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Generation and Characterization of a Recombinant Moloney Murine Leukemia Virus Containing the v-*myc* Oncogene of Avian MC29 Virus: In Vitro Transformation and In Vivo Pathogenesis

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A new retrovirus consisting of the v-*myc* oncogene sequences of avian MC29 virus inserted into the genome of Moloney murine leukemia virus (M-MuLV) was generated. This was accomplished by constructing a recombinant DNA clone containing the desired organization, introducing the recombinant DNA into mouse NIH 3T3 cells, and superinfecting the cells with replication-competent M-MuLV. The construction was designed so that an M-MuLV *gag-myc* fusion protein would be produced. The resulting virus, M-MuLV(*myc*), morphologically transformed uninfected NIH 3T3 cells. Stocks of M-MuLV(*myc*)–M-MuLV were infected into secondary mouse embryo cultures. M-MuLV(*myc*) induced striking growth and proliferation of hematopoietic cells. These cells were of the myeloid lineage by morphology, phagocytic properties, and surface staining with Mac-1 and Mac-2 monoclonal antibodies. They resembled mature macrophages, although they displayed minor properties of immaturity. The myeloid cells were transformed in comparison with uninfected myeloid cells since they were less adherent and had unlimited proliferative capacity and reduced growth factor requirements. The transformed myeloid cells with proliferative potential were actually myeloid progenitors which apparently underwent terminal differentiation to macrophages. It was possible to derive a permanent line of factor-independent macrophages from M-MuLV(*myc*)-transformed myeloid cells. M-MuLV(*myc*) also immortalized and morphologically transformed mouse embryo fibroblasts. These in vitro properties closely resembled the biological activity of MC29 virus in avian cells and suggested that the nature of the v-*myc* oncogene was an important determinant in transformation specificity. Neonatal NIH Swiss mice inoculated intraperitoneally with M-MuLV(*myc*)–M-MuLV only developed lymphoblastic lymphoma characteristic of the M-MuLV helper alone, and no acute fibrosarcomas or myeloid tumors resulted. In light of the strong myeloid transformation observed in vitro, the absence of acute in vivo myeloid disease was noteworthy. Interestingly, when a derivative of M-MuLV(*myc*) carried by a nonpathogenic amphotropic MuLV helper was inoculated, T lymphomas developed with long latency. Molecular hybridization confirmed that these tumors contained M-MuLV(*myc*).

Retroviruses which cause solid tumors or acute neoplasms carry transforming genes (oncogenes) which were transduced from normal-cell genetic information (3). Viral oncogene proteins generally differ from their cellular homologs by point mutations, deletions, or fusions to viral protein (24). These alterations are frequently necessary for the transforming potential of the viral oncogene. Acute transforming retroviruses induce specific diseases when inoculated into animals. For instance, the avian retrovirus MC29, which carries the v-*myc* oncogene, induces myelocytomatosis (a myeloid neoplasm) in chickens, whereas Rous sarcoma virus (carrying the v-*src* oncogene) causes sarcomas.

Several possible factors could influence the disease specificity of an acute transforming retrovirus. (i) The nature of the oncogene could determine disease specificity; (ii) viral protein attached to the oncogene protein could be important (39); or (iii) retroviral regulatory sequences (particularly in the long terminal repeat ([LTR]) could be important (49). In addition, more than one of these factors could interact for a given retrovirus.

To investigate the molecular mechanisms of disease specificity, we have generated new transforming retroviruses by recombinant DNA techniques by inserting well-characterized oncogenes of avian acute transforming viruses into the genome of Moloney murine leukemia virus (M-MuLV). This system offers several advantages. (i) Several avian retroviral oncogenes have been extremely well characterized and studied, and the viruses have been molecularly cloned and sequenced; (ii) M-MuLV, which predominantly causes T-cell lymphomas (42), has also been molecularly cloned and sequenced (2, 46); and (iii) characterization and culture of mouse hematopoietic cells is well developed (8, 10, 17, 34).

In previous experiments, we generated an M-MuLV carrying the v-*src* gene of Rous sarcoma virus (16). This virus, M-MuLV(*src*), induced sarcomas in newborn mice, and no other neoplastic diseases were observed, indicating that the nature of the v-*src* oncogene was the dominant determinant in disease specificity for the virus.

In the experiments reported here, an M-MuLV carrying the v-*myc* oncogene from avian MC29 virus was developed. In chickens, MC29 predominantly transforms cells of the myeloid lineage. The myeloid transformation is rather specific to more differentiated myeloid cells—myelocytes, which are immediate precursors to terminally differentiated macrophages. Several other avian retroviruses carrying transduced *myc* also exist (e.g., CMII, MH2, and OK10), and different isolates induce carcinomas or myeloid tumors. In contrast, the cellular c-*myc* oncogene has been associated with several lymphoid tumors. In avian leukosis virus-induced B lymphomas the c-*myc* gene is activated by insertion of the avian leukosis provirus at an adjacent location

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(23). A similar mechanism may be important in the development of some MuLV-induced leukemias as well (5, 20, 45). In addition, some nonretroviral B-lymphoid leukemias in mice and humans (e.g., Burkitt's lymphoma and murine plasmacytomas) show activation of c-*myc* by chromosomal translocation into an actively transcribed immunoglobulin locus (12). Thus, generation of an M-MuLV carrying the v-*myc* oncogene is of considerable interest. The generation and characterization of such a virus is reported here.

MATERIALS AND METHODS

Cell culture. The cell lines used in these experiments were as follows: mouse NIH 3T3 fibroblasts (52); A9, an NIH 3T3 clone productively infected with M-MuLV (15); rat XC cells (26). All cell lines were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% calf serum. Mouse embryo cultures were prepared with 14- to 19-day-old embryos from BALB/c mice. After removal of heads, appendages, and red organs, tissue was finely minced and treated with 0.05% trypsin (Irvine Scientific) in TD (0.14 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Sigma 7-9), pH 7.5, at 37°C to obtain a single cell suspension. Cells were plated at 2×10^6 cells per 9-cm (diameter) dish. While still subconfluent, cells were prepared for freezing and stored in liquid nitrogen (17). Mouse embryo cells were thawed and subpassaged twice prior to use in infections. Mouse embryo cells were grown in Dulbecco modified Eagle medium plus 10% fetal calf serum.

Recombinant DNA cloning. Starting materials used in the construction of pM-MuLV(myc) were (i) p63-2, a pBR322 derivative which contains an M-MuLV proviral clone (31), and (ii) the 2.9-kilobase (kb) *Bam*HI fragment including v-*myc* sequences of MC29 also cloned into pBR322 (43), kindly provided by Premkumar Reddy. The v-*myc* containing clone was grown in the *Escherichia coli* GM119 (*dcm*$^-$ *dam*$^-$) in the presence of ampicillin to obtain unmethylated DNA. (*Bcl*I sites are methylated in wild-type *E. coli*.) The molecular cloning techniques used generally followed standard protocols (33).

DNA transfections. DNA was transfected into tissue culture cells as described previously (14, 28). Briefly, 10 μg each of pM-MuLV(myc) and pSHL72 DNAs were cotransfected together with 5 μg of calf thymus DNA into 10^6 NIH 3T3 cells by calcium phosphate precipitation (19). (pSHL72 confers kanamycin resistance to mammalian cells [4].) Approximately 4 h later, plates were treated with 25% dimethyl sulfoxide in phosphate-buffered saline and then refed with growth medium. On the following day, cultures were passaged 1:5 with medium containing the antibiotic G418 (GIBCO). G418-resistant colonies, as well as individual transformed foci resulting from infection with helper virus, were isolated by one or more cycles of selective trypsinization in cloning cylinders and grown as individual cultures (16).

Viral infections. Confluent cultures of cells were passaged into 5-cm (diameter) dishes at 1:10 for NIH 3T3 cells or 1:5 for mouse embryo cultures. Viral infections were performed as described previously (15); cells were pretreated with 20 μg of Polybrene per ml prior to virus adsorption to enhance infection.

RNA analysis. Cytoplasmic RNA was isolated as described previously (13). RNA dot blots (51) were prepared by spotting RNA in serially decreasing 1:2 dilutions onto nylon membranes (GeneScreen Plus; New England Nuclear Corp.) which had been equilibrated in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (31). Blots were prehybridized overnight at 65°C in 1 M NaCl–10% dextran sulfate–1% sodium dodecyl sulfate (SDS) and then hybridized with 4×10^5 cpm of probe per ml plus 150 μg of denatured salmon sperm DNA per ml overnight at the same temperature. The probes used are indicated in the figure legends. After hybridization, blots were washed twice for 5 min at room temperature in 2× SSC, twice for 30 min at 65°C in 2× SSC–1% SDS, and twice for 30 min at room temperature in 0.1× SSC. Washed blots were analyzed by autoradiography with Kodak XAR-5 film.

DNA analysis. DNA was transferred from agarose gels to nylon membranes by capillary blotting as directed by the manufacturer (New England Nuclear). Blots were prehybridized in 50% formamide–1 M NaCl–10% dextran sulfate–1% SDS at 42°C for 6 h. Labeled probe plus denatured salmon sperm DNA was then added, and hybridization was overnight at the same temperature. Washing of blots and autoradiography were as for RNA dot blots.

Labeling of cell proteins and immunoprecipitation. Subconfluent cultures were incubated at 37°C for 1 h in phosphate-free medium plus 2% calf serum. Cells were then labeled for 1 h at 37°C with ^{32}P; (Amersham Radiochemicals) in phosphate-free medium. Proteins were extracted, immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis on 10% gels as described previously (16, 21). The antibodies used were rabbit anti-M-MuLV p30 (37) or rabbit antibody to a synthetic v-*myc* C-terminal peptide (kindly provided by Robert Eisenman).

Agar assays. A bottom layer of 5 ml of Dulbecco modified Eagle medium–10% calf serum containing 0.5% Bacto-Agar (32) was poured into 5-cm dishes followed by overlay with 4 ml of medium containing 0.4% Bacto-Agar and 1×10^4 to 2×10^4 cells. The cultures were then incubated for 2 weeks. They were fed with 2 ml of 0.4% Bacto-Agar medium after 1 week. Colonies were counted at 14 days.

Titration of viral stocks. Infectivity of viral stocks was measured by titration by the XC plaque assay (44) as described previously (15). For titration of transforming virus, NIH 3T3 cells were infected with serial dilutions of M-MuLV(*myc*) viral stock. Foci of transformed cells were counted 12 to 16 days postinfection.

Inoculation of mice and pathology. Neonatal NIH Swiss mice 1 to 2 days old were inoculated intraperitoneally with 0.2 ml of viral stock. Moribund animals were killed, and tissue specimens were prepared for pathological analysis as described previously (38).

RESULTS

Construction of pM-MuLV(*myc*). As the first step in these experiments, a recombinant DNA clone was constructed of an M-MuLV provirus containing inserted v-*myc* oncogene sequences [pM-MuLV(*myc*)]. Details of the construction are shown in Fig. 1. The construction of pM-MuLV(*myc*) placed the *myc* sequences into the same reading frame as the remaining 5′ portion of the M-MuLV *gag* gene. An M-MuLV *gag*–MC29 *myc* fusion protein (also with 26 amino acids of avian retroviral *gag* protein immediately amino terminal to the *myc* sequences) would be expected.

Preparation of cells expressing pM-MuLV(*myc*). pM-MuLV(*myc*) was first introduced into mouse NIH 3T3 cells by cotransfer with the kanamycin resistance expression plasmid pSHL72 (4) by calcium phosphate precipitation. The cultures were then grown in the presence of the antibiotic G418, which selects for cells carrying the kanamycin resist-

ance gene. G418-resistant cell colonies were isolated and screened for expression of *myc*-specific RNA by RNA dot blot analysis (51) with a radioactive v-*myc* DNA probe. One *myc* RNA-positive clone, NP4-6, was selected for further analysis.

The *myc*-positive NP4-6 cells were also tested for expression of viral protein. Since MC29 v-*myc* protein, as well as activated c-myc protein, is phosphorylated (21), NP4-6 cells were tested for M-MuLV(*myc*) *gag-myc* protein by labeling with ^{32}P; and immunoprecipitation (Fig. 2). The NP4-6 cells contained a new *gag*-specific protein with an electrophoretic mobility corresponding to 110 kilodaltons (kDa). Immunoprecipitation with an anti-v-*myc* serum yielded a *myc*-specific protein of the same mobility, consistent with the expected *gag-myc* fusion protein. A *gag-myc* fusion protein resulting from $Pr65^{gag}$ fused to v-*myc* would have a predicted

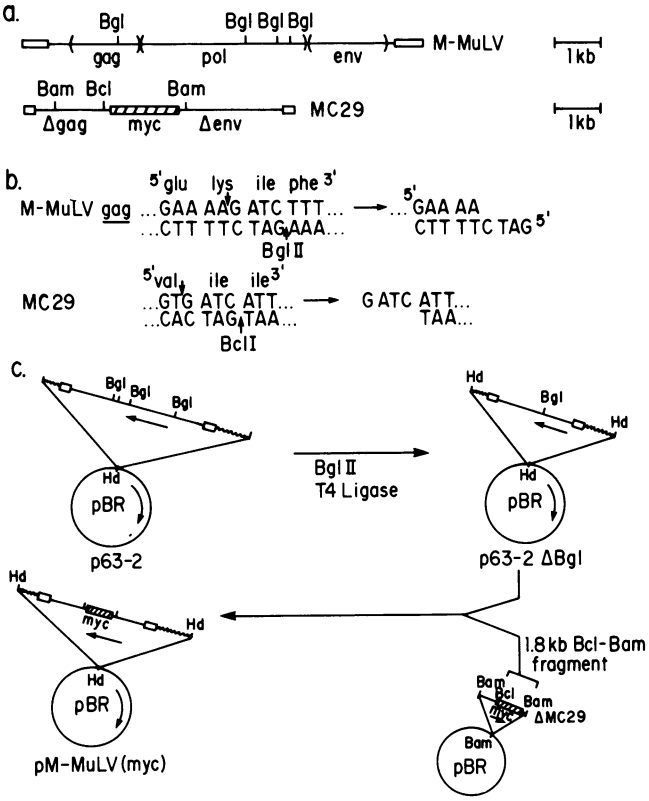

FIG. 1. Construction of pM-MuLV(*myc*). (a) Restriction maps of M-MuLV and MC29 proviral DNAs. Viral LTRs are indicated by open bars. v-*myc* sequences are shown as a cross-hatched bar. Coding regions for the viral structural genes are also shown. (b) Nucleic and amino acid sequences around the restriction enzyme sites used in inserting v-*myc* into the M-MuLV *gag* gene. *Bcl*I and *Bgl*II generate the same cohesive ends, making direct ligation possible. Furthermore, such a ligation inserts v-*myc* coding sequences in the same reading frame as the M-MuLV *gag* gene. (c) Cloning steps for pM-MuLV(*myc*). The starting plasmids were: p63-2, an integrated M-MuLV provirus cloned into a derivative of pBR322 (lacking the *Sal*I site) at the *Hin*dIII (Hd) site (31); and a pBR322 subclone of MC29 containing the internal *Bam*HI (Bam) fragment (43) (lower right plasmid). Internal *Bgl*II (Bgl) fragments were deleted from p63-2, and the 1.8-kb v-*myc*-containing a *Bcl*I (Bcl)-*Bam*HI fragment of MC29 was cloned in at the *Bgl*II site. A clone containing the v-*myc* sequences in the correct orientation was identified by secondary restriction enzyme digestion and designated pM-MuLV(*myc*).

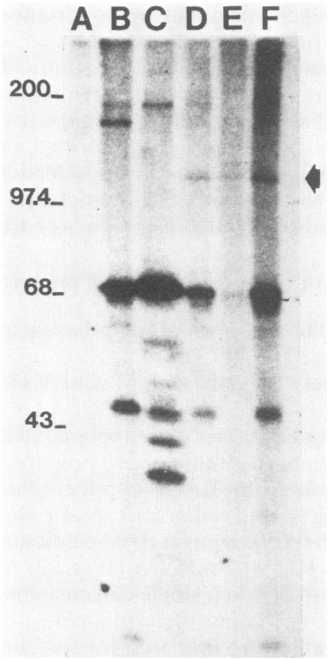

FIG. 2. Immunoprecipitation from infected and transfected cells. Culture dishes (9-cm diameter) of subconfluent cells were labeled with 1.33 mCi of ^{32}P; for 1 h as described in Materials and Methods. Cell extracts were prepared and incubated with either rabbit anti-p30 serum (37) or rabbit anti-v-*myc* serum followed by collection of immunoprecipitates with fixed *Staphylococcus aureus*. Immunoprecipitated protein was released from the fixed bacteria and analyzed by SDS-polyacrylamide gel electrophoresis in a 10% gel (27), followed by drying of the gel and autoradiography. Positions of stained molecular mass markers are indicated on the left. Lanes: A and B, uninfected NIH 3T3 cells; C, M-MuLV-infected NIH 3T3 cells (clone A9 [15]); D to F, NP4-6 cells containing pM-MuLV(*myc*) DNA; A, immunoprecipitation with rabbit preimmune serum; B, C, D and F, immunoprecipitation with anti-p30 serum; E, immunoprecipitation with anti-v-*myc* serum; F, NP4-6 cells treated for 90 min with 1 μg of tunicamycin per ml prior to labeling. Infected A9 cells (lane C) showed extensive phosphate labeling of the $Pr65^{gag}$ polyprotein protein precursor (the actual mobility of this polyprotein is closer to 68 kDa for M-MuLV [11], as well as several cleavage intermediates of lower molecular mass. This was expected (9). Uninfected NIH 3T3 cells also showed unexpected labeling of $Pr65^{gag}$. This presumably represents labeling of *gag* polyprotein encoded by a replication-defective endogenous retrovirus; the NIH 3T3 cells were virus negative as measured by XC syncytial overlay and by reverse transcriptase production (data not shown). NP4-6 cells showed an additional labeled protein of 110 kDa (arrow). This protein was immunoprecipitable by either anti-p30 or anti-v-*myc* antibody (lanes D and E), and mobility was not affected when cell labeling was done in the presence of tunicamycin (lane F).

molecular mass of 97 kDa, whereas $gPr80^{gag}$ fused to v-*myc* would be 112 kDa. However, when NP4-6 cells were treated with tunicamycin (which inhibits glycosylation) prior to labeling, the molecular mass of the 110-kDa NP4-6 protein did not change (lane F). This suggests that the *gag-myc* protein was not glycosylated and represented a fusion of $Pr65^{gag}$ and v-*myc*. The apparent discrepancy in molecular mass may reflect properties of the v-*myc* protein (1, 21).

The organization of pM-MuLV(*myc*) preserved the M-MuLV *env* coding sequences, as well as the splice donor and acceptor sites for spliced *env* mRNA. Thus, it was possible that NP4-6 cells also encoded M-MuLV *env* protein. However, NP4-6 cells did not form syncytia when overlayed with

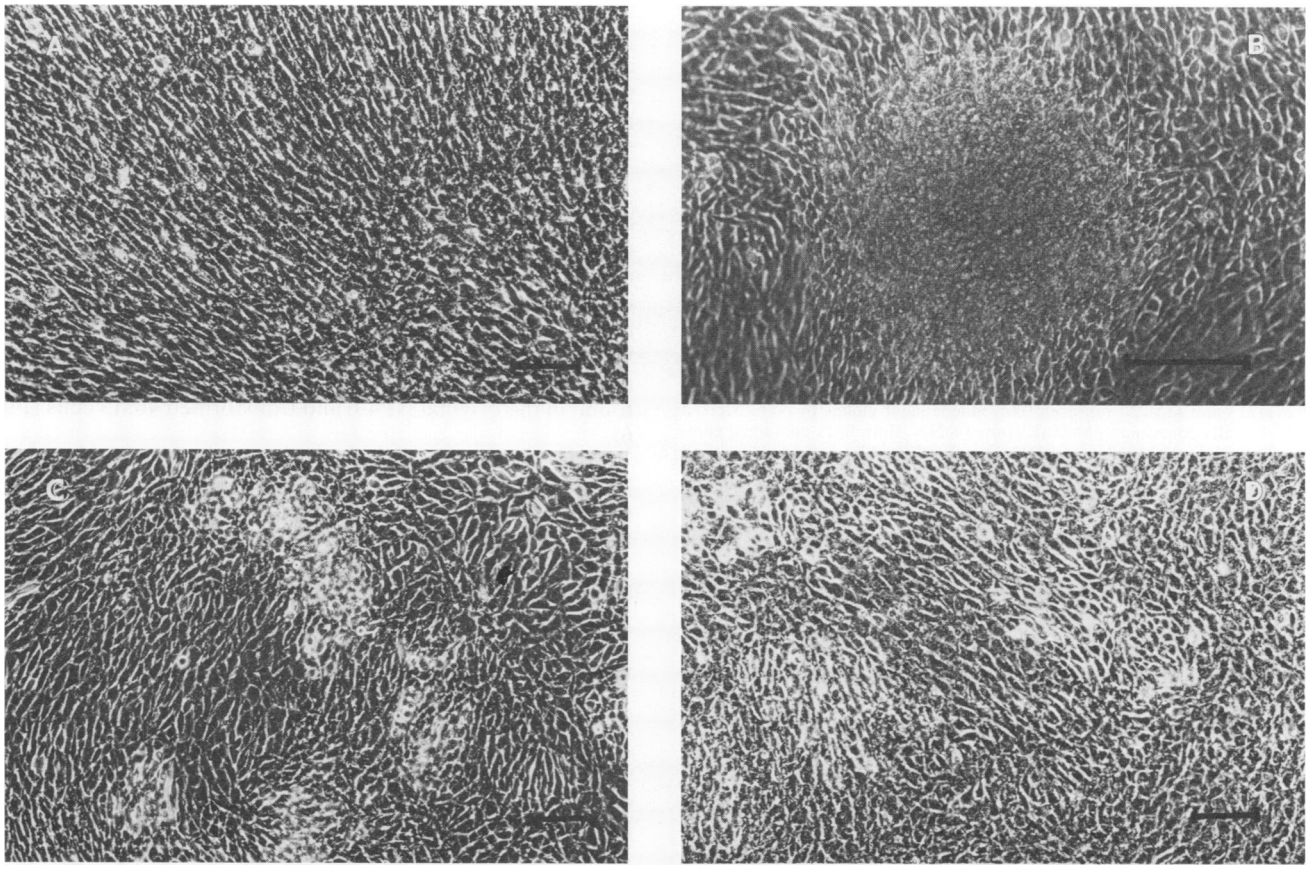

FIG. 3. Focus formation in infected NP4-6 cells. (A) Uninfected NP4-6 cells. (B) NP4-6 cells infected with M-MuLV. NP4-6 cells (5×10^5 per 9-cm dish) were infected with 5×10^4 XC PFU of M-MuLV. Numerous foci (~2,000 to 3,000/9-cm dish) of rounded, densely clustered refractile cells overgrowing the monolayer were evident 15 days after infection. (C) Enrichment for transformed cells. Individual foci were selectively trypsinized in cloning rings and transferred to a new culture dish. Whereas some nontransformed cells were still present, the frequency of transformed cell foci was increased. C shows one such culture, 46M5, 10 days after transfer. (D) NIH 3T3 cells infected with undiluted cell-free supernatants from 46M5 cells. Infection resulted in the formation of foci similar to those in the originally infected NP4-6 cells. Bars = 100 μm.

rat XC cells (44), indicating that they produced little, if any, M-MuLV *env* glycoprotein.

Infection of NP4-6 cells with M-MuLV and focus formation. To recover infectious M-MuLV(*myc*), NP4-6 cells were infected with replication-competent wild-type M-MuLV. Infection was feasible, since the NP4-6 cells were XC negative (*env*-positive cells would be resistant to M-MuLV infection). Whereas the NP4-6 cultures resembled the parental NIH 3T3 cells morphologically, the M-MuLV-infected cells developed foci of transformed cells which overgrew the monolayer (Fig. 3). The transformed cells tended to round up off the dish and form dense clusters of refractile cells. Foci of transformed cells were enriched by selective trypsinization in cloning rings, and one such culture was designated 46M5; a single cell clone of 46M5 was designated M5B2. The transformed 46M5 cells also produced virus [M-MuLV(*myc*)] which could transform uninfected NIH 3T3 cells (Fig. 3D).

The titers of tissue culture supernatants from the 46M5 (and M5B2) cells were determined on NIH 3T3 cells for both transforming activity and replication-competent M-MuLV by focus formation and XC plaque assays. Virus stocks obtained from 46M5 cells contained 50- to 100-fold more M-MuLV helper virus than transforming virus, with typical focus-forming titers of 3×10^3 to 4×10^3 FFU/ml. M5B2 produced sixfold higher titers of transforming virus.

The M-MuLV(*myc*)-transformed cells were also tested for another property of transformed cells, growth in agar suspension. The transformed 46M5 and subcloned M5B2 cells both showed high-efficiency growth in agar (Table 1). In contrast, nontransformed NP4-6, parental NIH 3T3, and M-MuLV-infected NIH 3T3 cells showed no growth in agar.

The appearance of transformed foci on M-MuLV-infected NP4-6 cells was surprising since the NP4-6 cells themselves contained and expressed *myc* RNA and protein. Subsequent analysis by Southern blot hybridization revealed that the NP4-6 cells contained an incomplete copy of M-MuLV(*myc*) DNA. This incomplete provirus lacked extreme 3′ sequences, including the entire 3′ LTR, but all of the 5′ sequences, including the M-MuLV *gag* and MC29 *gag* and v-*myc* sequences, were intact. During infection with wild-type M-MuLV, recovery of the 3′ LTR of M-MuLV(*myc*) presumably occurred by recombination with the infecting M-MuLV. Subsequent cycles of infection with the complete M-MuLV(*myc*) might produce higher levels of viral expression and result in transformation of the infected cell. Restoration of 3′ MuLV LTRs to defective proviruses during infection with replication-competent MuLV has been documented by Goldfarb and Weinberg (18). Indeed, Southern blot analysis of cells transformed with the infectious M-MuLV(*myc*) derived from 46M5 cells confirmed the restoration of the 3′ LTR (see below).

TABLE 1. Growth of M-MuLV(*myc*) cultures in soft agar[a]

Expt no.	Culture	No. of colonies/2 × 10^4 cells
1	3T3	57
	A9	0
	NP4-6	0
	46M5	1,516
2	3T3	46
	M5B2	2,622
3	3T3	0
	MM213[b]	1,012
	MM220[b]	371

[a] Cell cultures were seeded in 5-cm dishes in 0.4% agar as described in Materials and Methods. Colonies were counted after 14 days.

[b] MM213 and MM220 represent two independent clones of cotransferred NIH 3T3 cells containing pM-MuLV(*myc*) DNA. Both lines contained complete copies of pM-MuLV(*myc*) DNA by Southern blot hybridization, and they expressed *myc* RNA by RNA dot blot hybridization.

After discovery of the incomplete M-MuLV(*myc*) provirus in NP4-6 cells, other colonies of pM-MuLV(*myc*) DNA-containing cells were isolated, and several which contained a complete copy of pM-MuLV(*myc*) DNA were identified. As expected, several of these colonies themselves showed transformed phenotypes (morphological transformation, growth in agar, or both (Table 1, experiment 3). Infection of these cells with M-MuLV also resulted in release of infectious transforming virus with properties identical to those of the M-MuLV(*myc*) released from infected 46M5 cells. The data presented here describe experiments with virus from 46M5 cells since the most extensive experiments were performed with this virus. However, the major biological properties of 46M5 virus were confirmed with the more recently derived viruses.

Infection of mouse embryo cultures with M-MuLV(*myc*). Since M-MuLV(*myc*)–M-MuLV transformed NIH 3T3 cells, it was also of interest to infect cells from mouse embryo cultures. Land et al. (29) suggested that the v-*myc* sequences from MC29 virus may immortalize primary rat embryo fibroblasts without morphologically transforming them. Therefore, secondary mouse embryo cultures from 14- to 19-day-old BALB/c mouse embryos were infected with M-MuLV(*myc*)–M-MuLV and monitored microscopically. The most prominent result was the appearance of transformed colonies of small (10 to 20 μm), highly refractile cells, which were nonadherent or loosely adherent to the underlying fibroblast monolayer (Fig. 4B). With time, the highly refractile cells completely overgrew the culture (Fig. 4C; see below). The size and nonadherent nature of these cells suggested that they were of hematopoietic lineage. In addition to the colonies of loosely adherent transformed cells, foci of proliferating fibroblasts also appeared in the monolayer (Fig. 4D). Control secondary mouse embryo cultures (uninfected or infected with M-MuLV alone) did not show equivalent colonies of loosely adherent cells or proliferating fibroblasts in the same time scale (Fig. 4A).

Identification of M-MuLV(*myc*)-transformed myeloid cells. The transformed refractile cells in the M-MuLV(*myc*)-infected mouse embryo cultures were identified as relatively mature macrophages by a number of criteria. Phase-contrast microscopy of the infected cultures indicated that the colonies also contained nonrefractile adherent cells with the morphology typical of macrophages (Fig. 4E). Cytocentrifugation and staining of the nonadherent cells revealed appearance typical of mature macrophages, although there were some features indicative of immaturity (nucleoli and pseudopods [Fig. 4F]). In addition, both adherent and nonadherent refractile cells exhibited phagocytosis of India ink particles (50). Finally, the majority of the cells showed positive staining with Mac-1 and also with Mac-2 monoclonal antibodies as measured by flow cytometry (47).

It was important to establish that the transformed myeloid cells were infected with M-MuLV(*myc*). A myeloid cell line derived from the M-MuLV(*myc*)-infected cultures (MAC 7) (see below), as well as the entire M-MuLV(*myc*)-infected mouse embryo culture (I-10), was analyzed for the presence of *myc* RNA by RNA dot blot analysis, and they contained relatively high levels of *myc*-specific RNA (Fig. 5). The cells were also analyzed for presence of M-MuLV *gag-myc* fusion protein, and they showed the 110-kDa *gag-myc* protein found in the original NP4-6 and transformed 46M5 cells (Fig. 6).

M-MuLV(*myc*)-infected embryo cultures were also analyzed for M-MuLV(*myc*) proviral DNA by Southern blot hybridization (Fig. 7). The total infected culture was analyzed, as well as the MAC7 macrophage line. Cleavage with *Sac*I (and with several other diagnostic restriction enzymes [data not shown]) confirmed that the cells contained M-MuLV(*myc*) proviral DNA. These results also confirmed the recovery of the 3′ LTR on M-MuLV(*myc*) during M-MuLV infection of the NP4-6 cells discussed above.

In one additional experiment, culture supernatant from the MAC7 macrophage cell line was used to infect fresh mouse embryo cultures. Myeloid colonies appeared with approximately the same time course and frequency as for the mouse embryo cells infected with M-MuLV(*myc*). Thus, M-MuLV(*myc*)-transformed myeloid cells produced infectious M-MuLV(*myc*).

Unlimited growth of transformed myeloid cells. The myeloid colonies which developed in the M-MuLV(*myc*)-infected mouse embryo cultures contained rapidly dividing cells (Fig. 4B and C). Typical infected cultures showed 10 to 20 myeloid colonies at 8 days postinfection, and by 12 to 14 days refractile cells had reached saturation density (2 × 10^6 per 5-cm dish). When incubated for longer periods, uninfected mouse embryo cultures also developed colonies of refractile myeloid cells at low frequency (e.g., two colonies at 10 days in one experiment). However, the myeloid colonies from M-MuLV(*myc*)-infected and control cultures differed. First, the morphology of the myeloid colonies from control cultures was somewhat different in that the refractile cells were larger in size, less densely spaced, and more firmly attached to the fibroblast monolayer. The adherent macrophages in these uninfected cultures also had a more vacuolated appearance. In addition, cells in the myeloid colonies from control cultures did not proliferate beyond the colony stage. In contrast, cells in the myeloid colonies from infected cultures had virtually unlimited proliferative capacity.

The unlimited growth potential of the M-MuLV(*myc*)-transformed myeloid cells was demonstrated in two ways. First, M-MuLV(*myc*)-infected cultures which had reached saturation density for the myeloid cells (as well as confluency for the underlying fibroblasts) were serially passaged 1:2 by trypsinization and replating. It was possible to passage an infected culture by this procedure more than 30 times over a period of 1 year. At the end of this period, the culture (a mixture of immortalized fibroblasts and myeloid cells) still exhibited undiminished myeloid cell proliferation.

In the second experiment, M-MuLV(*myc*)-infected cultures which contained myeloid cells at saturation density

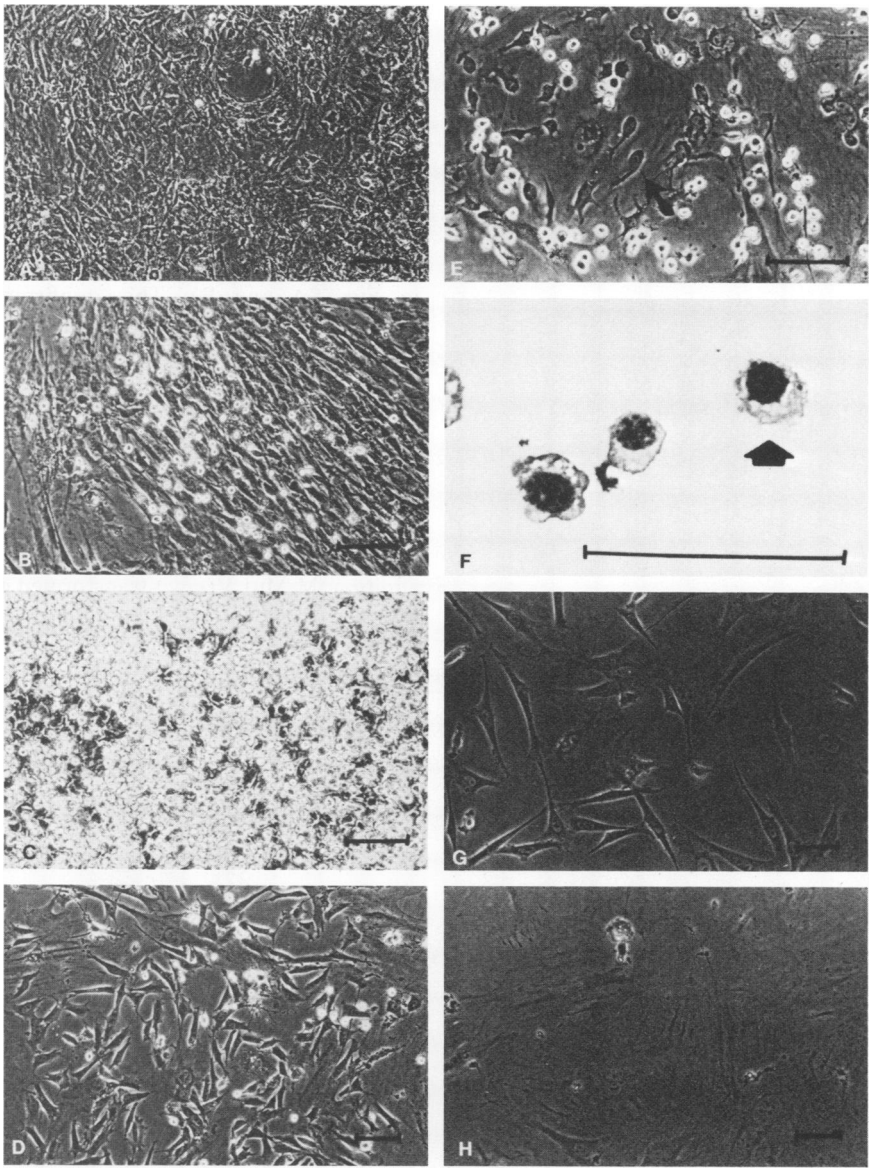

FIG. 4. Transformation of mouse embryo cultures. Secondary mouse embryo cultures were transferred at a 1:5 dilution into 5-cm tissue culture dishes and infected with M-MuLV(*myc*)–M-MuLV (1.5×10^3 FFU of M-MuLV(*myc*) and 10^5 XC PFU of M-MuLV per culture). (A) Uninfected cells. (B) A colony of transformed myeloid cells which appear as small refractile cells from the M-MuLV(*myc*)-infected culture 6 days postinfection. (C) The same culture 12 days postinfection, at which time the refractile myeloid cells had reached saturation density (2×10^6 cells per 5-cm dish). (D) A focus of transformed fibroblasts, smaller in size and reduced in stress fibers, in contrast to surrounding normal fibroblasts. A few transformed myeloid cells are also evident in the photograph. (E) A higher-magnification view of a transformed myeloid colony. In addition to the refractile myeloid cells, adherent macrophages attached to the underlying fibroblasts were also evident (arrow). (F) Cytology of transformed nonadherent cells. Nonadherent cells were harvested from an M-MuLV(*myc*)-infected mouse embryo culture by gentle aspiration. Cell suspensions were cytocentrifuged onto glass slides and stained with Wright-Giemsa. The arrow indicates a cell with nucleoli and pseudopods. (G) Morphology of a colony of M-MuLV(*myc*)-immortalized mouse embryo fibroblasts in the same assay. The M-MuLV(*myc*)-immortalized fibroblasts were smaller in size and more refractile, and they lacked the stress fibers present in spontaneously immortalized cells. (H) Morphology of a colony of spontaneously immortalized mouse embryo fibroblasts from an uninfected culture seeded at low density. Bars = 100 μm.

were depleted of refractile cells, many of which were nonadherent or loosely adherent. This was accomplished by gentle washing and aspiration of the infected culture with a stream of growth medium. Cultures treated in this fashion repeatedly regenerated the refractile macrophages, which eventually again reached saturation density. Microscopic counting of myeloid cells from a repeatedly depleted culture indicated that in one infected culture they underwent at least 65 doublings from the initially infected cells.

Growth factor requirements of myeloid cells. For these studies, loosely adherent and nonadherent myeloid cells were removed from an infected culture as described above and tested for growth under several conditions. Culture of harvested myeloid cells in fresh growth medium resulted in complete lysis or cell death within 3 to 4 days. Thus, other factors were necessary for survival or growth. M-MuLV(*myc*)-transformed myeloid cells could be successfully cultured on UV light-irradiated feeder layers of second-

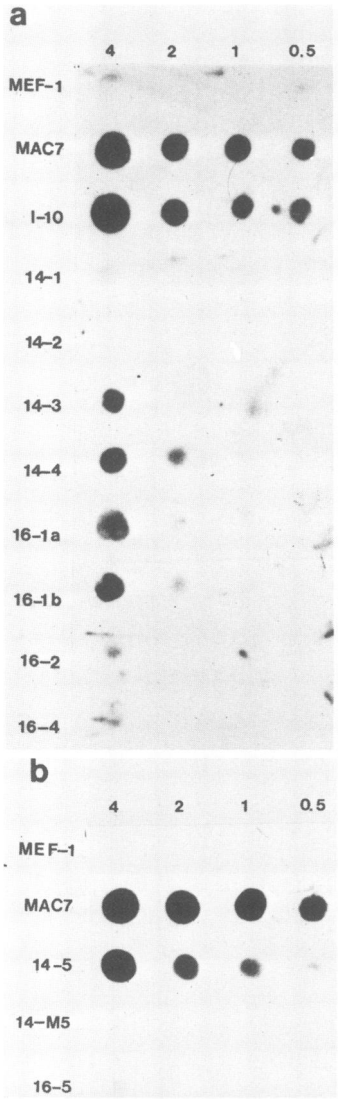

FIG. 5. Tests for *myc* RNA in cell lines. Total cytoplasmic RNA was extracted from cell lines and dot blotted onto nylon membranes (GeneScreen Plus; 4, 2, 1, and 0.5 μg). The dot blots were hybridized with a nick-translated v-*myc*-specific probe (panel A, the 1.8-kb *Bcl*I-*Bam*HI fragment from cloned MC29 [Fig. 1c]) or a radioactive RNA probe (panel B, transcribed in vitro from an SP-6 expression vector). Cell lines 14-1 through 14-5 and cell lines 16-1 through 16-5 are individual immortalized fibroblast colonies from mouse embryo cultures infected with M-MuLV(*myc*). MEF-1 is a spontaneously immortalized fibroblast line obtained from an uninfected mouse embryo culture. I-10 is an M-MuLV(*myc*)-infected mouse embryo culture containing both macrophages and fibroblasts. MAC7 is a permanent macrophage line derived from M-MuLV(*myc*)-transformed myeloid cells.

ary mouse embryo cultures. Figures 8A, B, and C depict growth on a feeder layer, indicating that mouse embryo cultures can supply the necessary factors. Since secondary mouse embryo cultures contain cell types other than the predominant fibroblasts (e.g., the myeloid cells detected by M-MuLV(*myc*) transformation), it was possible that one of the minority cell populations supplied the growth factors. However, the myeloid cells could also be grown on NIH 3T3 fibroblast feeder layers or on a line of spontaneously immortalized mouse embryo fibroblasts (MEF-1).

Harvested myeloid cells could also be cultured without a feeder layer but in medium conditioned by secondary mouse embryo, NIH 3T3, or MEF-1 cells. The cells demonstrated proliferative capacity, although at a somewhat reduced level in comparison with growth on feeder layers (Fig. 8D).

The ability to transfer and grow loosely adherent and nonadherent myeloid cells allowed more detailed examination of their properties and behavior. First, when loosely adherent and nonadherent transformed myeloid cells were added to a feeder layer of an irradiated mouse embryo culture, a portion of them rapidly became adherent (arrows in Fig. 8A and B). This confirms that the adherent macrophagelike cells associated with the original myeloid colonies were related to the nonadherent cells. Second, behavior of transformed myeloid cells harvested from M-MuLV(*myc*)-infected cultures could be compared with normal myeloid cells harvested from control cultures. Most M-MuLV(*myc*)-transformed myeloid cells remained refractile and loosely adherent when harvested and cultured in conditioned medium (Fig. 8D). In contrast, normal myeloid cells in the same medium adhered strongly to the tissue culture plastic and became less refractile (Fig. 8E). In addition, M-MuLV(*myc*)-transformed cells showed a 10-fold increase in cell number 4 days after transfer into conditioned medium, whereas uninfected myeloid cells actually de-

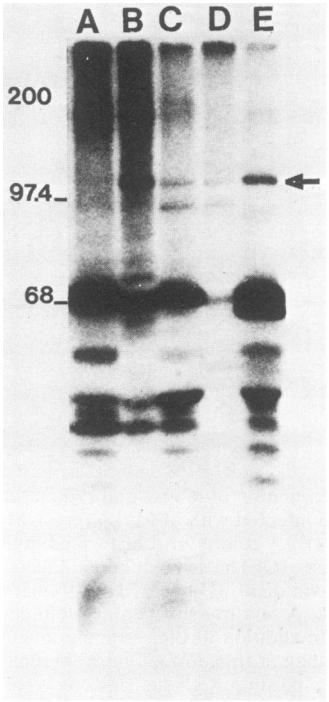

FIG. 6. Immunoprecipitation from transformed myeloid cells. Cells were labeled with ^{32}P; and immunoprecipitated with anti-p30 or anti-v-*myc* serum as described in the legend in Fig. 2. Lanes: A, wild-type M-MuLV-infected NIH 3T3 cells (clone 43D); B, NP4-6 cells; C and D, transformed 46M5 cells; E, MAC7 cells—a permanent line of myeloid cells derived from M-MuLV(*myc*)-transformed myeloid cells. All immunoprecipitations were with anti-p30 serum except lane D, which was immunoprecipitated with anti-v-*myc*. The 110-kDa *gag-myc* fusion protein was present in transformed myeloid cells (arrow). 46M5 cells contained a smaller (95-kDa) *gag-myc* fusion protein in addition to $p110^{gag\text{-}myc}$, which may have resulted from proteolytic cleavage. Other investigators have found that both MC29 *gag-myc* protein and normal-cell c-*myc* protein are metabolically labile (22).

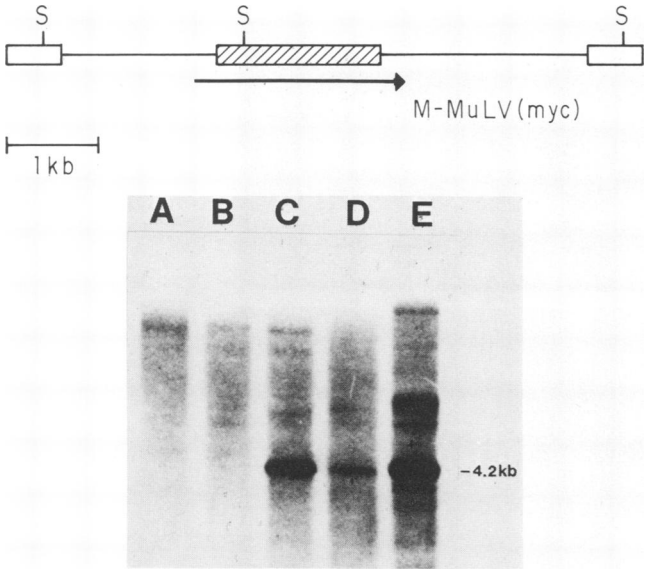

FIG. 7. Viral DNA analysis in M-MuLV(*myc*)-transformed cells. High-molecular-weight DNA was extracted from cells, digested with *Sac*I restriction endonuclease (as well as *Cla*I and *Sma*I; data not shown), and analyzed by electrophoresis in a 0.8% agarose gel and Southern blot hybridization with nick-translated v-*myc* probe. A map of pM-MuLV(*myc*) DNA is shown in A. The arrow indicates the direction of transcription. *Sac*I digestion yielded two *myc*-specific fragments of 2.2 and 4.2 kb; the 4.2-kb fragment is diagnostic for the 3′ portion of the provirus. B shows an autoradiogram of the Southern blot. Lanes: A, DNA from uninfected NIH 3T3 cells; B, NP4-6 cells; C, I-10 M-MuLV(*myc*)-infected mouse embryo culture containing both fibroblasts and macrophages; D, MAC7 macrophage cell line derived from M-MuLV(*myc*) infection; E, pM-MuLV(*myc*) plasmid DNA also cut with *Sac*I (2 pg) mixed with *Eco*RI-digested NIH 3T3 DNA as the carrier. I-10 and MAC7 cells show the appropriate *myc*-containing fragments for M-MuLV(*myc*). The figure also demonstrates that the integrated pM-MuLV(*myc*) DNA in the original NP4-6 cells lacked the 3′ LTR. The lack of the 3′ LTR in NP4-6 cells was confirmed by digestion with *Sma*I and *Cla*I as well (data not shown).

creased 90% in number. Thus, the M-MuLV(*myc*)-infected myeloid cells were transformed in terms of both adherence and growth properties.

Target cells for myeloid transformation. M-MuLV(*myc*)-induced appearance of macrophages could result from one of two possibilities: (i) M-MuLV(*myc*)-induced proliferation of normally terminally differentiated macrophages or (ii) increased proliferation of macrophage precursor cells due to M-MuLV(*myc*) infection. Experiments were performed to distinguish these two possibilities. First, the proliferative capacities of completely nonadherent cells from M-MuLV(*myc*)-infected cultures were studied. These cells could be obtained by gentle aspiration of infected cultures without washing to remove loosely adherent cells, and they exhibited a uniform morphology of mature macrophages (Fig. 9A). Nonadherent macrophages showed no proliferative capacity (Table 2). In contrast, nonadherent plus loosely adherent cells harvested by gentle pipetting showed considerable proliferative capacity. Thus, mature macrophages in the M-MuLV*myc*)-infected cultures could not proliferate. Rather, macrophage precursor cells apparently acquired increased proliferation.

A second experiment was performed to characterize the loosely adherent myeloid cells. Loosely adherent plus nonadherent cells were used as starting material. The cells were passed through 15-μm mesh Nitex cloth, which separates cells on the basis of size or rigidity. (Mature macrophages are amorphous in shape.) The cells retained on the Nitex cloth were enriched for proliferative capacity in comparison with those which passed through (Table 2). The cells which passed through the Nitex morphologically resembled mature macrophages (Fig. 9B), whereas cells retained on the Nitex consisted of some mature macrophages but additionally blastlike hematopoietic cells with high nucleus-cytoplasm ratios (Fig. 9C).

Although the M-MuLV(*myc*)-transformed myeloid cells were growth factor dependent, it was possible to derive a permanent line of factor-independent myeloid cells from the infected cultures. Loosely adherent plus nonadherent cells were maintained at high cell density in conditioned medium (approximately 2×10^6 cells per 5-cm dish) and refed biweekly. After several weeks, the cultures were fed with unconditioned medium. Although cell number initially dropped after change to unconditioned medium, the number of cells in the culture gradually increased owing to outgrowth of factor-independent cells. The resulting cell line, MAC7, was passaged 1:2 in standard growth medium with fetal calf serum more than 30 times and could achieve high saturation density (Fig. 9D). The morphology of MAC7 cells is similar to that of normal macrophages (Fig. 9E and F, respectively). Molecular analyses confirmed that MAC7 cells were infected with M-MuLV(*myc*) and that they expressed the appropriate M-MuLV(*myc*)-specific RNA and protein (Fig. 5 and 6). Presumably a secondary event occurred in the original factor-dependent cells to confer independence.

Immortalization and transformation of fibroblasts. The results described above indicated that M-MuLV(*myc*) could transform both permanent and primary fibroblasts. M-MuLV(*myc*) was also tested for the capacity to immortalize primary mouse embryo fibroblasts. The assay was to score for colony formation after seeding of cells at low density (41) (Table 3). In contrast to primary rat embryo fibroblasts, which show negligible levels of spontaneous immortalization, mouse embryo fibroblasts showed a measurable level of spontaneous immortalization (3×10^{-4} to 6×10^{-4}). Infection with M-MuLV alone actually reduced the immortalization frequency somewhat, perhaps owing to a mild cytopathic effect. Infection with M-MuLV(*myc*)–M-MuLV resulted in increased immortalization—twofold in comparison with uninfected cells and threefold in comparison with M-MuLV-infected cells. There might be some concern that the M-MuLV(*myc*)-infected cultures only showed two to three times as many colonies as control cultures. However, this was more a reflection of the spontaneous immortalization of mouse embryo fibroblasts than the immortalization potential of M-MuLV(*myc*). Rassoulzadegan et al. (41) reported similar frequencies of colony formation for polyomavirus large-T antigen in rat embryo fibroblasts, although the magnitude of the effect appeared greater owing to the negligible frequency of spontaneous colony formation in control cultures.

Cytoplasmic RNA from individual immortalized colonies from the M-MuLV(*myc*)-infected culture was also analyzed by RNA dot blot analysis with v-*myc* probe (Fig. 5). Of 10 clones analyzed, 5 contained elevated levels of *myc* RNA. These results are consistent with those in Table 3.

Immortalized cells in M-MuLV(*myc*)-infected cultures also showed a different morphology compared with immortalized cells from control mouse embryo cultures. M-MuLV(*myc*)-transformed cells were considerably smaller

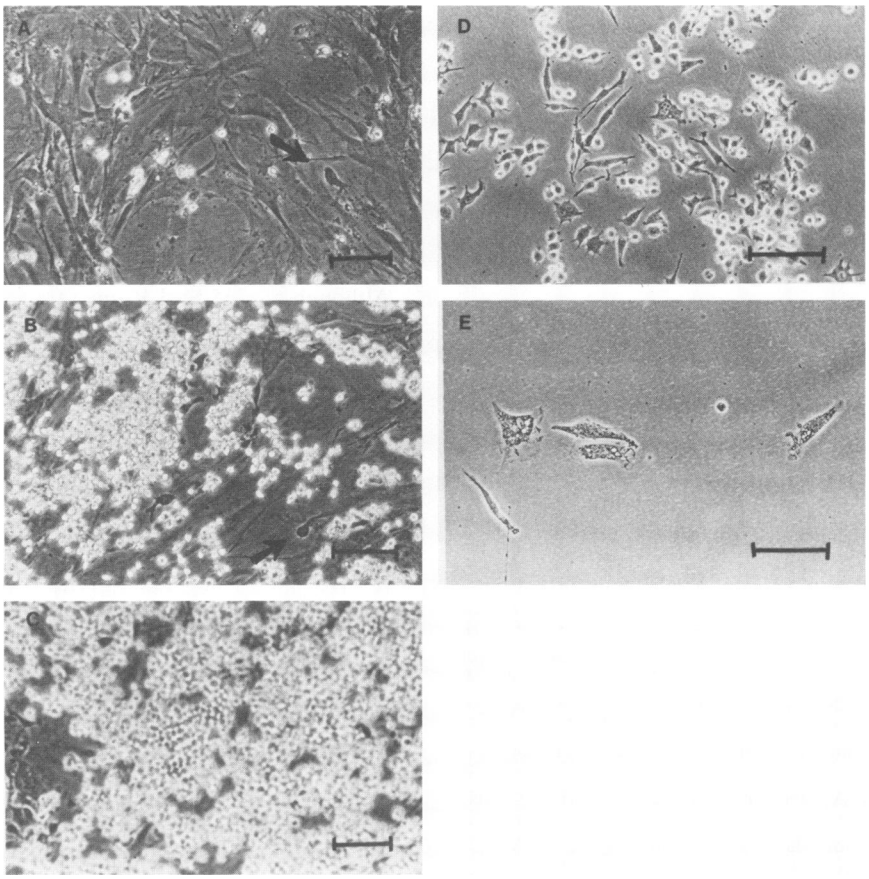

FIG. 8. Growth of harvested myeloid cells. Nonadherent plus loosely adherent myeloid cells were harvested from an M-MuLV(*myc*)-infected mouse embryo culture by gently washing the culture with growth medium and aspirating the dislodged cells. The harvested cells were placed into a tissue culture dish containing a UV-irradiated feeder layer of mouse embryo cells. The culture is shown 3 (A), 6 (B), and 9 (C) days after seeding. The refractile myeloid cells grew until they reached saturation density. In addition to the refractile cells, adherent macrophagelike cells were evident within 6 h of seeding (arrows in A and B). (D) Myeloid cells were harvested from an M-MuLV(*myc*)-infected mouse embryo culture and seeded into a tissue culture dish lacking a feeder layer but containing growth medium conditioned by mouse embryo cells (see Materials and Methods). The culture is shown 4 days after seeding. Both adherent and nonadherent refractile cells were evident. (E) Myeloid cells were harvested from an uninfected culture in the same manner as for infected myeloid cells and cultured under the conditions described for D. Cytocentrifugation and morphological staining of these cells verified that they were essentially mature macrophages. The culture is shown 4 days after seeding. The myeloid cells became tightly adherent to the tissue culture plastic and showed extensive vacuolation. Equal numbers of myeloid cells were seeded onto the tissue culture dishes shown in D and E. Bars = 100 μm.

and highly refractile and lacked the stress fibers present in immortalized fibroblasts from control cultures (Fig. 4G and H). In addition, M-MuLV(*myc*)-transformed cells appeared to be highly motile since they grew in a dispersed pattern. In fact, by 6 weeks cells in M-MuLV(*myc*)-infected cultures were evenly dispersed, whereas in control cultures discrete colonies of nontransformed cells were evident. Thus, M-MuLV(*myc*) could apparently both immortalize and transform embryo fibroblasts.

Inoculation of mice with M-MuLV(*myc*). To test the pathogenicity of M-MuLV(*myc*), neonatal NIH Swiss mice were inoculated intraperitoneally with M-MuLV(*myc*)–M-MuLV. Inoculated animals showed no evidence of acute neoplasm (death within less than 4 weeks). Most (12 of 14) of the animals developed lymphoblastic lymphoma with the same time course as control animals inoculated with helper M-MuLV alone, although one animal which died at 6 weeks demonstrated bone marrow aplasia and a possible monocyte-macrophage tumor. Thus, M-MuLV(*myc*) apparently either was not pathogenic in animals or its pathogenicity was slow in comparison with the disease induced by the M-MuLV helper.

To investigate the pathogenicity of M-MuLV(*myc*) further, a stock carried by a nonpathogenic amphotropic MuLV(Am-MuLV) helper (strain 4070) was generated. It was necessary to start with a virus which would not recombine with the Am-MuLV to produce pathogenic M-MuLV. To achieve this, the M-MuLV *env* sequences were deleted from pM-MuLV(*myc*); infectious transforming virus, M-MuLV(*myc*)′–Am-MuLV, was recovered by infection of expressing cells with Am-MuLV as described above. Although relatively low-titered stocks of M-MuLV(*myc*)′–Am-MuLV were obtained, they were inoculated into newborn NIH Swiss mice (approximately 10 FFU per animal). Inoculated animals developed lymphoblastic leukemias at high frequency (10 of 18 in the first 6 months) with latencies between 6 and 24 weeks (mean time, 16 weeks). Control animals inoculated with Am-MuLV developed no disease

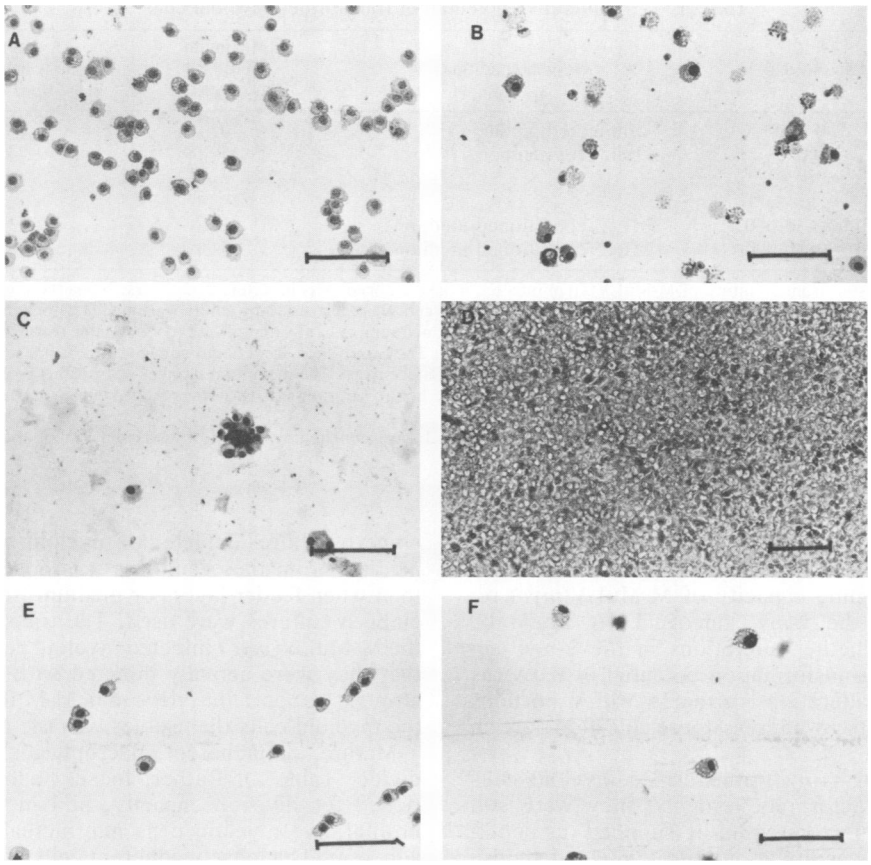

FIG. 9. Subpopulations of harvested myeloid cells. (A) Nonadherent myeloid cells were removed from an M-MuLV(*myc*)-infected mouse embryo culture by gentle aspiration without washing (which removes loosely adherent cells). The cells were subjected to cytocentrifugation onto glass slides and stained with Wright-Giemsa. The cells showed the morphology of mature macrophages. (B and C) Nonadherent plus loosely adherent myeloid cells (removed from a transformed culture by gentle washing) were filtered through Nitex cloth (15-μm mesh). Cells that passed through the Nitex are shown in B. Cells retained on the Nitex were recovered by back-washing the Nitex cloth and are shown in C. (D) Light microscopy of a dense culture of the MAC7 permanent macrophage line. (E) Cytocentrifugation of MAC7 cells showing macrophage morphology. (F) Cytocentrifugation of normal myeloid cells. Bars = 100 μm.

within the first 6 months, as expected. High-molecular-weight DNA from two representative tumors was examined by Southern blot hybridization (Fig. 10). Hybridization with a v-*myc* DNA probe confirmed that both of the animals contained M-MuLV(*myc*) proviral DNA in the tumors (Fig. 10A). Furthermore, hybridization with a cDNA probe for the mouse T-cell receptor beta chain gene showed monoclonal or oligoclonal T-cell receptor gene rearrangements in the tumors. This indicated that both of the tumors were T lymphoid. Molecular analyses of all of the tumors examined revealed characteristic T-cell receptor gene rearrangements. Thus, M-MuLV(*myc*)–Am-MuLV induced T-lymphoid leukemia but with long latency.

DISCUSSION

The generation of a recombinant murine leukemia virus carrying the v-*myc* oncogene of avian MC29 virus is described here. This virus, M-MuLV(*myc*), encodes an M-MuLV *gag*–v-*myc* fusion protein of 110 kDa. M-MuLV(*myc*) morphologically transformed NIH 3T3 fibroblasts, and it both transformed and immortalized secondary mouse embryo fibroblasts. Furthermore, M-MuLV(*myc*) showed strong transformation of myeloid cells when infected into secondary mouse embryo cultures. The predominant transformed myeloid cell resembled mature macrophages by a variety of criteria. Despite the myeloid and fibroblast transformation exhibited in vitro, inoculation of M-MuLV(*myc*) did not induce rapid myeloid leukemias or fibrosarcomas. Rather, the virus induced T-lymphoid leukemia.

The fibroblast transformation ability of M-MuLV(*myc*) was interesting since others have suggested that MC29 v-*myc* protein may immortalize but not morphologically transform fibroblasts (29). Our results might have been due to higher levels of v-*myc* expression as an outcome of viral infection. Recently other investigators have also reported conditions in which avian v-*myc* or murine c-*myc* can transform or immortalize fibroblasts or both (25, 53).

M-MuLV(*myc*) infection of mouse embryo cultures resulted in transformed myeloid cells by three criteria: (i) adherence properties of the resulting macrophages, (ii) unlimited growth potential in tissue culture, and (iii) reduced growth factor requirement. Myeloid cells from uninfected mouse embryo cultures differed in all three of these properties.

The strong myeloid transforming ability of M-MuLV(*myc*) was particularly noteworthy since the parental MC29 virus

TABLE 2. Proliferative potential of transformed myeloid cells

Subpopulation[a]	No. seeded	Growth conditions	Maximum no. of transfers[b]	Avg no. of days between transfer[c]	Calculated doublings[d]
Nonadherent	500,000	Uninfected feeder	0		<1
Loosely adherent + nonadherent	500,000	Uninfected feeder	5	3	10
Nitex filtrate	20,000–30,000	MEC[e]-conditioned medium	1	43	6
Nitex retained	20,000–30,000	MEC[e]-conditioned medium	3	18	18

[a] Myeloid cells were harvested from a culture of M-MuLV(*myc*)-infected mouse embryo cells in different manners. Nonadherent cells were harvested from cultures by gentle aspiration without washing. Nonadherent plus loosely adherent cells were harvested by gentle washing. The nonadherent plus loosely adherent cells were further separated by passage through 15-μm mesh Nitex cloth to produce retained cells and filtrate (cells passing through).

[b] Seeded cells were allowed to grow until they reached 10^6 cells per 5-cm dish. They were then seeded into new cultures at the same indicated numbers, and culture was continued. This was repeated until no further growth of myeloid cells occurred. The maximum number of transfers is indicated.

[c] For Nitex filtrate and Nitex-retained populations, more doublings had to occur before saturation density was reached because the number of cells seeded was lower. Therefore, the time to reach the first transfer was longer.

[d] The total number of doublings occurring before growth capacity was exhausted was calculated by the following formula: number of doublings = log (fold cell increase)/log 2.

[e] MEC, Mouse embryo culture.

causes myeloid tumors in birds, and it can transform macrophages in cultured avian cells. This suggests that the in vitro myeloid transforming capacity of M-MuLV(*myc*) reflected the nature of the v-*myc* oncogene. It would be interesting to know whether alterations in the v-*myc* sequences can alter the transformation potential of the virus and likewise whether alterations in the M-MuLV portions can do so. Experiments to address these questions are in progress.

Although the M-MuLV(*myc*)-transformed myeloid cells had reduced growth factor requirements, they were still factor dependent. The factors could be supplied (as conditioned medium or by cocultivation) by uninfected mouse embryo cultures or permanent fibroblast lines (36, 48). The exact nature of the required growth factor(s) was not investigated in detail. Other investigators have identified a variety of factors involved in growth and differentiation of myeloid precursor cells (7, 35), and it will be interesting to test defined growth factors for their effects on transformed myeloid cells.

Since the transformed myeloid cells were detected in mouse embryo cultures (which actually predominantly contained fibroblasts), it was formally possible that M-MuLV(*myc*) infection and transformation of another cell type played a primary or secondary role in development of the transformed myeloid cells. For instance, if M-MuLV(*myc*) induced mouse embryo cells to produce higher amounts or new kinds of myeloid growth factors (see, e.g., Leutz et al. [30]), myeloid proliferation could be a secondary event. However, growth of transformed myeloid cells on irradiated feeder layers of M-MuLV(*myc*)-infected mouse embryo cultures depleted of myeloid cells or in conditioned medium from these cultures was not significantly different than when feeder layers or medium from uninfected mouse embryo cultures were used. The transformed properties of the M-MuLV(*myc*)-infected myeloid cells, as well as the fact that they were actually infected with M-MuLV(*myc*), also strongly support the view that M-MuLV(*myc*) infection of the myeloid cells themselves was the primary event.

TABLE 3. Low-density seeding of infected mouse embryo cultures

Expt no.	Infecting viral stock	No. of colonies/ 8×10^4 cells[a]
1	M-MuLV(*myc*)–M-MuLV[b]	48
	M-MuLV	14
	Uninfected	26
2	M-MuLV(*myc*)–M-MuLV	25
	Uninfected	15

[a] Colonies were counted 4 weeks after seeding 40,000 to 50,000 cells onto 9-cm dishes.

[b] The source of M-MuLV(*myc*)–M-MuLV viral stock was 46M5 cells.

Mature, nonadherent macrophages had no capacity to divide (Table 2). Rather, loosely adherent cells were enriched for division capacity, and in fact the bulk of the proliferative myeloid cells may actually have been tightly adherent. The loosely adherent cells of Table 2 only showed limited proliferative capacity, yet infected cultures passaged by trypsinization showed unlimited myeloid proliferative potential. Also, when M-MuLV(*myc*)-infected mouse embryo cultures were depleted of nonadherent and loosely adherent cells they exhibited myeloid proliferative capacity identical to that of nondepleted cultures.

Whereas the initially transformed myeloid cells were factor dependent, it was possible to derive a factor-independent macrophage line, MAC7, from them. Presumably a secondary event occurred in the transformed myeloid cells to allow growth in unconditioned medium. This might reflect loss of growth factor requirement or production of autocrine growth factors. In this regard, M-MuLV(*myc*) infection of an interleukin 3-dependent myeloid cell line induced interleukin 3 independence (40). M-MuLV(*myc*) might also provide a useful means for generating both factor-dependent and factor-independent myeloid cells for other studies.

Whereas these experiments demonstrated the myeloid transforming capacity of M-MuLV(*myc*), they did not rule out transformation potential for other hematopoietic lineages. The myeloid transformation was fortuitously observed during infection of mouse embryo cultures. No particular measures were taken to favor hematopoietic cell growth. Thus, it is possible that hematopoetic target cells for other lineages (e.g., erythroid and lymphoid) were no longer present in secondary mouse embryo cultures. Additionally, if M-MuLV(*myc*)-transformed erythroid or lymphoid cells still required factors which could not be supplied by infected mouse embryo cultures, the culture conditions may not have supported their growth. Futher tests for hematopoietic progenitor proliferation are in progress.

In light of the myeloid and fibroblast transformation ob-

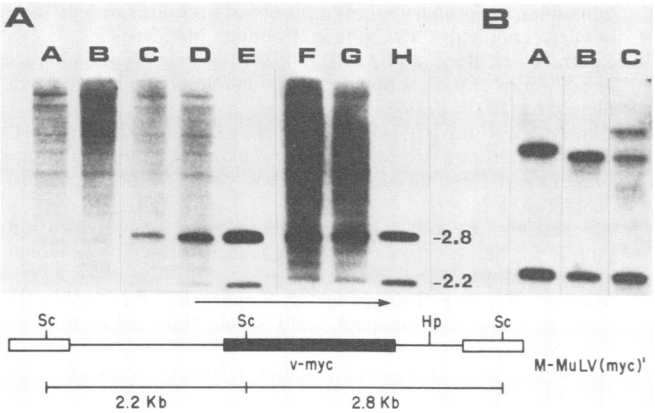

FIG. 10. Southern blot analysis of M-MuLV(*myc*)' tumor DNA. (A) Thymus and spleen DNAs were digested with *Sac*I (Sc) and analyzed by electrophoresis on a 0.8% agarose gel and Southern blot hybridization to a v-*myc*-specific probe. A restriction map of M-MuLV(*myc*)' showing the expected *Sac*I restriction fragments is shown below. The arrow indicates the direction of transcription. Lanes: A, uninoculated mouse spleen; B, Am-MuLV-inoculated mouse spleen; C and D and F and G, spleen and thymus, respectively, of two moribund M-MuLV(*myc*)'–Am-MuLV-inoculated mice; E and H, *Sac*I-digested pM-MuLV(myc)' DNA (2 pg). (B) DNAs were digested with *Hpa*I (Hp) and analyzed as described above with a T-cell receptor-specific probe (cDNA clone kindly provided by Stephen Hedrick). Lanes: A, uninoculated mouse spleen showing expected germ line $C_{\beta 1}$ and $C_{\beta 2}$ bands; B and C, thymus DNAs of two moribund M-MuLV(*myc*)'–Am-MuLV-inoculated mice treated as described for A. M-MuLV(*myc*)'–Am-MuLV-inoculated mouse thymus DNAs show discrete bands indicating clonal populations of T cells which are rearranged at the T-cell receptor beta chain locus.

served in vitro, it was noteworthy that M-MuLV(*myc*) did not induce acute leukemias or solid tumors at high frequency when inoculated into newborn mice. In the case of the myeloid cells, it is possible that M-MuLV(*myc*) infected myeloid cells in vivo but that the cells underwent terminal differentiation. In such a case, no neoplasm would result. This is consistent with the apparent terminal differentiation of the M-MuLV(*myc*)-infected macrophages. In vitro transformed macrophages also did not induce tumors upon inoculation into syngeneic BALB/c mice.

The induction of T-lymphoid leukemia by M-MuLV(*myc*) was extremely interesting. The activation of c-*myc* protooncogenes in nonviral B lymphomas and lymphomas induced by nontransforming retroviruses is well documented (5, 6, 20, 45). It is noteworthy that M-MuLV [the parent of M-MuLV(*myc*)] also induces T lymphoma, and the disease specificity is a reflection of the enhancer sequences in the M-MuLV LTR. In fact, M-MuLV-induced T lymphomas frequently show activation of c-*myc* by adjacent M-MuLV LTR insertion (20, 45). It will be interesting to generate and test an M-MuLV(*myc*) which contains an LTR with a different cell type specificity.

Two lines of evidence suggest that the mechanism of M-MuLV(*myc*)-induced leukemogenesis is different from that of most acutely transforming retroviruses, even though it carries an oncogene. First, the long latency of disease appearance does not resemble the rapid disease appearance of acute transforming retroviruses. Second, the tumors were monoclonal, whereas neoplasms induced by acute transforming retroviruses are frequently polyclonal. Leukemogenesis by M-MuLV(*myc*) may actually be more similar to leukemogenesis by nonacute retorviruses, in which activation of c-*myc* is frequently an important event.

Further characterization of leukemias induced by M-MuLV(*myc*) is in progress. Recent results indicate that the T lymphomas differ in relative differentiation state in comparison with tumors induced by M-MuLV, in that many may represent more immature thymocytes. In addition, some inoculated animals have shown lymphoid tumors with two different phenotypes. Recent preliminary results also indicate that M-MuLV(*myc*) may also induce tumors of epithelial origin in some of the inoculated mice.

While these experiments were in progress, Vennstrom et al. (53) also reported generation of an M-MuLV carrying v-*myc*. The molecular organization of their virus differed significantly from that of the M-MuLV(*myc*) described here in two important ways. First, the v-*myc* oncogene was obtained from avian OK10 virus rather than MC29. Second, the construction resulted in synthesis of an unfused 55-kDa, v-*myc* protein rather than the 110-kDa M-MuLV *gag-myc* fusion protein encoded by M-MuLV(*myc*). Despite these differences, the virus of Vennstrom et al. had properties very similar to those of M-MuLV(*myc*). It transformed fibroblasts in vitro, and it transformed macrophages as well. Thus, the similar biological behavior underscores the influence of the v-*myc* sequences in the in vitro transforming potential of these two viruses.

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