mutations, indicated by arrows (see text). (D) Relative positions of deletion mutations, denoted by a gap in the diagram.

mutations within the *gag-myc* gene showed that several mutations within the 5′ portion of the v-*myc* gene encode proteins either completely defective for transformation or compromised in their ability to transform chicken embryo fibroblasts but not macrophages. Mutations within the 3′ end of the v-*myc* gene, encoding the basic/helix-loop-helix region, were defective for transformation of both fibroblasts and macrophages. To further investigate the properties of defective variants, eight were subcloned into the replication-competent avian expression vector RCAS (28). Cells infected with transformation-defective, replication-competent viruses readily expressed functionally defective Myc proteins. Further, transfection of chicken fibroblasts with the *dl*91-137 variant blocked subsequent transformation of those cells by wild-type MC29. Our results provide additional evidence for the hypothesis that Myc proteins function as regulators of transcription in a variety of cell types. In addition, our data clearly point out the necessity of putative regulatory domains within the amino-terminal half of the Myc protein.

MATERIALS AND METHODS

Cells, viruses, and plasmids. Chicken embryo cell (CEC) cultures were prepared from 10-day-old embryos (SPAFAS, Norwalk, Conn.) as described previously (12) and maintained in Dulbecco's modified Eagle medium plus 4% fetal calf serum, 2% chicken serum, and 1% penicillin-streptomycin (GIBCO/Life Technologies, Grand Island, N.Y.). COS-1 cells (19) were maintained in Dulbecco's modified Eagle medium plus 10% fetal calf serum and 1% penicillin-streptomycin. Plasmid pMC29 (26) contains a nonpermuted copy of the MC29 P110$^{gag\text{-}myc}$ coding sequence flanked by avian retroviral long terminal repeat sequences and was modified by removal of the unique *Bgl*II site in the polylinker sequence (26). Plasmid pΔMst contains a subgroup A, transformation-defective provirus derived from the Prague A strain of Rous sarcoma virus (RSV) (26). Plasmid pΔMst(B) contains a subgroup B proviral genome generated by substitution of the envelope (*env*) gene sequences of pMst(A) with *env* sequences from a proviral clone of Prague B RSV. For transfection experiments, calcium phosphate-DNA precipitates (12, 23) were prepared by mixing either pMC29 or mutant pMC29 DNA with pΔMst(B) DNA (4:1 ratio), using a total of 1 μg of DNA per 60-mm culture dish. The cells were maintained at 37°C and passaged every 2 to 3 days. Virus stocks were prepared from the supernatants of transfected cultures 14 to 21 days posttransfection. COS-1 cells were transfected by calcium phosphate precipitation (23)

with the glycerol shock modification (26), using 10 μg of viral DNA per 100-mm culture dish.

Isolation of linker insertion and deletion mutations in the P110$^{gag\text{-}myc}$ coding sequence. For linker insertion mutagenesis (27, 54), 10 μg of pMC29 DNA was digested with 8 U of *Alu*I (New England Biolabs, Beverly, Mass.) in the presence of ethidium bromide (10 μg/ml) for 60 min at 37°C. The full-length linear plasmid DNA was purified by agarose gel electrophoresis (34), and the DNA was treated with 10 U of calf intestinal alkaline phosphatase (Boehringer Mannheim, Indianapolis, Ind.). Following ligation with a 250-fold molar excess of a *Bgl*II linker (New England Biolabs) and transformation of *Escherichia coli*, ampicillin-resistant colonies were picked, and the plasmid DNA was screened for the insertion of a *Bgl*II restriction site. Insertion mutations at 13 of 15 *Alu*I restriction sites in the P110$^{gag\text{-}myc}$ gene of MC29 were isolated, including insertions at 10 of the 11 sites in the v-*myc* coding region (arrows in Fig. 1C). The unique *Bgl*II sites from pairs of insertion mutations were used to generate in-frame deletions spanning the P110$^{gag\text{-}myc}$ coding sequence (Fig. 1D). Generation of the *dl*P19-P10 and *dl*245-346 deletions required removal of the *Bgl*II termini with mung bean nuclease prior to ligation, and *dl*138-245 was generated by filling in the *Bgl*II ends with the Klenow fragment of *E. coli* DNA polymerase I prior to ligation. The sequences for all insertion and deletion mutations were confirmed by dideoxy DNA sequencing.

PCR. The polymerase chain reaction (PCR) was performed by using a Gene Amp kit (Perkin-Elmer Cetus, Norwalk, Conn.). Plasmid DNA (1 μg) and oligonucleotide primers (25-mers, 1 μM) were initially denatured at 92°C for 3 min. The samples were then subjected to 30 rounds of amplification.

Cloning of *myc* variants into the RCAS vector. A three-step cloning strategy was used to clone the *myc* portion of wild-type and variant MC29 DNA into the replication-competent avian vector RCAS (28) (Fig. 2). Synthetic oligonucleotide primers, a 5′ *Nco*I site, and a 3′ *Hin*dIII site were introduced by PCR (Fig. 2). Insertion of an *Nco*I site altered the fourth nucleotide of the *myc* coding sequence from C to G, resulting in a proline-to-alanine change at residue 2. Several regions of high G+C content within the *myc* gene significantly reduced the yield of PCR product. Since the largest region of high G+C content was missing in the *dl*138-245 variant, this DNA was used as template for the PCR reaction (Fig. 2). The second step involved cloning of the PCR product into the shuttle vector Cla12Nco (28) and isolation of plasmid p*dl*138-245-C (Fig. 2). Plasmid pMyc-C was generated by joining *Pst*I-*Sph*I fragments from p*dl*138-245-C and the original pMC29 plasmid (Fig. 2). DNA fragments with insertion and deletion mutations in the *myc* coding sequence were then introduced into pMyc-C, and the sequences of the plasmids were confirmed by DNA sequence analysis. The final step of the cloning procedure (Fig. 2) involved transfer of the *Cla*I fragment, containing the *myc* gene, to the RCAS vector (28).

CEC infection and growth in soft agar. Cells were seeded at a density of 5×10^5/60-mm plate and allowed to attach for at least 6 h prior to infection. Dilutions of MC29 or MC29 variant virus stock were added to the cell monolayer, and cultures were incubated at 37°C for 1 h, refed, and maintained at 37°C for 24 h. To assess growth in soft agar, the cells were trypsinized and plated in soft agar at a density of approximately 2×10^5/60-mm dish, using a modification of the procedure of Pierce and Aaronson (44). The plates were incubated at 37°C, and 0.5 ml of growth medium was added every 5 days. Colonies formed within 2 to 3 weeks after plating.

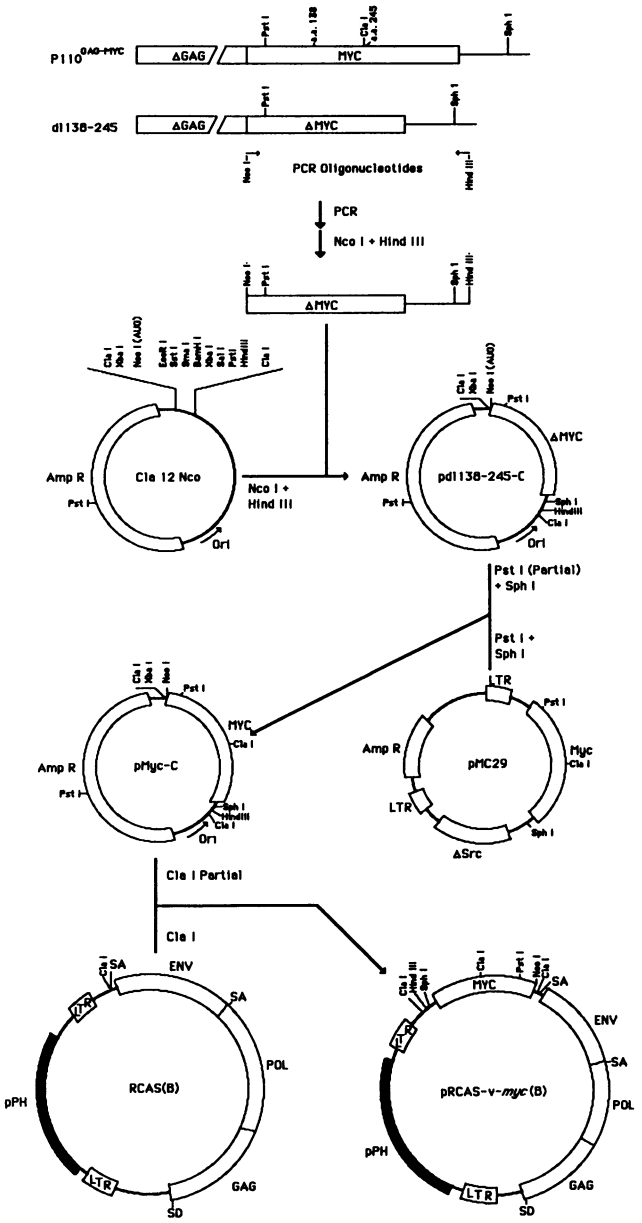

FIG. 2. Cloning of MC29 v-*myc* and v-*myc* variants into RCAS. The PCR strategy and cloning strategy are described in Materials and Methods. LTR, long terminal repeat; SA, splice acceptor; SD, splice donor.

Western immunoblot analysis of proteins. Cell extracts were prepared as follows. Cells were washed twice with phosphate-buffered saline, lysed in 1 ml of boiling Laemmli sample buffer (31), passed three times though a 26-gauge needle, boiled for 3 min, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration of each lysate was measured by using the BCA protein assay (Pierce, Rockford, Ill.). Approximately 50 μg of protein was loaded for each sample. Following electrophoresis, the proteins were transferred to nitrocellulose filters (Schleicher Schuell, Keene, N.H.), and

the filters were blocked with BLOTTO (5% nonfat dried milk in TBS [10 mM Tris, 150 mM NaCl, pH 7.2]) for at least 30 min at 25°C. The filters were incubated for 2 h at 25°C in 10 ml of BLOTTO containing 10 μl of primary antibody, washed five times with TBS, and then incubated with 10 ml of BLOTTO containing 100 μl of ^{125}I-labeled (10 μCi/ml) protein A (Amersham, Arlington Heights, Ill.) for 1 h. The filters were washed five times with TBS, air dried, and autoradiographed.

RESULTS

MC29 insertion and deletion variants express stable Gag-Myc fusion proteins. To confirm the expression and determine the size of the proteins encoded by individual MC29 variants, cell lysates from transfected COS-1 cells were subjected to Western blot analysis (Fig. 3). Extracts from cells transfected with the wild-type DNA contained a 110-kDa protein reactive with the anti-Gag serum (Fig. 3A, lane 2; Fig. 3B, lane 2). Extracts from cells transfected with each of the pMC29-derived variant plasmids also contained an immunoreactive protein, indicating that the *gag-myc* reading frame was intact and that the mutant constructs were not otherwise defective in their ability to direct the synthesis of variant Gag-Myc proteins. The mobility of the Gag-Myc proteins encoded by the insertion variants was similar to that of the wild-type P110$^{gag\text{-}myc}$ in all cases (Fig. 3A, lanes 3 to 12). Western blot analysis of extracts prepared from COS-1 cells transfected with individual deletion variants revealed Gag-Myc proteins of the predicted electrophoretic mobility (Fig. 3B, lanes 3 to 11). Two notable exceptions were the *dl*43-57-encoded protein (Fig. 3B, lane 5) which exhibited a slightly faster mobility than expected, and the *dl*347-410 protein (Fig. 3B, lane 11), which had a slightly slower mobility than expected from a protein with a net deletion of 60 amino acids. The deletion in the latter construct removed much of the basic region of the protein, which may explain the aberrant migration pattern.

Cell-type-specific transformation of CEC is altered by mutations in MC29 Gag-Myc proteins. Morphological transformation of CEC cultures occurs within 10 to 14 days after cotransfection of cultures with pMC29 and pΔMst(B) [or pΔMst(A)] DNA (Fig. 4). MC29-transformed cells were rounded and more refractile than their normal counterparts (Fig. 4A and B), grew to higher density, and exhibited increased acidification of the growth medium (data not shown). MC29-infected cells also formed colonies in soft agar (Fig. 4F), whereas helper virus-infected cells did not (Fig. 4E). When primary CEC cultures were infected with pMC29 and pΔMst(B), two different types of colonies were observed (Fig. 5): small, compact colonies (Fig. 5B) and large, diffuse colonies (Fig. 5C). Wild-type MC29 induced the formation of approximately three times as many small colonies as diffuse colonies (Table 1). The small colonies consisted of transformed fibroblasts. When these colonies were picked from the soft agar and plated in microtiter dishes, the cells formed monolayers with a morphology typical of MC29-transformed fibroblasts (data not shown). The large colonies were composed primarily of transformed macrophages. When picked and propagated in microtiter dishes, the cells resembled cultures of MC29-transformed macrophages derived from chicken peripheral blood (data not shown) or MC29-infected bone marrow cells (26). The cells derived from the large diffuse soft agar colonies had greater ability to phagocytize latex beads than did cells from fibroblast colonies (data not shown).

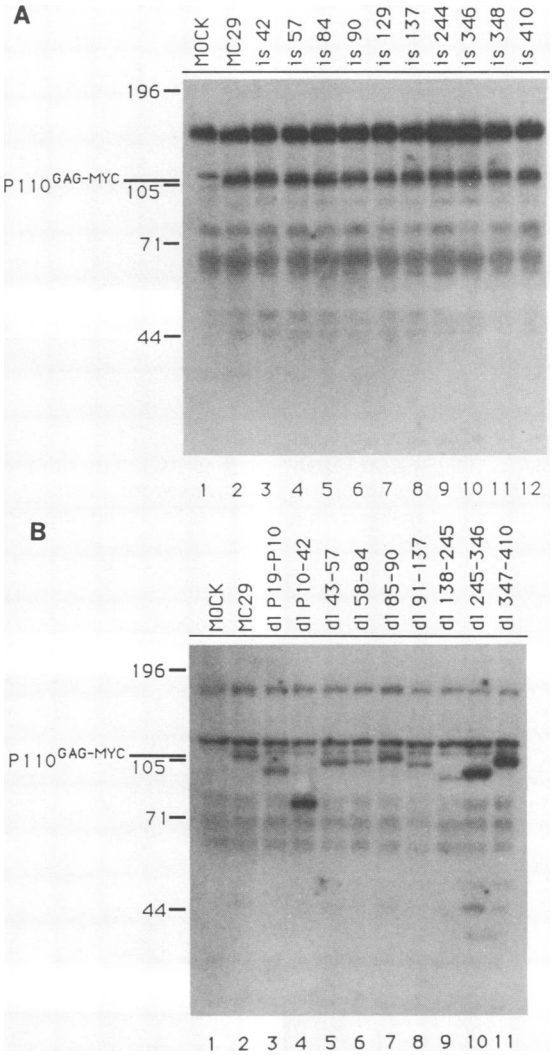

FIG. 3. Western blot analysis of v-Myc proteins encoded by individual insertion and deletion variants. COS-1 cells were transfected with pMC29 or plasmid DNA containing individual MC29 variants, and extracts were subjected to Western blot analysis as described in Materials and Methods. (A) Western blot of wild-type and insertion variants. Lanes: 1, mock-transfected control extract; 2, MC29; 3 to 12, insertion variants. (B) Western blot of MC29 and deletion variants. Lanes: 1, mock-transfected control extract; 2, MC29; 3 to 11, deletion variants.

The ability of each variant to transform CEC cultures and induce the formation of colonies in soft agar was tested; the results of these analyses are illustrated in Fig. 4 and summarized in Tables 1 and 2. Three general patterns of transformation and growth in agar were exhibited by the insertion mutations. First, the insertion variants *is*129, *is*137, *is*244, and *is*410 readily induced morphological transformation of CEC cultures, and cells infected with these variants formed fibroblast and macrophage colonies in soft agar similar to that observed for MC29-infected cells. Interestingly, cells infected with *is*410 formed slightly more macrophage colonies in soft agar relative to the wild type; however, the number and the size of the colonies formed by this mutant were somewhat reduced (Table 2). Second, variants with linker insertions in the 5′-terminal portion of the v-*myc*

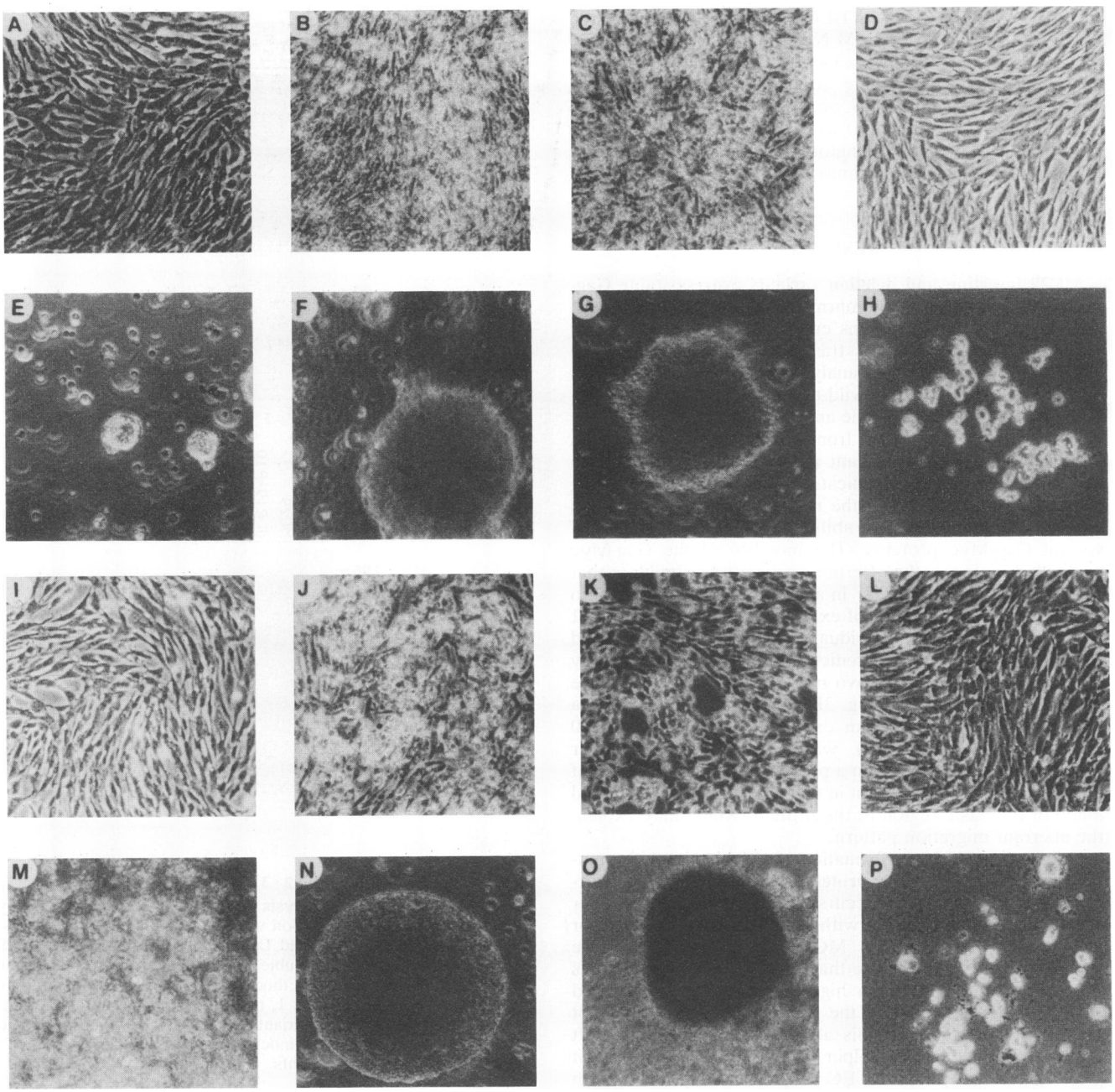

FIG. 4. Morphology of wild-type- and variant-infected CEC cultures and soft agar assay of wild-type- and variant-infected cells CEC cultures were infected and cultured (A to D and I to L) as described in Materials and Methods or seeded in soft agar (E to H and M to P). (A and E) ΔMst(B); (B and F) MC29; (C and G) *is*57; (D and H) *is*346; (I and M) *dl*P10-42; (J and N) *dl*58-84; (K and O) *dl*138-245; (L and P) *dl*347-410.

coding sequence (*is*42 and *is*57; Fig. 4C and G), *is*84, and *is*90 (data not shown) exhibited what was termed a delayed phenotype. These variants retained the ability to transform CEC cultures; however, the appearance of foci of morphologically altered cells was delayed, becoming apparent 2 to 3 days after foci were observed in wild-type MC29-infected cells. A common feature of cells infected with these amino-terminal variants was the formation of a higher proportion of macrophage colonies in soft agar relative to wild-type MC29 (Table 2). Finally, linker insertions within the putative DNA binding region, e.g., following the codons for residue 346 (Fig. 4D and H) or 348 (data not shown), abolished CEC transformation and growth in soft agar.

Deletion of virtually all of the *gag* sequences, *dl*P19-P10, did not alter any of the parameters of transformation of CEC or formation of colonies in soft agar (Table 1). However, variants with deletion mutations in the 5′ half of *myc*, *dl*58-84 (Fig. 4J and N), *dl*138-245 (Fig. 4K and O), *dl*43-57, and *dl*85-90 (data not shown), induced transformation with a delayed onset similar to that observed for the variants with

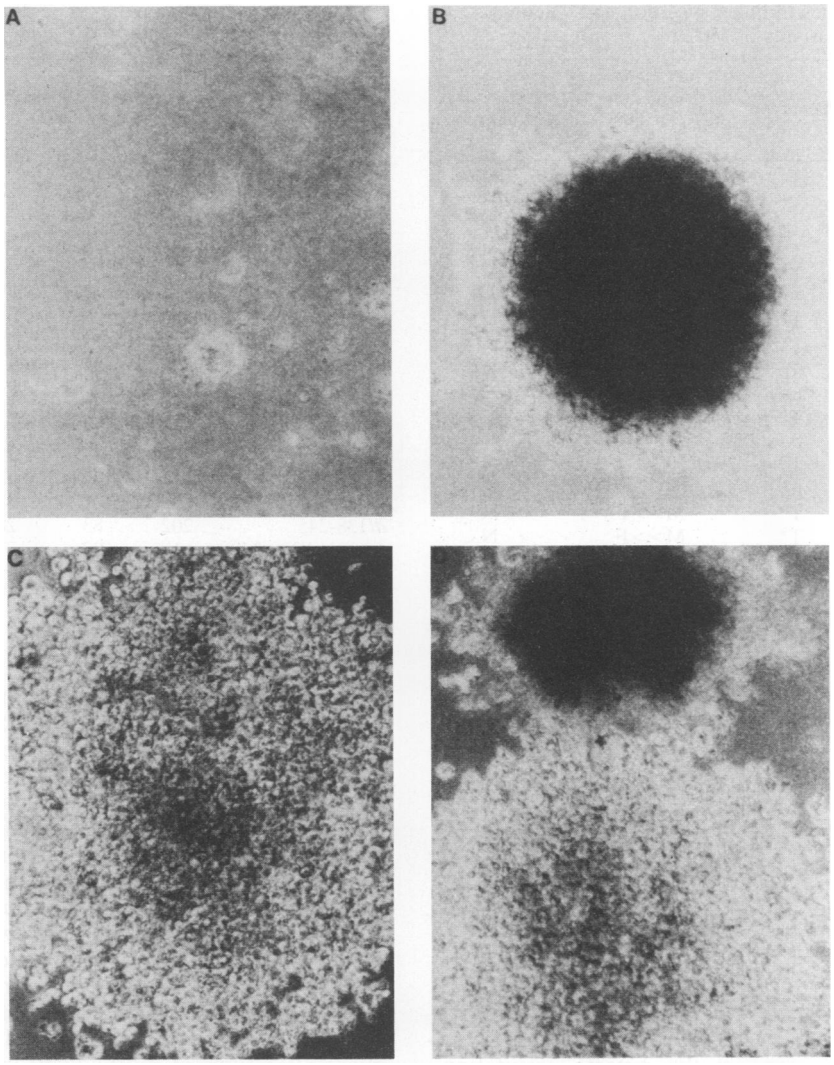

FIG. 5. Morphology of colonies formed in the dual macrophage/fibroblast soft agar assay. (A) Uninfected CEC; (B) fibroblast colony; (C) macrophage colony; (D) fibroblast and macrophage colony.

insertions in the 5′ portion of v-*myc*. Cells infected with 5′ deletion mutants also exhibited a distinctly enhanced ability to form macrophage colonies in soft agar (Fig. 4; Table 2). The cultures infected with *dl*P10-42 (Fig. 4I) appeared morphologically indistinguishable from uninfected or helper virus-infected CEC; however, when plated in soft agar, *dl*P10-42-infected cells formed large macrophage colonies (Fig. 4M). Variants bearing large deletions in the carboxyl-terminal part of the protein, *dl*347-410 (Fig. 4L and P) and *dl*245-346 (data not shown), as well as amino-terminal deletion *dl*91-137 (data not shown) were defective for transformation. As previously reported, the variant CH201 (*dl*239-249) readily transformed CEC cultures. However, when these cells were plated in soft agar, only fibroblast colonies were observed (26) (Table 2).

To determine the intracellular localization of proteins encoded by each of the MC29 variants, COS-1 cells were transfected with viral DNA and immunostained with anti-Gag serum. pMC29-encoded P110$^{gag\text{-}myc}$ was localized to the nucleus, as were all of the variant proteins except the *dl*245-346-encoded protein, which was found exclusively in the cytoplasm (data not shown). This latter mutation removes a sequence similar to the nuclear localization signal previously defined by Dang and Lee (15) for human c-*myc*.

Cloning of *myc* variants into the RCAS vector. Transformation-defective variants replicated poorly when introduced into embryo cells along with a replication-competent helper virus (17a). Therefore, to assess the biological and biochemical properties of transformation-defective v-Myc proteins in CEC cultures, the wild-type v-*myc* gene and several of the variant v-*myc* genes were transferred to the replication-competent avian retroviral vector RCAS(B) (28). Only the Myc portion of P110$^{gag\text{-}myc}$ was subcloned into the expression vector (Fig. 2) due to size constraints for viral packaging and also to eliminate the possibility of recombination between homologous regions containing *gag* sequences. The wild-type clone was designated RCAS-v-*myc*(B), denoting that only the v-Myc portion of MC29 P110$^{gag\text{-}myc}$ was contained in the vector and that it contained a subgroup B *env* gene. In addition to the wild-type v-*myc* gene, eight additional variants were cloned into RCAS(B) (*is*346, *dl*91-137, *dl*245-346, and *dl*347-410) as well as amino terminal

TABLE 1. Properties of linker insertion and deletion variants of MC29

Variant	Phenotype[a]	Colony formation in soft agar[b]	Intracellular localization[c]
pMC29 (wild type)	T	F>>M	N
*is*42	T*	M>F	N
*is*57	T*	M>>F	N
*is*84	T*	M>F	N
*is*90	T*	F>M	N
*is*129	T	ND	N
*is*137	T	F>M	N
*is*244	T	F>M	N
*is*346	td	—	N
*is*348	td	—	N
*is*410	T	M>F	N
*dl*P19-P10	T	ND	N
*dl*P10-42	td	M>>>F	N
*dl*43-57	T*	M>>F	N
*dl*58-84	T*	M>>F	N
*dl*85-90	T*	M>F	N
*dl*91-137	td	—	N
*dl*138-245	T*	M>>F	N
*dl*245-346	td	—	C
*dl*347-410	td	—	N
CH201	T	F>>>M	N

[a] T, transformation of CEC; T*, delayed transformation of CEC; td, transformation defective.

[b] Based on the percentage of macrophage and fibroblast colonies reported in Table 2. M>F, 50 to 70% macrophage colonies; M>>F, 71 to 90% macrophage colonies; M>>>F, 91 to 100% macrophage colonies; F>M, 50 to 70% fibroblast colonies; F>>M, 71 to 90% fibroblast colonies; F>>>M, 91 to 100% fibroblast colonies; ND, not different from wild-type pMC29; —, no growth in soft agar.

[c] N, nuclear; C, cytoplasmic.

TABLE 2. Relative numbers of fibroblast and macrophage soft agar colonies

Variant	No. of colonies[a]			Expt. no.	% of total colonies scored as:	
	Mϕ	Fib	Total, Mϕ + Fib		Mϕ	Fib
ΔMst(B)	0	0	0	6	0	0
MC29	359	1,246	1,605	6	22	78
*is*42	200	125	325	2	61	39
*is*57	224	38	262	2	85	15
*is*84	106	70	176	2	60	40
*is*90	148	221	369	2	40	60
*is*137	36	86	122	2	30	70
*is*244	239	414	653	2	37	63
*is*410	60	48	108	2	55	45
*dl*P10-42	576	19	595	4	97	3
*dl*43-57	142	54	196	2	72	38
*dl*59-84	111	26	137	3	81	19
*dl*85-90	52	31	83	2	63	37
*dl*138-245	202	83	285	2	71	29
Ch201(*dl*239-249)	18	692	710	4	2	98

[a] The number of macrophage (Mϕ) or fibroblast (Fib) colonies was determined as described in the text.

mutations *is*57 and *dl*58-84 and two deletion mutations in the middle of the v-*myc* gene, *dl*138-245 and CH201. An RCAS variant with the v-*myc* gene in the reverse orientation, RCAS-*cym*(B), was isolated and used as a negative control.

Analysis of the biological properties of the RCAS-v-*myc* variants. The biological activity of individual RCAS variants was tested by transfection of CEC cells. In each case, the RCAS variants exhibited the same transforming properties and intracellular localization as did their MC29$^{gag\text{-}myc}$ counterparts (Table 1). To confirm the synthesis of v-Myc proteins in RCAS-infected cells, lysates of infected CEC cultures were resolved by SDS-PAGE and subjected to Western immunoblotting using a rabbit polyclonal antiserum directed against a carboxyl-terminal peptide of MC29 P110$^{gag\text{-}myc}$ (24). RCAS-v-*myc*(B) encoded a protein with an apparent molecular size of 54 kDa (Fig. 6A, lane 4). Uninfected cells (lane 1) and cells infected with the RCAS(B) virus (lane 2) or RCAS-*cym*(B) (lane 3) contained only endogenous c-Myc proteins, visible following long exposure of the film (data not shown). With the exception of the extract from cells infected with RCAS-*dl*347-410, each of the extracts contained Myc-related proteins of the predicted size (lanes 5 to 12). The RCAS-*dl*347-410-encoded protein was not detected by anti-Myc C12 antisera but was observed when extracts were immunoblotted with a polyvalent antiserum directed against bacterially produced human c-Myc protein (56) (Fig. 6B, lane 2, open arrow). The steady-state levels of expression of v-*myc*-encoded protein and the proteins encoded by RCAS-*is*57 (lane 5), RCAS-*dl*58-84 (lane 7), RCAS-*dl*91-137 (lane 8), and RCAS-CH201 (lane 12) were similar. In contrast, proteins encoded by the transformation-defective variants RCAS-*is*346 (lane 6) and RCAS-*dl*347-410 (Fig. 6B, lane 2) were detected at significantly lower levels.

Expression of *dl*91-137 inhibits transformation by wild-type MC29. A series of double-infection experiments was performed to determine whether expression of mutant Myc proteins inhibited or altered the course of transformation by wild-type MC29. CEC cultures were first infected with mutant viruses of subgroup B, and 14 days later cultures were challenged by superinfection with wild-type MC29 virus of subgroup A, MC29(A). Cells initially infected with RCAS-*dl*138-245 were transformed and showed no subsequent alterations in morphology upon superinfection with wild-type MC29 (Fig. 7D). Cells initially infected with the nontransforming virus, RCAS(B) (Fig. 7A), or cells infected with the transformation-defective variant RCAS-*is*346 (Fig. 7B), RCAS-*dl*245-346 (Fig. 7E), or RCAS-dl347-410 (Fig. 7F) became transformed when superinfected with the wild-type MC29(A) virus. However, cells infected first with the transformation-defective RCAS-*dl*91-137 virus and then challenged with MC29(A) retained their normal morphology (Fig. 7C) even with prolonged culturing. RCAS-*dl*91-137(B)-infected cells were efficiently transformed by RSV(A) (Fig. 7K), showing that prior expression of a defective Myc protein did not block subsequent transformation by a cytoplasmic oncoprotein. In each case, the efficiency of infection with the initial virus was assessed by measuring the resistance of these cells to transformation by a superinfecting virus of the same subgroup (Fig. 7H).

Lysates from doubly infected cells were analyzed by SDS-PAGE and Western blot to verify the stable expression of both mutant and wild-type Myc protein (Fig. 8). As expected, uninfected control cells (Fig. 8A lane 3) and MC29(A)- or RCAS(B)-infected cells (lane 4) superinfected with MC29(A) contained only MC29(A)-encoded P110$^{gag\text{-}myc}$. Cultures initially infected with RCAS-*dl*138-245 (lane 6) or RCAS-*dl*245-346 (lane 7) and superinfected with MC29(A) contained mutant Myc proteins as well as

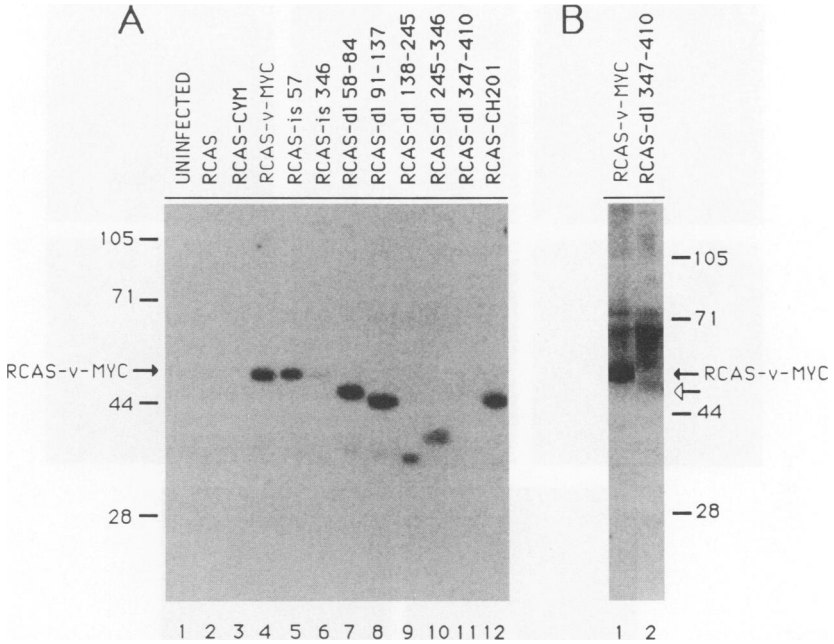

FIG. 6. Western blot analysis of RCAS-v-*myc* variant proteins. (A) CEC were infected with RCAS-v-*myc* and variant viruses as indicated, and extracts were subjected to SDS-PAGE and immunostained with anti-Myc C12 serum (24) as described in Materials and Methods. (B) Immunoblot with antibacterial Myc serum (56). Extracts from RCAS-v-*myc* (lane 1) and RCAS-*dl*347-410 (lane 2) were subjected to analysis as described above.

P110$^{gag\text{-}myc}$. However, in cells infected first with RCAS-*dl*91-137 and later with MC29(A), P110$^{gag\text{-}myc}$ was not detected, whereas *dl*91-137-encoded protein was clearly present (lane 5). Thus, the absence of transformation of these cells appeared to be due to a restriction in the stable expression of wild-type Gag-Myc protein imposed by prior infection with the *dl*91-137 variant.

DISCUSSION

In this report, we show that mutations (e.g., insertions and deletions) within the amino-terminal 245 amino acids of the Myc-specific region of P110$^{gag\text{-}myc}$ significantly alter the course of transformation of CEC in culture and appear to differentially affect the efficiency of formation of macrophage and fibroblast colonies in soft agar. In addition, we show that the deletion mutation *dl*91-137, when stably expressed in CEC, blocks subsequent infection and transformation by wild-type MC29. Further, we confirm earlier reports that structural alteration of the basic/helix-loop-helix or leucine zipper region of the v-Myc protein blocks transformation of both fibroblasts and macrophages.

Insertion and deletion mutations in the amino-terminal 245 amino acids of v-Myc exhibit distinct phenotypes. First, variants encoding v-Myc proteins with amino acid insertions at residues 90, 129, 137, and 244 efficiently transform CEC cultures, and cells infected with these variants form both fibroblast and macrophage colonies with an efficiency similar to that of wild-type MC29 virus. Hence, we conclude that the minimal structural alterations induced by amino acid insertions in these mutants have little effect on Myc function. Second, deletion of residues 91 to 137 blocks efficient transformation of CEC cultures, indicating that this region of v-Myc is critical for v-Myc-mediated events leading to cellular transformation. Finally, cells infected with variants encoding v-Myc proteins with insertions at amino acid 42, 57, 84, or 90 or variants with deletions of residues 43 to 57, 58 to 84, 85 to 90, or 138 to 245 (Tables 1 and 2) yielded a higher number of macrophage colonies than did wild-type MC29-infected cells (Table 1 and 2). In addition, these variants exhibited a delayed transformation phenotype. The delayed appearance of foci did not appear to result from differences in virus titer or culture conditions (unpublished observations). The *dl*P10-42 variant, a deletion of all of the P27^{gag} moiety and the first 42 residues of v-Myc, was defective for morphological transformation of CEC cultures. However, *dl*P10-42 was very efficient in inducing transformation of macrophages present in CEC cultures. It is interesting to speculate that the altered ratios of fibroblast and macrophage colonies formed in soft agar and perhaps the delayed phenotype exhibited by amino-terminal variants may reflect the inefficient interaction of the structurally altered v-Myc proteins with cellular factors required to mediate efficient fibroblast transformation. We would further argue that such an interaction requires elements of structure predominately within region 1 of Myc (Fig. 1A) and may be influenced (as in the case of *dl*P10-42) by adjacent *gag* sequences. The efficient transformation of macrophages by these same variants would suggest that specific factors either are missing from macrophages or are not required to mediate efficient transformation of macrophages or macrophage precursors. While an attractive possibility, other explanations are equally viable.

Deletion mutagenesis studies on the human c-Myc protein have previously demonstrated the functional importance of sequences in the highly conserved Myc boxes I and II (Fig. 1A and B) (53). Insertion or deletion of sequences in Myc box I reduce but do not abolish the efficiency of transformation of rat embryo cells in a *ras-myc* complementation assay and the transformation of the immortalized Rat-1 cell line. Deletion of residues within Myc box II, e.g., residues 106 to

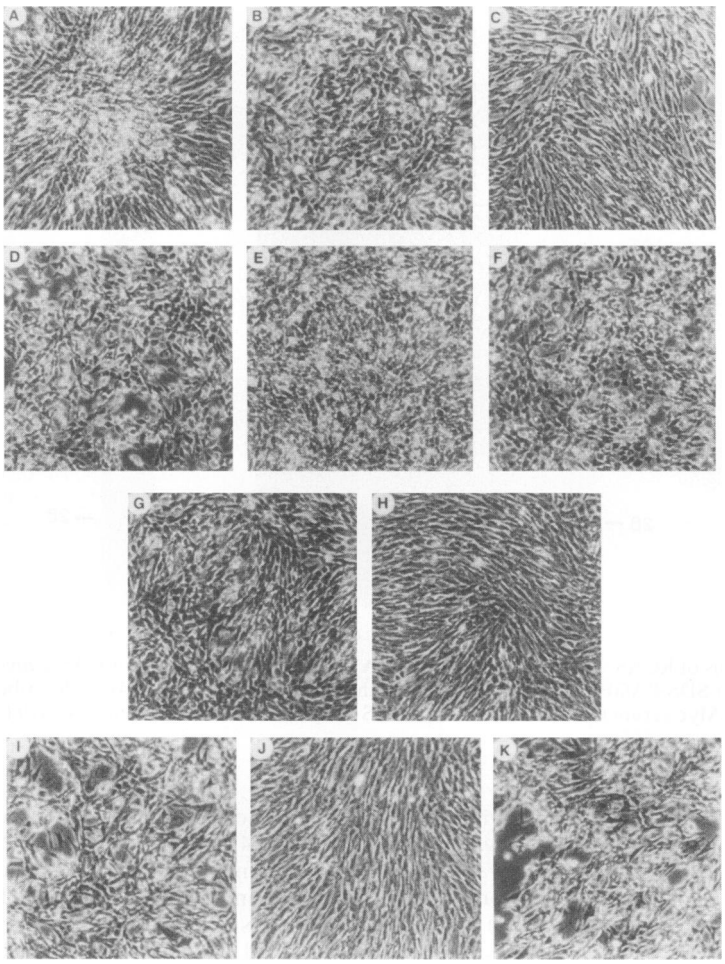

FIG. 7. Morphology of doubly infected CEC cultures. CEC cultures were infected with variant viruses and cultured for 2 weeks. The cells were superinfected with wild-type viruses, cultured for 2 weeks, and photographed. The virus used to superinfect the initial cultures is indicated at the left. (A, G, and I) Uninfected cells; (B) RCAS-*is*346(B); (C) RCAS-*dl*91-137(B); (D) RCAS-*dl*138-245(B); (E) RCAS-*dl*245-346(B); (F) RCAS-*dl*347-410(B); (H) RCAS(B); (J) ΔMst(A); (K) RCAS-*dl*91-137(B).

143 of human c-Myc (53), as well as smaller deletions in this region (51) completely block the transformation of rodent cells. Further, the c-Myc deletion variant D106-143 is defective for autoregulation of c-Myc (42) and inhibits c-*myc*/EJ *ras*-mediated transformation of rat embryo cells (15a). The notion that Myc box II specifies a particularly critical region of the Myc protein is further supported by the observation that deletion of resides 91 to 137 of v-Myc blocks transformation of both chicken fibroblasts and macrophages. The dominant negative phenotype of *dl*91-137 and the failure to detect MC29-encoded proteins following superinfection suggests that expression of the defective *dl*91-137 gene product antagonizes the subsequent activity of $P110^{gag\text{-}myc}$, leading to inhibition of growth of coinfected cells. At present, it is unclear what the mechanism for this process is.

The carboxyl-terminal half of the Myc protein, region 4 (Fig. 1), has been extensively altered by site-directed mutagenesis (14, 17, 26, 39, 42, 52, 53). The studies reported here confirm the functional significance of the basic/helix-loop helix and leucine zipper regions. The transformation-defective phenotype of insertion mutants *is*346 and *is*348 arises from the alteration of the first alpha helix of the basic/helix-loop-helix region, whereas the deletion mutation present in *dl*347-410 removes virtually all of this region. Similar mutations of human c-*myc*, In370, In373, D371-412, and D414-433, also abolish transforming activity (53). Interestingly, the insertion variant *is*410 is competent for transformation, although infected cells form smaller colonies in soft agar and the number of fibroblast colonies relative to macrophage colonies is reduced (Tables 1 and 2). The *is*410 mutation displaces the carboxyl-terminal leucine of the zipper motif (Leu-411). Enrietto (17) has described an MC29 deletion variant, S90.9, which lacks the carboxyl-terminal nine amino acids of $P110^{gag\text{-}myc}$, including Leu-411. This mutant is partially defective for transformation of chicken fibroblasts but exhibits wild-type transformation of chicken macrophages. Crouch et al. (14) have analyzed mutations that cause premature termination of the chicken c-Myc protein within the leucine zipper. RCANc-*myc*Δ7, a variant that lacks terminal Leu of the zipper (Leu-434 in c-Myc), increases cell growth rate and density-independent growth of chicken cells. However, this variant is somewhat compromised in its ability to induce the growth of cells in soft agar compared with the wild-type protein. RCANc-*myc*Δ10, a variant that lacks an additional three residues amino terminal to Leu-434, is completely defective for transformation. Nei-

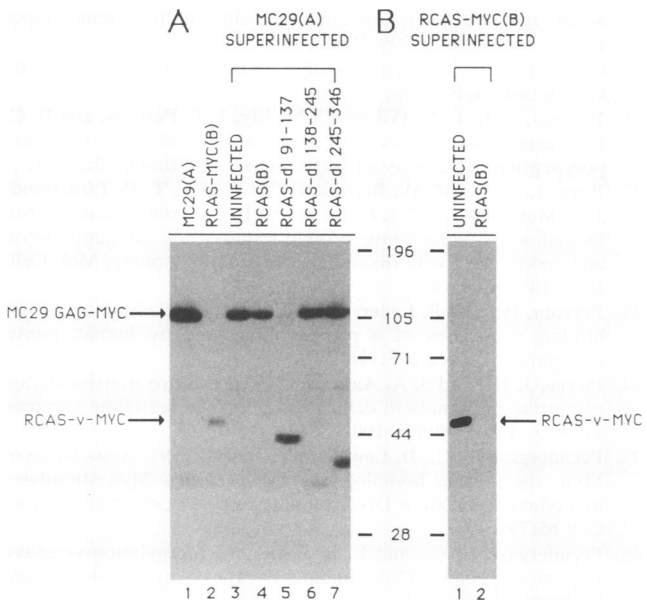

FIG. 8. Western blot analysis of doubly infected CEC. CEC cultures were infected with variant viruses and cultured for 2 weeks. The cells were superinfected with wild-type viruses and cultured for 2 weeks, and extracts were subjected to analysis as described for Fig. 6. (A) Lanes: 1, MC29(A)-infected cells; 2, RCAS-v-*myc*; 3, CEC cells superinfected with MC29(A); 4, RCAS(B)-infected cells superinfected with MC29(A); 5, RCAS(B)-*dl*91-137-infected cells superinfected with MC29(A); 6, RCAS(B)-*dl*138-245 infected cells superinfected with MC29(A); 7, RCAS(B)-*dl*245-346 infected cells superinfected with MC29(A). (B) Lanes: 1, uninfected cells infected with RCAS(B)-v-*myc*; 2, RCAS(B)-infected cells superinfected with RCAS(B)-v-*myc*.

ther the RCANc-*myc*Δ7 or the RCANc-*myc*Δ10 variant has been tested for macrophage transformation.

The presence of a basic/helix-loop-helix and leucine zipper motifs in v-Myc, c-Myc, and other Myc protein family members has implicated these proteins in critical features of transcriptional regulation. The demonstration that carboxyl-terminal fragments of c-Myc bind to a specific DNA sequence, CACGTG (10, 46), strengthens the argument for Myc as a transcription factor. Recently, proteins (Max and it murine homolog, Myn) have been identified that interact with c-Myc through the C-terminal basic/helix-loop-helix–leucine zipper region, forming stable heterodimers (11, 45). Max (and Myn) contain a basic/helix-loop-helix region and a leucine zipper and as heterodimers with Myc protein bind efficiently to the CACGTG motif. Since Max/Myn are relatively small proteins, approximately 180 amino acids, and their interaction with Myc is mediated by the 80 residues within the basic/helix-loop-helix–leucine zipper region, it is tempting to speculate that the amino-terminal portion of the Myc protein dictates additional regulatory interactions, possibly with other components of the transcription complex. Myc box II thus appears to be of particular importance for Myc function. Delineating the function of this region as well as other regions within Myc's regulatory domain should provide useful insights into the role of Myc family members in the control of cell growth and differentiation.

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