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Synergism of v-*myc* and v-Ha-*ras* in the In Vitro Neoplastic Progression of Murine Lymphoid Cells

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Murine bone marrow was either singly or doubly infected with retroviral vectors expressing v-*myc* (OK10) or v-Ha-*ras*. The infected bone marrow was cultured in a system that supports the long-term growth of B-lineage lymphoid cells. While the v-*myc* vector by itself had no apparent effect on lymphoid culture establishment and growth, infection with the v-Ha-*ras* vector or coinfection with both v-*myc* and v-Ha-*ras* vectors led to the appearance of growth-stimulated cell populations. Clonal pre-B-cell lines stably expressing v-Ha-*ras* alone or both v-*myc* and v-Ha-*ras* grew out of these cultures. In comparison with cell lines expressing v-Ha-*ras* alone, cell lines expressing both v-*myc* and v-Ha-*ras* grew to higher densities, had reduced dependence on a feeder layer for growth, and had a marked increase in ability to grow in soft-agar medium. The cell lines expressing both oncogenes were highly tumorigenic in syngeneic animals. These experiments show that the v-*myc* oncogene in synergy with v-Ha-*ras* can play a direct role in the in vitro transformation of murine B lymphoid cells.

The *myc* oncogene has been correlated with the genesis of murine, human, and avian B-cell neoplasias. The physical structure and expression of c-*myc* has been elucidated in neoplastic versus normal cells. Chromosomal translocation is associated with the activation of c-*myc* in murine plasmacytomas and human Burkitt lymphomas (reviewed in references 28 and 38), while retroviral insertion is responsible for the activation of c-*myc* in avian leukosis virus-induced B-cell lymphomas (20, 37). Avian retroviruses that express v-*myc* induce a wide range of cancers in vivo including tumors of the kidney and liver and myelocytomatosis (reviewed in reference 17). These retroviruses have been the focus of investigations of in vitro *myc*-induced oncogenesis. The only hematopoietic cells transformed by these retroviruses in vitro are macrophages. The strategies employed in these in vitro experiments generally selected for the rapid transformation of target cells by v-*myc*.

Two recent in vivo experimental investigations of *myc* oncogene causality in lymphoid neoplasia have been reported. One utilized the v-*myc*-expressing avian retrovirus HB1 (16). This mutant of MC29 induces lymphomas in vivo. When bursal lymphoblasts are infected in vitro with HB1, they can form preneoplastic bursal structures when introduced into chickens (32). A c-*myc* oncogene linked to immunoglobulin enhancers for expression induces lymphomas in transgenic mice carrying that gene (2). It is likely that the in vivo expression of this c-*myc* construct induces a preneoplastic state that progresses to malignancy.

In vivo observations of *myc* causality in lymphoid transformation leave unclear the secondary genetic alterations that might interact with *myc* expression to bring about complete transformation. The role of the *myc* oncogene in the transformation of B lymphoid cells is better studied in the context of an in vitro neoplastic progression. Oncogenesis may proceed as either an ordered series of events involving the expression of specific genes involved in the regulation of cellular growth or as an additive effect of events involving such genes (recently reviewed in references 5, 24, and 64). An experimental demonstration of a requirement for two complementing oncogenes in transformation may be a reflection of this process. A synergy between Ha-*ras* (EJ) and v-*myc* (MC29) or Ha-*ras* (T24) and E1A has been demonstrated in the in vitro transformation of primary rat embryo fibroblasts (27, 46).

The concept that this synergy might be important for the induction of lymphoid neoplasia is suggested by several observations. *myc* genes derived from lymphoid tumors are incapable of transforming fibroblasts. Although c-*myc* is activated in chicken B-cell lymphomas and in murine plasmacytomas, a separate gene is responsible for the transformation of NIH 3T3 cells by transfected DNA from those tumors (1, 9). Similarly, the Ramos Burkitt lymphoma has an activated N-*ras* oncogene (31) in addition to a rearranged c-*myc* gene (1). Lymphoid neoplasms have been induced by a murine retrovirus expressing both *raf* (*mil*) and v-*myc* (MC29), while animals inoculated with a virus expressing only *raf* (*mil*) develop fibrosarcomas (42). The Ha-*ras* oncogene is a good candidate for synergy with v-*myc* in lymphoid transformation because it has been shown to transform lymphoid cells in vitro, albeit at a low frequency (39). A synergy between v-*myc* and Ha-*ras* should be studied in a culture system that allows for the isolation of cells expressing intermediate transformed phenotypes. The long-term B-cell culture system of Whitlock and Witte (65) meets that requirement. This culture system supports the growth of B cells and their precursors (25, 65, 66). This allows for growth of a diversity of lymphoid targets in the B-cell lineage.

The v-*myc* oncogene can act in synergism with v-Ha-*ras* to effect the neoplastic transformation of murine pre-B cells in vitro. Those cells expressing v-Ha-*ras* alone display an intermediate level of transformation. The ability to recognize and isolate cells of intermediate phenotypes in lymphoid transformation may provide a unique opportunity to follow

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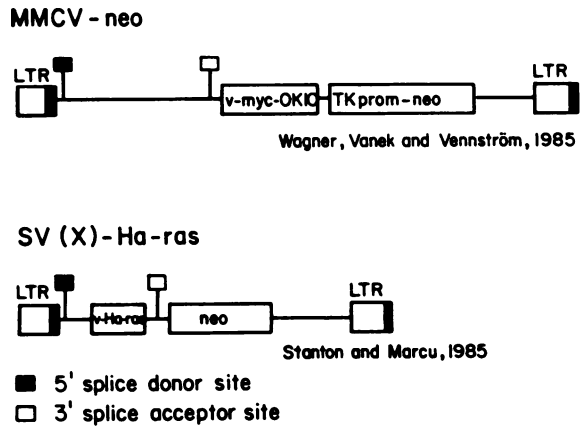

FIG. 1. Structure of the retrovirus vectors. LTR, Long terminal repeat.

the neoplastic progression in vitro through the experimental manipulation of those cells.

MATERIALS AND METHODS

Viruses. MMCV-neo (62) (Fig. 1) contains long terminal repeats derived from segments of Moloney murine leukemia virus and Harvey sarcoma virus. The virus carries the 2.5-kilobase (kb) *Bam*HI-*Eco*RI fragment of OK10 inserted with *Bam*HI linkers. *myc* is expressed as a subgenomic mRNA from the 5′ viral long terminal repeat. MMCV-neo also contains the dominant selectable Tn*5* *neo* gene expressed from the herpesvirus thymidine kinase promoter.

SV(X)-Ha-ras (Fig. 1) was constructed by insertion of the 0.7-kb *Sac*I-*Pst*I fragment encoding v-Ha-*ras* (12) into the *Bam*HI site of pZIP-NEO SV(X)1 (7) with *Bam*HI linkers. This vector contains Moloney murine leukemia virus long terminal repeats and expresses the Tn*5* *neo* gene through a subgenomic mRNA from the 5′ viral long terminal repeat.

Virus stocks consisted of culture medium from NIH 3T3 cell lines that had been cotransfected with a proviral clone of Moloney murine leukemia virus and either pMMCV-neo or pSV(X)-Ha-ras. Virus titers of 3.2×10^6 to 1.0×10^7 G418-resistant colonies per ml were obtained for MMCV-neo on NIH 3T3 cells; titers of 1.9×10^5 to 7.2×10^5 were obtained for SV(X)-Ha-ras.

Cell culture and viral infections. Bone marrow from 3- to 5-week-old BALB/c mice was cultured by the procedure of Whitlock and Witte (65) with the addition of a viral infection step. Bone marrow was suspended at 2.0×10^6 cells per ml in RPMI 1640 medium supplemented with 5% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol. To this was added an equal volume of virus stock and Polybrene (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 8 μg/ml. After 3 h of incubation at 37°C, the cells were pelleted and resuspended at 10^6 cells per ml in RPMI 1640 supplemented with 5% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol. Either 4 or 5 ml of this suspension was plated per 6-cm culture dish. Cultures were fed twice weekly with the same medium. Once a week approximately 80% of the spent medium was replaced with fresh medium. The cultures were expanded by transfer of nonadherent cells to feeder cultures of adherent bone marrow cells (66). Growth in soft-agar medium was performed as described by Whitlock et al. (66).

Nucleic acid isolation. Cytoplasmic RNA was isolated by a sodium dodecyl sulfate-urea procedure as described by Schwartz et al. (48). High-molecular-weight DNA was isolated from nuclei collected in the preceding procedure. The nuclei were washed once in 10 mM Tris hydrochloride (pH 7.5)–10 mM KCl–1 mM $MgCl_2$–5 mM $CaCl_2$. Nuclei were washed a second time in the same solution supplemented with 0.05% Triton X-100 and then suspended in 50 mM Tris hydrochloride (pH 7.5)–100 mM NaCl–10 mM EDTA. Sodium dodecyl sulfate was added to 0.05% to lyse the nuclei. The nuclear lysate was incubated with RNase A (40 μg/ml) at 37°C for 1.5 h. The lysate was then incubated overnight at room temperature with proteinase K (200 μg/ml). The lysate was extracted alternately with phenol-chloroform-isoamyl alcohol (1:1:0.1, vol/vol/vol) and chloroform. DNA was collected from the aqueous phase by ethanol precipitation at room temperature.

Nucleic acid hybridization probes. Hybridization probes were prepared by nick translation (45) through the incorporation of 5′-[α-^{32}P]dATP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). The v-Ha-*ras* probe was mp10-v-H-ras, the replicative form of phage M13mp10 containing a 0.46-kb *Eco*RI fragment corresponding to v-Ha-*ras*-encoding sequences (15). The v-*myc* probe was mp10-v-myc, the replicative form of phage M13mp10 containing a 1.5-kb *Pst*I fragment corresponding to the v-*myc*-encoding sequences of retrovirus MC29 (61). The J_H heavy-chain probe consisted of the genomic 1.9-kb *Bam*HI-*Eco*RI fragment, which corresponds to the J_{H2}, J_{H3}, and J_{H4} regions that are 5′ to the Mu heavy-chain constant-region gene (13). The C_K probe was mpC_K12, the replicative form of phage M13mp10 containing a genomic 0.48-kb *Hpa*I-*Bgl*II fragment extending from a point about 50 base pairs within 5′ terminus of the kappa light-chain constant-region gene to the 3′ poly(A) addition site (49). The T_β probe consisted of the genomic 1.85-kb *Xba*I fragment, which corresponds to exons 2 and 3 of the $T_{\beta 1}$ constant-region gene (30). This fragment was derived from pA10cat2XX1.95.

Metabolic labeling and immunoprecipitation. Cells were metabolically labeled and immunoprecipitates were analyzed as described by Whitlock et al. (66). Cells (10^7) were labeled in 2 ml of medium containing 200 μCi of [^{35}S]methionine (1,170 Ci/mol; Amersham). Cells were incubated at 37°C for 3 h. Cleared lysates were incubated overnight at 4°C with either 15 μl of normal rabbit serum or anti-*ras* peptide serum. The anti-*ras* peptide serum was raised against peptide 15 of Tanaka et al. (57) (amino acid residues 160 to 179 of v-Ha-*ras*).

For labeling with $^{32}P_i$ (carrier free; ICN Pharmaceuticals Inc., Irvine, Calif.), a similar protocol was followed. Cells were incubated at 37°C for 2 h in medium containing 1 mCi of $^{32}P_i$. The samples were incubated with either 25 μl of anti-v-*myc* peptide serum or 25 μl of the same serum blocked with the immunizing peptide. The anti-v-*myc* serum was raised against peptide v-*myc* 12C of Hann et al. (18) (the 12 C-terminal amino acids of MC29 v-*myc*).

Cytological analyses. Cells were cytocentrifuged onto a microscope slide and allowed to air dry overnight. The smeared cells were incubated with one of the following monoclonal antibodies: goat anti-mouse immunoglobulin M-horseradish peroxidase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); rat anti-Mac-I (M1/70; Hybritech); rat anti-B220 (RA3-6B2). Mouse anti-rat immunoglobulin-glucose oxidase (TAGO) was used in a secondary incubation for Mac-I detection. Goat anti-rat immunoglobulin-horseradish peroxidase (Jackson Laboratory) was used in a secondary incubation for B220 detection. Glucose oxidase-labeled cells were detected by microscopy after reaction

with Glucose Oxidase Substrate Kit II (Vector Laboratories) and counterstaining with Mayer hematoxylin (Sigma). Horseradish peroxidase-labeled cells were detected after reaction with 3-amino-9-ethylcarbazole (Sigma) and counterstaining with Mayer hematoxylin.

The presence of nonspecific esterase, chloroacetate esterase, and myeloperoxidase was determined by cytochemical staining, as described by Yam et al. (67). Sudan black B staining was performed as described by Davey and Nelson (10). All these stains were performed with Sigma research kits.

Phagocytosis was assayed by the method of Raschke et al. (43).

Tumor challenges. Cells were washed twice in RPMI 1640 and were then resuspended in the same as 8×10^6 cells per ml. BALB/c mice, 4 to 10 weeks old, were injected intraperitoneally with 0.25 ml of the cellular suspension. Animals were observed for a maximum of 10 weeks postinjection. Animals were sacrificed and autopsied when they became moribund or at 10 weeks.

RESULTS

Infection of explanted bone marrow with retroviruses expressing v-*myc* or v-Ha-*ras*. A critical test for the synergy of v-*myc* and v-Ha-*ras* required a broad range of B lymphoid target cells and an efficient means of introducing the oncogenes into those cells. The infection of bone marrow with retroviruses rescued by Moloney murine leukemia virus met those rquirements, but required a strategy that would obviate the problem of helper virus block to superinfection and not depend on a temporal order of oncogene activity for transformation.

Two murine retrovirus vectors were utilized to introduce the v-*myc* and v-Ha-*ras* oncogenes into bone marrow cells (Fig. 1). MMCV-neo (62) expresses a v-*myc* derived from the avian retrovirus OK10. This v-*myc* sequence contains only two amino acid changes from the avian c-*myc* sequence (19, 63). SV(X)-Ha-ras expresses the v-Ha-*ras* of Harvey sarcoma virus. Bone marrow explants from BALB/c mice were infected with MMCV-neo, SV(X)-Ha-ras, or coinfected with both MMCV-neo and SV(X)-Ha-ras. These cells were then plated in liquid culture by the procedure of Whitlock and Witte (65). This bone marrow culture system supports the establishment of an adherent layer of stromal cells during the first 2 weeks of culture. This is accompanied by a decrease in nonadherent cells and the subsequent appearance and growth of nonadherent B cells and their precursors between 6 and 8 weeks after plating. A diverse B lymphoid population is maintained, ranging from stem elements to pre-B cells that have undergone heavy-chain-locus rearrangement, to more mature B cells that express surface immunoglobulin M (25, 65, 66). This typical progression was observed in cultures of cells that were mock infected, infected with Moloney murine leukemia virus, or infected with ZIP-NEO SV(X)1, the parent of SV(X)-Ha-ras. These cells grew to densities between 1×10^4 and 10×10^4 cells per ml. The infection of bone marrow cells with SV(X)-Ha-ras resulted in the appearance of nonadherent cells at about 2 weeks after plating. In 17 of 20 experiments, these cells grew to densities between 1×10^5 and 4×10^5 cells per ml. In three instances, cells grew to densities between 1×10^6 and 2×10^6 cells per ml. When a mixed infection of MMCV-neo and SV(X)-Ha-ras was performed, nonadherent cells appeared at about 2 weeks after plating. These cells grew to densities between 1×10^6 and 4×10^6 cells per ml in 6 of 12 experiments. In the other six experiments, cells grew to the lower densities observed after single SV(X)-Ha-ras infection. These cell populations proved to be singly infected with SV(X)-Ha-ras by analysis of their genomic DNA (see below). MMCV-neo infection generally resulted in cultures indistinguishable from controls in their time course of establishment and growth. An outgrowth of macrophages was observed in 4 of 19 experiments, as previously reported by Vennstrom et al. (60) with a similar retrovirus (data not shown). While MMCV-neo may have had more subtle effects on B-lineage cells, we focused on the growth-stimulated cells observed with SV(X)-Ha-ras or SV(X)-Ha-ras–MMCV-neo infection.

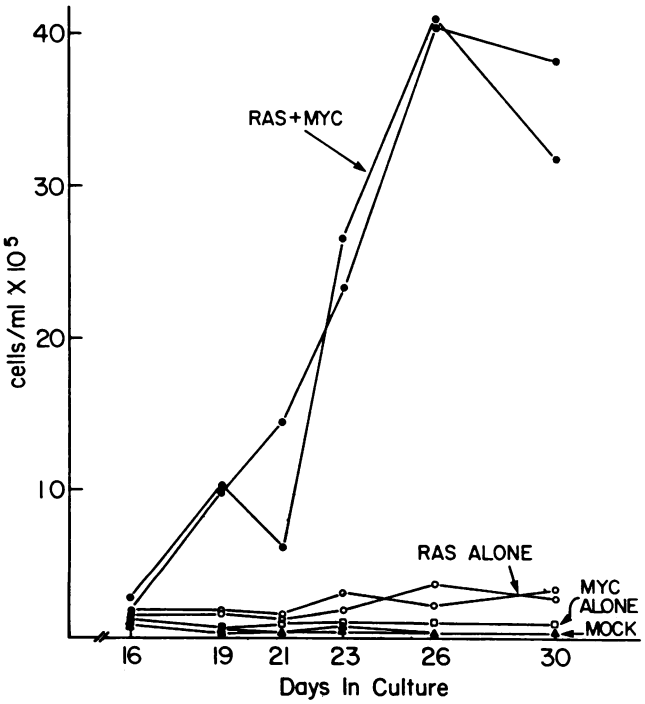

FIG. 2. Time course of growth in culture. Bone marrow was removed from the femurs of BALB/c mice, infected, and cultured as described in Materials and Methods. Cells were counted on the days indicated.

Time courses of the growth of nonadherent cells in a typical experiment are presented in Fig. 2. The dramatically higher densities of cell growth that resulted from coinfection suggest that v-*myc* and v-Ha-*ras* can act in synergism to transform cells from bone marrow. The rapid time course of cell growth (observable by day 16 and reaching peak levels on day 26) suggests that it is a primary effect of the retroviral oncogenes. Six populations, each growing out of a separate culture, were chosen for in-depth analysis: R1 and R2 arising from infection with SV(X)-Ha-ras alone and growing to low density (1×10^5 to 4×10^5 cells per ml); R3 and R4 arising from infection with SV(X)-Ha-ras alone and growing to high density (1×10^6 to 2×10^6 cells per ml); and RM1 and RM2 arising from coinfection with SV(X)-Ha-ras and MMCV-neo and growing to high density (1×10^6 to 4×10^6 cells per ml).

Growth-stimulated cells are clonal outgrowths that retain and express the retroviral oncogenes. Oncogene expression was examined in the growth-stimulated populations. For populations arising from coinfection with MMCV-neo and SV(X)-Ha-ras, we tested whether both viral genomes were present and expressed in a single cell: were these populations clonal outgrowths rather than mixed populations of singly infected cells? The transforming effects of *myc* and

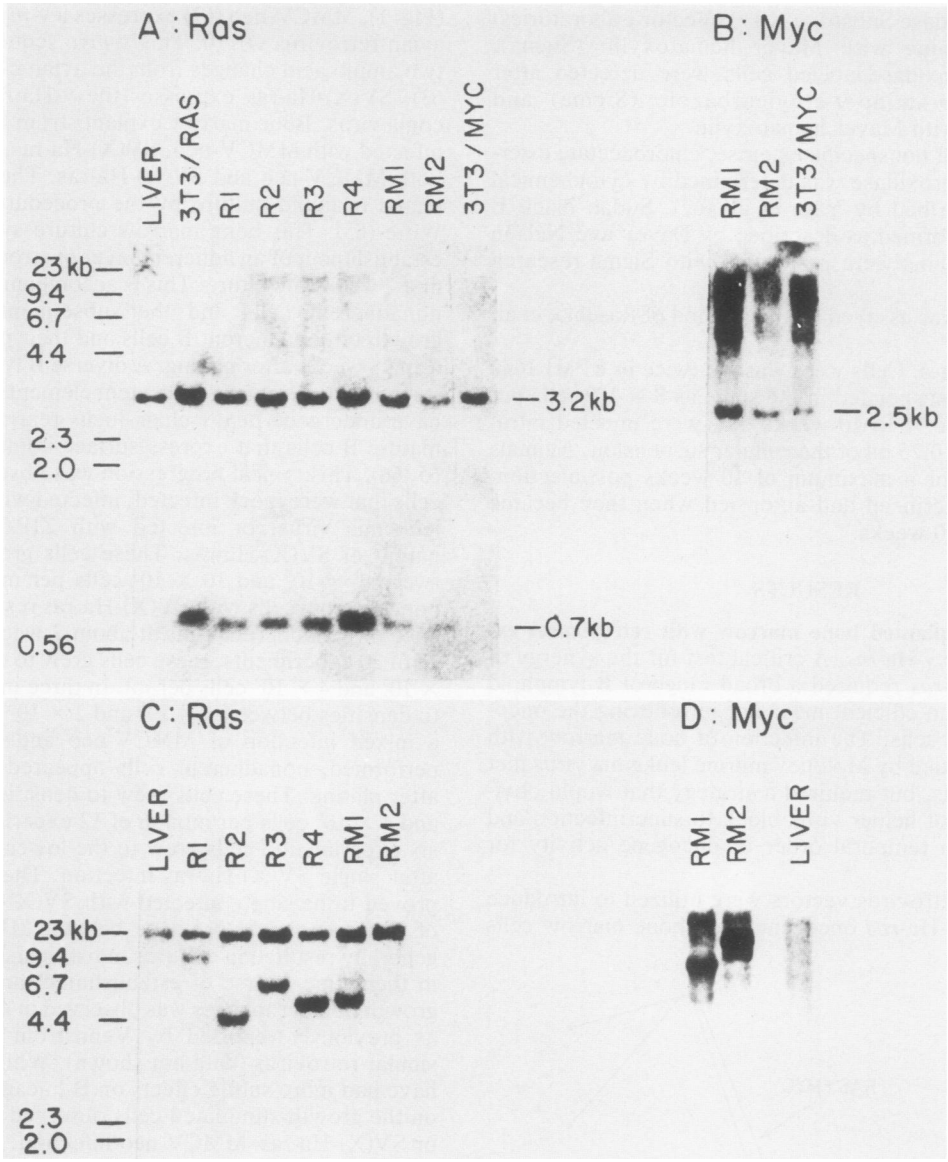

FIG. 3. Proviral integration. High-molecular-weight DNA samples were isolated from the nonadherent cells of cultures R1, R2, R3, R4, RM1, and RM2. DNA (10 μg) was digested with the indicated restriction enzymes, electrophoresed through 0.8% agarose, and transferred to nitrocellulose (51). The DNAs of BALB/c liver, NIH 3T3 cells producing SV(X)-Ha-ras (3T3/RAS), and NIH 3T3 cells producing MMCV-neo (3T3/MYC) served as controls. Size markers are the positions of an ethidium bromide-stained *Hin*dIII digest of bacteriophage lambda. (A) *Bam*HI digests were hybridized with a v-Ha-*ras* probe. The position of the retroviral v-Ha-*ras* is marked at 0.7 kb. The position of c-Ha-*ras* is indicated at 3.2 kb. (B) *Bam*HI digests were hybridized with a v-*myc* probe. The position of the retroviral v-*myc* is marked at 2.5 kb. (C) *Eco*RI digests were hybridized with a v-Ha-*ras* probe. (D) *Eco*RI digests were hybridized with a v-*myc* probe.

Ha-*ras* can be modulated by their levels of expression. Normal c-*myc* can act with Ha-*ras* (EJ) to transform rat embryo cells when it is expressed at a high level (29). Ha-*ras* (T24) can transform primary cells without a second oncogene when it is transcribed at a high level (52). Amplification of c-Ha-*ras*-1 can cause the malignant transformation of NIH 3T3 cells (40). We examined the copy number, level of transcription, and level of translation of the retroviral oncogenes.

MMCV-neo contains v-*myc* inserted as a *Bam*HI fragment of 2.5 kb. SV(X)-Ha-ras contains v-Ha-*ras* inserted as a 0.7-kb *Bam*HI fragment. These restriction fragments are diagnostic of the presence of their cognate viral genomes in infected cells. High-molecular-weight DNA was isolated from the nonadherent cells of cultures R1, R2, R3, R4, RM1, and RM2. For each of the infected cultures, a fragment of 0.7 kb was observed upon Southern hybridization analysis of *Bam*HI-digested DNA with a v-Ha-*ras* probe (Fig. 3A). Analysis with a v-*myc* probe of DNA isolated from RM1 and RM2 showed a 2.5-kb *Bam*HI fragment (Fig. 3B). Thus, both oncogenes reside in cultures RM1 and RM2. Cultures arising from coinfection with MMCV-neo and SV(X)-Ha-ras, but growing to lower density, possessed only v-Ha-*ras* (data not shown).

Southern hybridization analysis of *Eco*RI-digested DNA with v-Ha-*ras* or v-*myc* probes showed that the populations were clonal or pauci-clonal (Fig. 3C and D). Proviral integration produce a unique fragment defined by a 3′ *Eco*RI site

internal to either viral genome and a 5′ *Eco*RI site peculiar to the site of integration. Single restriction fragments of unique size were observed. This strongly suggests that each population resulted from the overgrowth of the progeny of a single infected cell. This may be the result either of infection and transformation being a rare event or of the progeny of a single infected cell dominating the population by the time of analysis. The latter is certainly the case, since the plating in soft-agar medium of cells immediately after infection proudced about 200 colonies per 5×10^6 cells. (5×10^6 cells is the number of cells plated on a 6-cm dish to produce a bone marrow culture.) For RM1 and RM2, in which a unique v-*myc* hybridizing fragment was observed in addition to one hybridizing to v-Ha-*ras*, Southern analysis showed residence of both viral oncogenes in the same cell. (For RM2, the v-Ha-*ras*-specific restriction fragment migrated as a close doublet with c-Ha-*ras* at about 23 kb.)

The observation of single *Eco*RI viral integration fragments in all populations studied, coupled with roughly equivalent intensities of hybridization between populations with both v-Ha-*ras* and v-*myc* probes (R4 may exhibit a twofold abundance of v-Ha-*ras*), suggests that the cells of R1, R2, R3, and R4 contain one or, at the most, two copies of the SV(X)-Ha-ras genome and that the cells of RM1 and RM2 contain one copy each of the SV(X)-Ha-ras and MMCV-neo genomes. It is unlikely that the copy number of integrated viral genomes plays a dramatic role in the differing growth properties of these cultures.

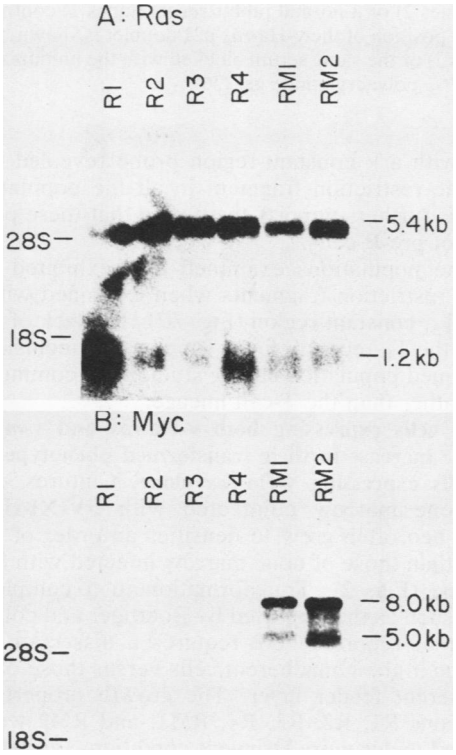

FIG. 4. Retroviral transcription. Cytoplasmic RNA was isolated from the nonadherent cells of cultures R1, R2, R3, R4, RM1, and RM2. Cytoplasmic RNA (20 μg) was denatured, electrophoresed in a formaldehyde–1.0% agarose gel (44), and transferred to nitrocellulose (58). Markers are the positions of ethidium bromide-stained 18S and 28S rRNAs. (A) Cytoplasmic RNAs were hybridized with a v-Ha-*ras* probe. The position of the SV(X)-Ha-ras genomic-length RNA is marked at 5.4 kb. The position of c-Ha-*ras* mRNA is marked at 1.2 kb. (B) Cytoplasmic RNAs were hybridized with a v-*myc* probe. The position of the MMCV-neo genomic-length RNA is marked at 8.0 kb. The position of subgenomic v-*myc* mRNA is marked at 5.0 kb.

Cytoplasmic RNAs of the transformed cell populations were examined by hybridization analysis (Fig. 4A and B). The expected genome-length retroviral transcript of 5.4 kb was observed in populations R1, R2, R3, R4, RM1, and RM2 with the v-Ha-*ras* probe. Hybridization analysis with the v-*myc* probe revealed the expected species, a genomic 8.0-kb species and a subgenomic 5.0-kb *myc* mRNA in both RM1 and RM2.

The SV(X)-Ha-ras genome is expressed as RNA in roughly equal abundance in all populations, suggesting that the differing growth properties of singly and doubly infected populations are not a function of differing levels of v-Ha-*ras* transcription. Interestingly, the level of c-Ha-*ras* mRNA (1.2 kb) in population R1 is elevated above that of the other populations; this does not correlate with any growth characteristic of this population.

The MMCV-neo-derived RNA species were expressed at a higher level in RM2 than in RM1. RM2 exhibited a more pronounced transformed phenotype than RM1 (see Fig. 8 and Table 1). The 2.4-kb c-*myc* mRNA was not detected with the avian v-*myc* probe under these hybridization conditions. Hybridization with a murine c-*myc* probe of similar specific activity revealed a 2.4-kb RNA to be expressed at levels at least 20-fold lower than that of v-*myc* mRNA, but at roughly equal levels among both v-Ha-*ras* and v-Ha-*ras*–v-*myc* populations (data not shown). Slot-blot analysis of graded amounts of cytoplasmic RNA confirmed the abundance of v-*myc*-specific RNA. It was at least 10 times more abundant in RM1 and RM2 than in our NIH 3T3 MMCV-neo-producing line (data not shown).

The nonadherent cells of cultures R1, R2, R3, R4, RM1, and RM2 were metabolically labeled with [^{35}S]methionine. Immunoprecipitation of cellular lysates with a v-Ha-*ras*-specific antiserum (57) showed that the v-Ha-*ras* p21 protein was present at roughly equivalent levels in all the transformed populations (Fig. 5A). No *ras* p21 protein was detected in uninfected NIH 3T3 cells (data not shown). This result further supports the conclusion that the differing growth properties of the infected populations are not related to the level of v-Ha-*ras* expression. The cells of cultures R1, RM1, and RM2 were metabolically labeled with $^{32}P_i$. Immunoprecipitation with a v-*myc*-specific antiserum (18) revealed a v-*myc* p57 protein in the lysates of RM1 and RM2 (Fig. 5B). Consistent with observations at the RNA level (Fig. 4B), more v-*myc* protein was observed in RM2 than in RM1.

Transformed populations consist of pre-B cells. The culture conditions utilized in these experiments were chosen because they favor the growth of B-lineage lymphoid cells (65). The four SV(X)-Ha-ras- and two SV(X)-Ha-ras–MMCV-neo-infected populations were examined by a variety of cytological procedures. Wright-Giemsa staining revealed all populations to have a blast-cell morphology with large nuclei and little cytoplasm (Fig. 6). While cytoplasmic immunoglobulin μ chain was not found in an immunoperoxidase detection procedure, all the cell populations expressed some level of B220, a B-lineage-specific marker (8). These data strongly suggest a pre-B-cell phenotype.

The transformed cell populations were also found to express low to moderate levels of Mac-I in an immunoglucose oxidase detection procedure. Mac-I is generally considered a marker for cells of the myeloid lineage (54), but has been observed in some B-cell lymphomas (11). It is

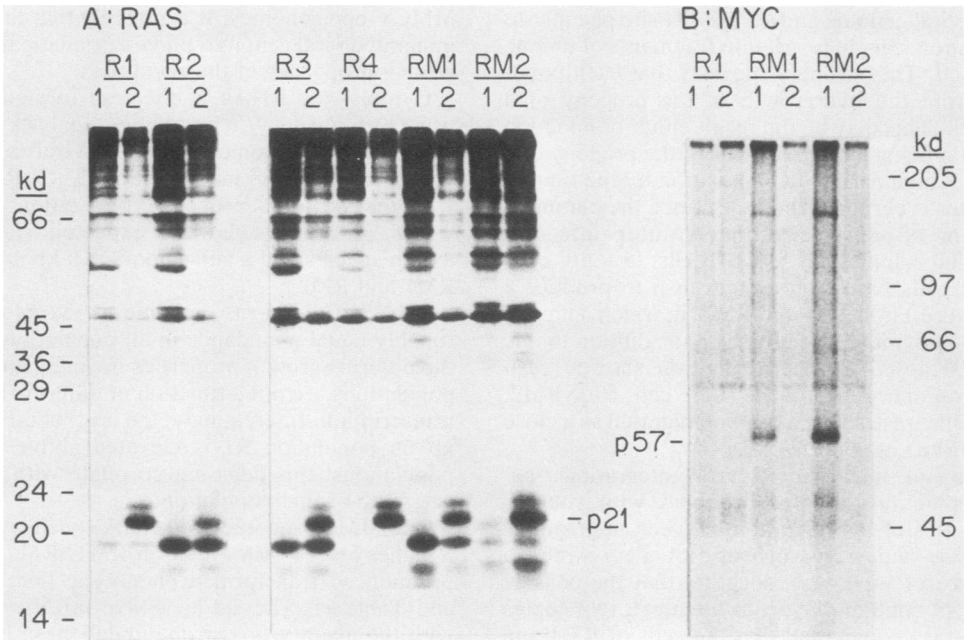

FIG. 5. Protein expression of viral oncogenes. Metabolically labeled cellular lysates were immunoprecipitated and electrophoresed as described in the text. Molecular size markers (in kilodaltons [kd]) are the positions of Coomassie blue-stained standards. (A) Lysates labeled with [^{35}S]methionine were immunoprecipitated with an anti-*ras* peptide serum (lanes 2) or a normal rabbit serum (lanes 1) control. Proteins were resolved through a sodium dodecyl sulfate–12% polyacrylamide gel (26). The position of the v-Ha-*ras* p21 doublet is shown. (B) Lysates labeled with $^{32}P_i$ were immunoprecipitated with an anti-v-*myc* peptide serum (lanes 1) or the same serum blocked with the immunizing peptide (lanes 2) as a control. Proteins were resolved through a sodium dodecyl sulfate–9% polyacrylamide gel (26).

suggested that cells early in the B-cell lineage share characteristics with cells of the myeloid lineage. Histochemical procedures revealed a low level of nonspecific esterase activity. This is generally associated with cells of the monocyte-macrophage lineage (67) but is sometimes detected at a low level in lymphoid cells. The populations were negative for nonspecific phagocytosis of latex beads, another marker of the monocyte-macrophage lineage (43). None of the populations studied were positive for granulocytic histochemical markers: chloroacetate esterase, myeloperoxidase, and sudan black B staining (10, 67).

While cellular morphology and expression of the B220 antigen indicated cells of the B lymphoid lineage, we further examined the transformed cell lines at the DNA level. The J_H heavy-chain (Fig. 7A), the κ light-chain (Fig. 7B), and the T_β-receptor (Fig. 7C) loci were examined by Southern hybridization analysis to determine whether any of these immune loci had undergone chromosomal rearrangement. Rearrangement of a D heavy-chain segment to a J_H heavy-chain segment is the first demonstrable step in the activation of the immunoglobulin heavy-chain locus. This rearrangement is detectable by the alteration of a germ line 6.2-kb *Eco*RI fragment that encompasses the J_H heavy-chain locus. Analysis of *Eco*RI-digested DNAs with a J_H heavy-chain probe revealed rearrangements in all the transformed cell populations (Fig. 7A). Since μ-chain protein was not detected, these rearrangements are probably between D and J_H gene segments. The presence of multiple rearrangements, some at an abundance less than that of the liver germ line control, suggests continuing rearrangement in these clonal or pauci-clonal populations.

Rearrangement of the κ light-chain locus is an event that follows the productive rearrangement of the immunoglobulin heavy-chain locus. Examination of *Bam*HI-*Eco*RI-digested DNAs with a κ constant-region probe revealed the 6.4-kb germ line restriction fragment in all the populations (Fig. 7B). This further supports the notion that these populations consist of pre-B cells.

All the populations examined also exhibited germ line *Hin*dIII restriction fragments when examined with a probe for the $T_{\beta 1}$ constant region (Fig. 7C). The lack of rearrangement in the T_B-receptor locus supports a conclusion that the transformed populations being studied are committed to the B-cell rather than the T-cell lineage.

Pre-B cells expressing both v-Ha-*ras* and v-*myc* show a dramatic increase in their transformed phenotype compared with cells expressing v-Ha-*ras* alone. Cultures established from bone marrow coinfected with SV(X)-Ha-ras and MMCV-neo often grew to densities an order of magnitude greater than those of bone marrow infected with SV(X)-Ha-ras alone (Fig. 2). Transformation in a complex culture system such as that reported by Boettiger and colleagues (6, 53) or that reported here requires a dissection of events occurring in the nonadherent cells versus those occurring in the adherent feeder layer. The growth properties of cell populations R1, R2, R3, R4, RM1, and RM2 were further examined under more stringent conditions: growth in liquid culture without an adherent feeder layer, growth in soft-agar medium, and growth as tumors in vivo. These experiments demonstrated that the differing growth properties of the nonadherent cell lines result from the expression of the oncogenes in these same cells rather than in cells of the adherent feeder layer.

The cell populations were cultured in the absence of adherent feeder layers. Even at high plating densities R1, R2, R3, and R4 failed to grow (Fig. 8). In contrast, populations RM1 and RM2 displayed order of magnitude increases in cell density under the same conditions (Fig. 8).

When plated in soft-agar medium over a feeder layer, RM1 and RM2 had cloning efficiencies of 7 and 36%, respectively (Table 1). This contrasts with much lower cloning efficiencies of R1, R2, R3, and R4. These populations had cloning efficiencies ranging from less than 0.02 to 0.46%. None of the populations studied could grow in soft-agar medium in the absence of a feeder layer (data not shown). The feeder dependence of cells expressing both oncogenes may be due to the dilution of autocrine effects at the low plating densities used in these soft-agar assays (a maximum plating of 5×10^3 cells in 10 ml of soft-agar medium on a 6-cm dish). While RM2 is capable of growth in soft-agar in the presence of G418, the other cell lines were not tested for drug resistance.

The abilities of the cell populations to form tumors in vivo when injected intraperitoneally into syngeneic BALB/c mice closely paralleled their cloning efficiencies in soft-agar medium (Table 1). The typical mouse injected with RM1 or RM2 developed a subcutaneous tumor at the site of injection and metastases throughout its lymph nodes and spleen. These animals became moribund or died within 6 weeks. The typical mouse injected with R1, R2, R3, or R4 survived for 10 weeks (the maximum time examined) without any ill

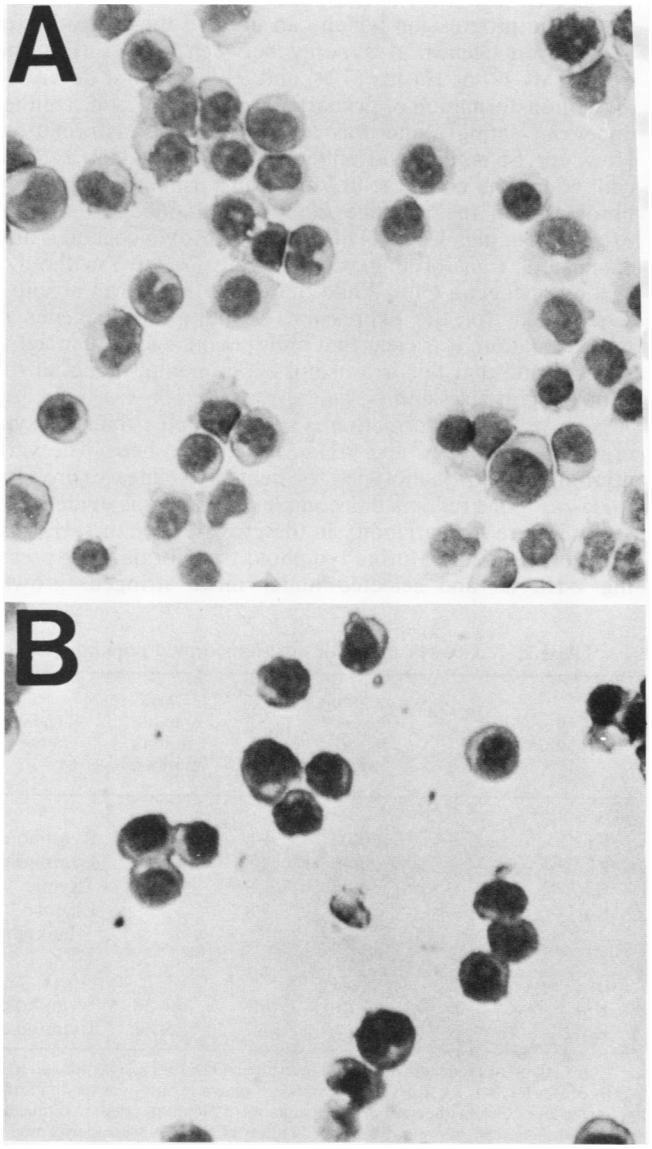

FIG. 6. Wright-Giemsa staining. (A) Nonadherent cells from culture R4; (B) nonadherent cells from culture RM2.

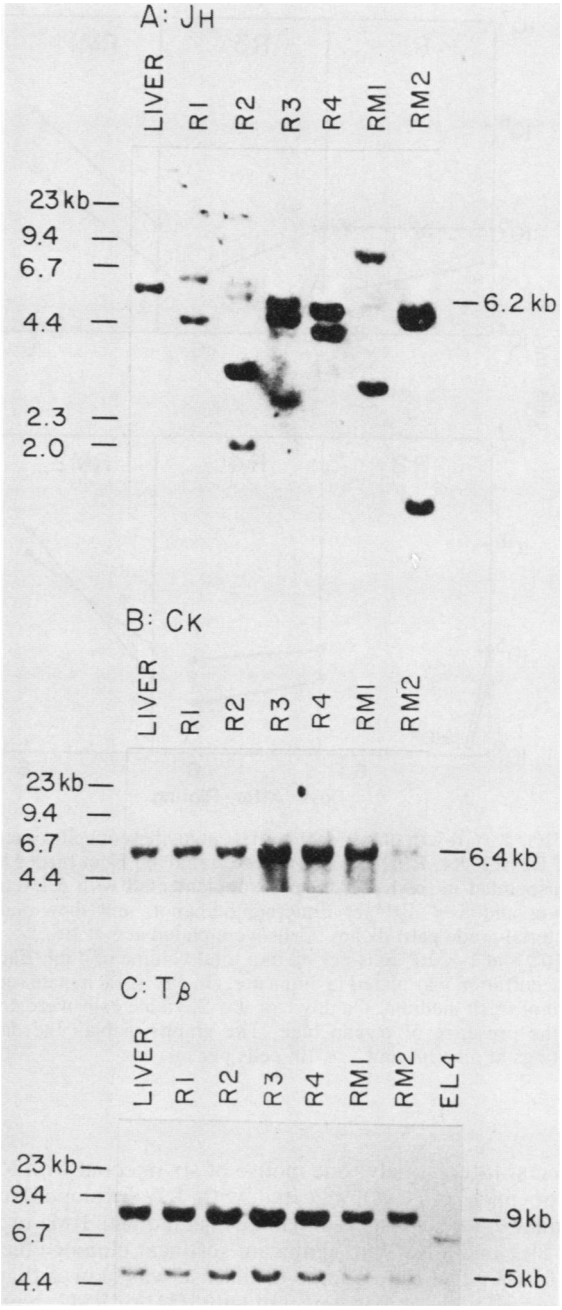

FIG. 7. Immune loci define a pre-B phenotype. High-molecular-weight DNA samples were isolated from the nonadherent cells of cultures R1, R2, R3, R4, RM1, and RM2. DNA was analyzed as described in the legend to Fig. 3. The DNA of BALB/c liver served as a control. Size markers are the positions of an ethidium bromide-stained *Hin*dIII digest of phage lambda. (A) *Eco*RI digests were hybridized with a J_H heavy-chain probe. The position of the germ line restriction fragment is marked at 6.2 kb. (B) *Bam*HI-*Eco*RI digests were hybridized with a C_K probe. The position of the germ like restriction fragment is marked at 6.4 kb. (C) *Hin*dIII digests were hybridized with a $T_{\beta 1}$ constant-region probe. The positions of the germ line restriction fragments are marked at 9 and 5 kb.

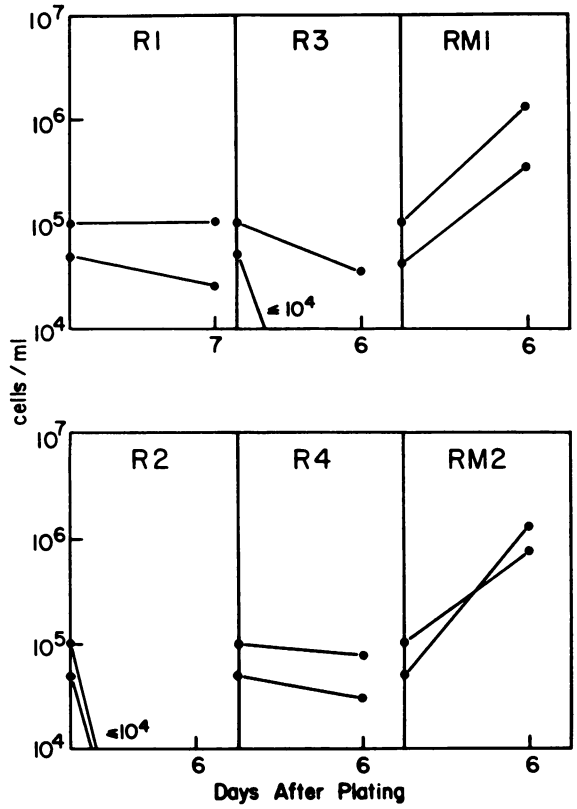

FIG. 8. Growth off of feeders. The nonadherent cells of cultures R1, R2, R3, R4, RM1, and RM2 were removed from feeder layers, resuspended in fresh RPMI 1640 supplemented with 5% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol, and then plated in bacterial-grade petri dishes. Cells were plated at 5×10^3, 1×10^4, 5×10^4, and 1×10^5 cells per ml in a total volume of 4 ml. Each cell concentration was plated in duplicate. On day 4, each plate received 2 ml of fresh medium. On day 6 or day 7, viable cells were counted in the presence of trypan blue. The graphs display the data for platings at 5×10^4 and 1×10^5 cells per ml.

effects. Interestingly, one mouse of six injected with R1 and three mice of seven injected with R2 developed tumors similar to those observed in mice injected with RM1 or RM2. R2 also had a low but significant soft-agar cloning efficiency of 0.46%. The time course of disease was slower than that observed with animals injected with RM1 or RM2, however. Whether this increased tumorigenicity in comparison with that of R3 and R4 reflects secondary alterations in R1 and R2, such as the activation of a second oncogene, is not known. The irregular occurrence of these tumors may reflect continued neoplastic progression in vivo. Since the cell lines examined in the tumor challenges are producing virus, it was necessary to determine the origins of the tumors. All the tumors examined, with two exceptions, contained the viral integration *Eco*RI fragments associated with their cognate injected cells (data not shown). The exceptions consisted of the following. One animal, upon macroscopic examination of its spleen, appeared to have died from *ras*-associated erythroleukemia. Another animal, apparently healthy when sacrificed, had a grossly enlarged thymus indicative of Moloney (helper virus) disease. The DNA of this thymic tumor lacked any viral integration *Eco*RI fragment complementary to the v-Ha-*ras* probe.

DISCUSSION

All the cell lines derived from the experiments reported here can be viewed as possessing intermediate transformed phenotypes. R1 and R2 are established in culture but show absolute dependence on an adherent feeder layer of stromal cells; R3 and R4 show extreme growth stimulation, but retain feeder dependence; RM1 and RM2 show extreme growth stimulation without feeder dependence, but can now grow in soft-agar medium in the presence of a feeder. Clearly, the coexpression of v-*myc* (OK10) with v-Ha-*ras* can induce a profound difference in the growth state of a lymphoid cell. The introduction of second oncogenes other than v-*myc* (OK10) into these cells might elicit transitions between these or alternative neoplastic states. A number of oncogenes have the ability to replace v-*myc* or v-Ha-*ras* in the synergistic transformation of primary fibroblasts (14, 21, 27, 36, 46, 47, 68). Various combinations of oncogenes display various degrees of tumorigenicity (27), so it would be of interest to examine them in this lymphoid system.

The data presented in this paper support the concept of a neoplastic progression which can involve the activation of multiple oncogenes. A synergy between Ha-*ras* (EJ) and v-*myc* (MC29) or Ha-*ras* (T24) and E1A was first described in the transformation of primary fibroblasts (27, 46). Neither oncogene acting alone was able to elicit transformation. However, Spandidos and Wilkie (52) found that Ha-*ras* (T24) can induce the complete in vitro transformation of primary fibroblasts in the absence of a second oncogene if it is expressed at high levels. The v-*myc* (OK10) oncogene is also capable of transforming primary cells in vitro without a second oncogene (60). While there may not be an absolute requirement for the expression of multiple oncogenes in transformation, it is clear that multiple oncogenes can act in synergy and that this is a useful experimental model of the neoplastic progression.

Neoplastic transformation is more complex than the synergy between v-*myc* and v-Ha-*ras* reported here. The variation in growth phenotypes for cellular isolates expressing v-Ha-*ras* alone reflects this complexity. There is evidence in the literature for variability in the growth state of v-Ha-*ras*-transformed cells. Murine lymphoid transformants expressing v-Ha-*ras* and selected under more stringent growth

TABLE 1. Growth properties of transformed populations[a]

Cell population	Growth off feeders	% Cloning efficiency in soft agar	Animals with tumors/ animals tested	Days until dead or moribund	Type of tumor
Ras					
R1	−	<0.02	1/6	71	Lymphoid
R2	−	0.46	3/7	44–56	Lymphoid
R3	−	0.05	1/9	72	Thymic
R4	−	<0.02	1/8	27	Erythro leukemia
Ras + myc					
RM1	+	7	6/7	30–38	Lymphoid
RM2	+	36	7/7	25–43	Lymphoid

[a] Growth off of feeders is described in the legend to Fig. 8. The nonadherent cells of R1, R2, R3, R4, RM1, and Rm2 were grown in soft-agar medium over a feeder layer (66). Either 500 or 5,000 cells were plated per dish in duplicate. Agar colonies were scored at 2 weeks. Tumor challenges were performed as described in Materials and Mehtods. Briefly, animals were injected intraperitoneally with 2×10^6 cells and were observed for a maximum of 10 weeks. Tumors were observed upon macroscopic examination of autopsied animals.

conditions can be highly tumorigenic (39). The transformation of preneoplastic Syrian hamster embryo cells with v-Ha-*ras* showed variability in tumor latency in vivo and anchorage-independent growth in vitro with no correlation to the level of v-Ha-*ras* expression (59). Land et al. (27) found that culture-established fibroblasts that had acquired Ha-*ras* (EJ) showed greater tumorigenicity than primary fibroblasts transformed with v-*myc* and Ha-*ras* (EJ). Thus, the synergy of *ras* and *myc* oncogenes is not necessarily equivalent to an endpoint of neoplastic progression. In fact, RM1 and RM2, although highly tumorigenic, still require factors provided by a feeder layer for growth in soft-agar medium.

Some genetic events have been identified that can occur secondarily to the introduction of oncogenes into cells. Syrian hamster embryo cells transformed with v-Ha-*ras* and v-*myc* consistently develop a monosomy for chromosome 15 (35). Plasmacytomas induced with pristane and Abelson murine leukemia virus show chromosomal rearrangements involving c-*myc* (34). Similarly, c-*myc* is amplified in NIH 3T3 cells transformed with Abelson murine leukemia virus (33). Rearrangements or amplifications of the c-*myc*, c-*abl*, and c-Ha-*ras* loci have not been observed for the cell lines reported here.

A high level of c-*myc* expression may be necessary for cotransformation of rat embryo cells with Ha-*ras* (EJ) (29). Our data with v-*myc* are consistent with this. Alternatively, c-*myc* mRNA is expressed at a similar level in both murine plasmacytomas and normal cells (1, 23, 50). Several investigators found normal c-*myc* expression to be suppressed in cells expressing activated c-*myc* (1, 3, 4, 55) or v-*myc* (41). In contrast, others have found normal c-*myc* expression to be unaffected (22, 56). We observed suppression of c-*myc* in our cell lines that express v-*myc*. Experiments directed at modulating v-*myc* and c-*myc* expression will better address this question.

It is clear from our work that multiple oncogenes can cooperate to induce the transformation of B lymphoid cells. The v-*myc* oncogene in synergy with v-Ha-*ras* can play a direct role in that transformation. Neoplasia appears to follow a progression in the alleviation of regulated growth, and intermediate phenotypes in that progression were isolated. The ultimate goal of these studies is to find molecular correlates that govern the transitions to and from stages within the neoplastic progression.

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