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Interaction Between *Raf* and *Myc* Oncogenes in Transformation In Vivo and In Vitro

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3611 MSV, a *raf*-oncogene-transducing murine retrovirus, induces fibrosarcomas and erythroid hyperplasia in newborn mice after a latency of 4–8 wk. In contrast, new recombinant murine retroviruses carrying the *myc* oncogene (J-3, J-5 construct viruses) do not induce tumors before > 9 wk. A combination of both oncogenes in an infectious murine retrovirus (J-2) induces hematopoietic neoplasms in addition to less prominent fibrosarcomas and pancreatic adenocarcinoma 1–3 wk after inoculation. The hematologic neoplasms consist of immunoblastic lymphomas of T and B cell lineage and erythroblastosis. If animals were inoculated with a variant of the J-3 virus, which induces altered foci in cultures of NIH 3T3 cells, carcinoma developed in the pancreas with a 2–6 mo latency. In parallel to the synergistic action of both oncogenes on hematopoietic cells in vivo, we find that *raf*-oncogene-induced transformation of bone marrow cells in culture is enhanced by the addition of *myc*, which by itself does not transform these cells when grown in standard media. We conclude that concomitant expression of *raf* and *myc* oncogenes in hematopoietic and epithelial cells alters their respective transforming activities. The contribution of v-*myc* in this synergism was examined by use of a series of recombinant murine retroviruses capable of expressing the avian v-*myc* to study the effect of altered *myc* expression on hematopoietic/lymphoid cells. With either interleukin 3- or interleukin 2-dependent cell lines, introduction of the recombinant viruses abrogated the requirement for IL 3 or IL 2 for growth, and associated with this was the suppression of c-*myc* expression. The findings suggest that *myc* is a component in the signal transduction pathway for IL 3 and IL 2 and support an autoregulatory mechanism of c-*myc* expression. In contrast to v-*myc*, expression of v-*raf* in primary lymphoid/hematopoietic cells has an immortilizing function without abrogating the requirement for IL 3 for growth. This suggests that v-*raf* and v-*myc* affect different components of growth regulation, as, for example, commitment (v-*myc*) and cell cycle progression (v-*raf*).

Key words: *raf*, *myc*, oncogene, synergism

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During the past several years, evidence has been accumulating which suggests that activation of cellular protooncogenes is the common denominator for tumor induction by chemical and biological carcinogens. Protooncogenes are a subset (~0.1%) of cellular genes which, after incorporation into retroviruses, or upon specific alteration within the cell in structure [1–4] or regulation [3,5], are capable of inducing tumorous growth.

Protooncogenes control the growth and affect the differentiation of eukaryotic cells. Long before the discovery of viral oncogenes and their cellular homologs [6], it was clear that malignantly transformed cells were genetically altered in regard to their growth factor requirements for propagation in culture [7,8]. Moreover, the fact that cells transformed by oncogene-transducing mammalian retroviruses were blocked for the binding of epidermal growth factor [9], and released transforming growth factors into the culture medium [10,11], suggested early on that several oncogenes might cause transformation by affecting the signal transmission pathway of growth factors. At least four of the approximately 25 known oncogenes derive from components of the growth factor signal transduction pathway: *erb*B, which derives from a portion of the receptor gene for epidermal growth factor (EGF) [12]; *sis*, which is homologous to part of the gene for platelet-derived growth factor (PDGF) [13–15]; *fms*, which is related to the receptor for colony-stimulating factor, CSF-1 [16]; and *myc*, which can relieve the requirement of fibroblasts for PDGF [17] and myeloid and lymphoid cells for IL-3 and IL-2, respectively [41,85]. Moreover, *myc* is induced [5] along with c-*fos* [18–20] and r-*fos* [21] by PDGF.

Recent findings have demonstrated that oncogenes may act synergistically in specific combinations when assayed with primary fibroblast cells in culture [22,23]. To examine the possible interaction of the *raf* and *myc* oncogenes in vivo in the development of tumors, we have used naturally occurring and recombinant murine retroviruses carrying *raf* and *myc* or only *myc* oncogenes. Several types of data suggest possible interaction of the *raf* and *myc* oncogenes in some tumors. A subclass of the small cell lung carcinomas, the small cell/large cell (SC/LC) carcinomas which have a particularly poor prognosis, have amplified *myc* gene DNA [24] and also express the c-*raf*-1 gene [25]. In addition, both familial renal carcinoma [26] and transfected DNA from primary stomach cancer [27] show translocations or rearrangements of the c-*raf*-1 locus, respectively, and presumably express c-*myc*. Moreover, the avian homolog of v-*raf*, v-*mil*, is naturally linked to v-*myc* in the carcinoma virus MH2 [28–32] where it presumably contributes to carcinoma induction since avian acute leukemia viruses which only contain v-*myc* have a much lower incidence of this tumor type [33,34]. It therefore became important to test the effect of v-*myc* on the oncogenicity of 3611 MSV. Our findings provide the first evidence for a synergistic action between the *raf* and *myc* oncogenes during lymphoma induction in vivo and suggest a mechanism that may underlie this phenomenon. The synergistic action of these two oncogenes has also been demonstrated in vitro where immortalization of primary murine bone marrow [84] or fetal liver cultures [Y. Weinstein, J. Cleveland, U. Rapp, J.N. Ihle, in preparation] requires expression of both *onc* genes.

The contributions of *raf* and *myc* to this synergism have been studied in vitro by examining their effects on hematopoietic/lymphoid cell differentiation, immortaliza-

tion, and factor dependence for growth. With either interleukin 3 (IL 3)- or interleukin 2 (IL 2)-dependent cell lines, the viruses expressing v-*myc* alone were capable of abrogating the requirement of IL 3 or IL 2 for growth. Associated with this abrogation was the suppression of c-*myc* expression.

EFFECT OF *RAF/MIL*- AND *MYC*-TRANSDUCING VIRUSES ON NIH 3T3 CELLS

In order to investigate potential interactions between the v-*raf* and v-*myc* oncogenes, we have made constructs [40,41] between DNA from p3611 MSV, pMH2, and pMC29 such that either or both genes were part of a transmissible viral genome (Fig. 1). To determine the biological activity of the various viruses, cloned DNAs were cotransfected with helper virus DNA (leuk strain of M-MuLV) onto NIH 3T3 fibroblast cells. Transfected cells were observed for the development of transformed foci and production of virus particles by assay for reverse transcriptase in the culture medium. Since neither of the two *myc* recombinants pHWJ-3 and pHWJ-5 induce foci of morphologically transformed cells, DNA from these constructs was cotransfected with a neomycin resistance marker (pSV2neo). Clones of antibiotic-resistant cells were examined for v-*myc* expression by immunoprecipitation with *myc*- and p30-specific antibodies and virus stocks were prepared from positive cells by rescue

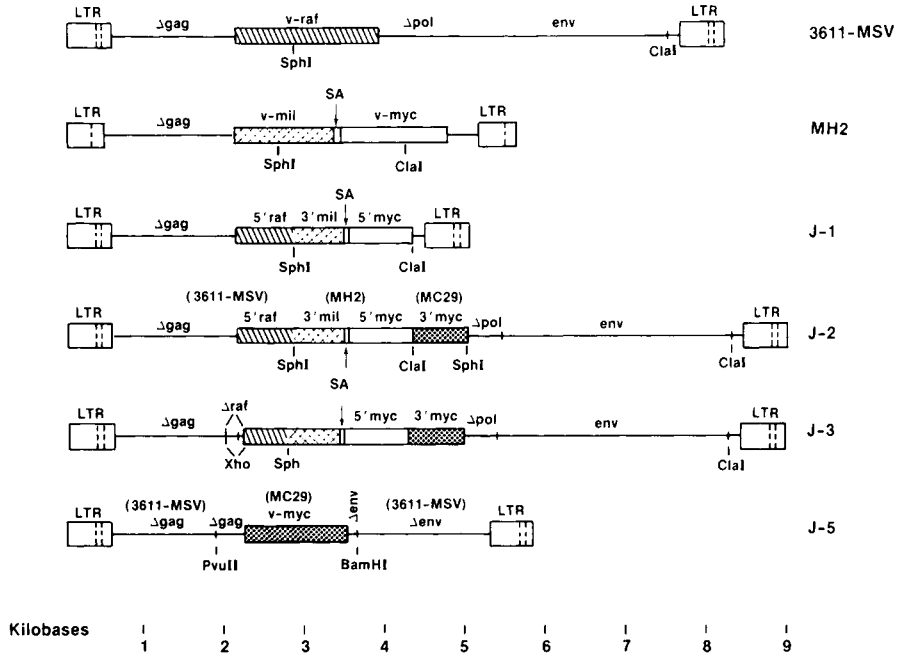

Fig. 1. Genomic organization of the defective viruses 3611-MSV and MH2 and of the construct viruses J-1 through J-5. Important restriction enzyme sites are indicated and the sizes of the various viruses are shown in kilobases. The origin of specific v-*onc* sequences in the constructs is indicated by specific shadings: ▨,3611-MSV v-*raf*; ▩, MH2 v-mil; □, MH2 v-*myc*; ▧, MC29 v-*myc*. SA indicates presence of splice acceptor sequences present in MH2 v-*myc*.

with ecotropic and amphotropic helper viruses (M-MuLV and 4070 A MuLV). All three v-*myc* recombinant viruses, J-2, J-3, and J-5, were produced at similar levels by helper-virus-infected cultures as determined by dot blot hybridization of viral RNA with a v-*myc*-specific DNA probe. In agreement with the hybridization data, indirect immunofluorescence assays for v-*myc* protein in acutely infected NIH 3T3 cells showed that all three viruses were produced at comparable titers of infectious virus.

In contrast to pHWJ-3 and pHWJ-5, constructs pHWJ-1 and pHWJ-2 do induce foci on NIH 3T3 cells. The fact that pHWJ-1 was transforming provided the first evidence for biological activity of the v-*mil* gene in MH2. Sequence comparisons between v-*mil* and v-*raf* have shown previously [32] that v-*mil* differs in 19 positions in its amino acid sequence from v-*raf*. Our transfection data show that 17 of these changes are compatible with transformation since they are located 3′ of the Sph I site at which the v-*mil* fragment was joined with v-*raf* in pHWJ-1 (Fig. 1). The morphology of transformed foci induced by J-1 virus differs, however, from those induced by 3611 MSV in that the foci are more flat and transformed cells are more commonly interspersed with nontransformed cells (Fig. 2). Addition of the hybrid MH2-MC29 v-*myc* gene to the hybrid v-*raf/mil* gene in pHWJ-2 has a dramatic effect on the fibroblast-transforming activity of the virus (Fig. 2). Foci of morphologically transformed cells are very dense, homogeneous, and consist of less elongated cells. We conclude that the 5′ half of MH_2 v-*myc* is biologically active and that concomitant expression of v-*raf/mil* and v-*myc* in NIH 3T3 fibroblast cells alters their transformed phenotype.

PATHOGENICITY IN NEWBORN NFS/N MICE

High-titer virus stocks of J-1 and -2 were prepared by cloning cells from individual foci and stocks of J-3 and -5 by rescue of transfected, neomycin-resistant cells with ecotropic and amphotropic helper MuLV as described above [40]. The tumor-inducing potential of construct viruses J-1 to J-5, as well as that of 3611 MSV and helper virus controls, was determined by intraperitoneal inoculation of newborn NFS/N mice. Consistent with the differential ability of these viruses to transform

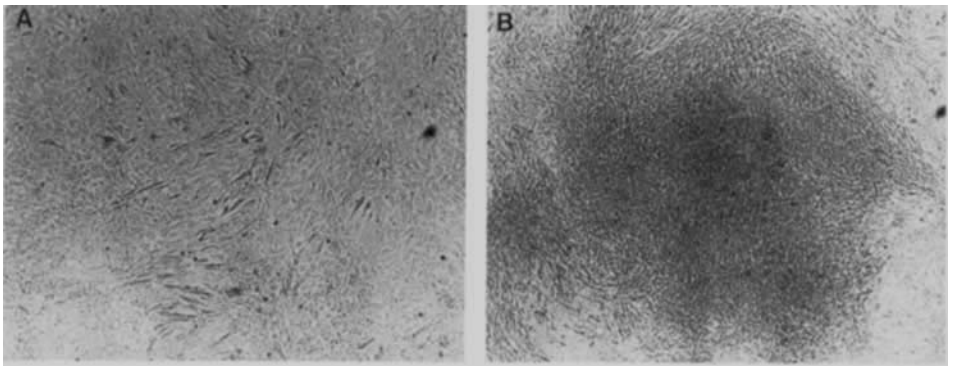

Fig. 2. Morphology of representative NIH/3T3 cell foci induced by J-1 (panel A) and J-2 (panel B). Conditions for infection were as previously described [38]. Comparison was between foci from dishes containing a comparable number of foci. Reproduced from previously published figure [40].

fibroblasts in culture, we observed a striking difference in their ability to induce tumors in newborn mice (Table I); 3611 MSV induced sarcomas and less frequent erythroid hyperplasia with a latency of 4–8 wk. Viruses derived from pHWJ-3 and -5 did not produce tumors with a latency < 9 wk, at which time the M-MuLV helper-virus-produced disease began. V-*myc* recombinant virus-specific RNA was present in such tumor cells (data not shown). Recently, using Cas-Br-M-MuLV as helper virus for infections with J-3 or J-5 we have again observed T cell lymphomas with a latency of 1½–4 mo, a disease which is not characteristic of Cas-Br-M-MuLV-infected animals [54]; therefore, the v-*myc*-transducing retroviruses do indeed appear to cause T cell lymphomas [Morse, Hartley, Yetter, Frederickson, Majumdar, Cleveland, and Rapp, submitted]. In contrast, the dual-oncogene-transducing virus J-2 induced lymphomas and erythroblastosis which killed the mice after an average latency of 2–3 wk, depending on the helper virus used (leuk M-MuLV or 4070 A, respectively). Viruses with the same in vitro and in vivo transforming properties as the input viruses were recovered from tumors induced by 3611 MSV, J-1, and J-2. We conclude from the pathogenicity pattern that concomitant expression of v-*raf* and v-*myc* in hematopoietic cells has a synergistic effect on the transformation of these cells in vivo.

Recently we have isolated a variant virus from the spleens of mice which were originally inoculated with J-3 virus. This virus induces altered foci (less transformed than J-2) on NIH 3T3 cells and upon injection into newborn mice causes immunoblastic lymphomas and adenocarcinoma of the pancreas with a latency of 2–6 mo [unpublished results]. Analysis of this variant virus indicates that this virus likely arose by recombination of the J-3 virus with the c-*raf*-1 locus; protein analysis

TABLE I. Tumor Development in Newborn NSF Mice*

Virus	Incidence	Latency (wk)	Pathology
3611			
M-MuLV	30/30	4–8	Fibrosarcoma, erythroid hyperplasia
4070	22/22	4–10	
J-1			
MuLV	20/20	7–10	Fibrosarcoma, erythroid hyperplasia
J-2			
M-MuLV	18/18	1–3	Lymphoma (T+B), erythroblastosis,
4070	12/12	2–4	fibrosarcoma, adenocarcinoma of pancreas, liver, and lung
J-3			
M-MuLV	12/12	9–16	Lymphoma (T)
4070	5/5	16	Lymphoma (T)
J-5			
M-MuLV	11/11	9–16	Lymphoma (T)
4070	8/8	16	Lymphoma (T)
Helper			
M-MuLV	20/20	9–16	Lymphoma (T)
4070	0/20	>52	N/A

*Newborn NFS/N mice were inoculated intraperitoneally with 2–5 × 10^4 focus-forming units (FFU) of 3611, J-1, or J-2 virus, and 1–2 × 10^3 v-*myc* fluorescence-inducing units (FIU) of J-3 or J-5 virus. Inoculated mice were observed for tumor growth and autopsied at late stages of disease. Tumors were evaluated by light microscopic examination of hematoxylin-eosin-stained tissue sections. NA = nonapplicable. Modified from previously published data [40].

indicates this recombination event allows the variant to make a gag-*raf* fusion protein unlike J-3, which makes no detectable *raf* products (data not shown).

HISTOPATHOLOGY AND FMF ANALYSIS OF TUMORS INDUCED BY J-2 AND CONTROL VIRUSES

Lesions induced in mice inoculated with J-2 virus consist of lymphoma, erythroblastosis, sarcoma, and dysplasia progressing to frank carcinoma, of pancreatic acinar cells [40]. Usually, neonatally inoculated mice have advanced lymphomas and erythroblastosis at 1–3 wk of age. By this time, sarcomatous growths are also seen in some animals. Pancreatic lesions appear sporadic, but since they are only discernable by histologic examination, the incidence may be higher than currently appreciated. Representative examples for the various histological lesions are shown in Figure 3.

Neoplasms appearing in mice inoculated with the 3611 virus are restricted to sarcoma, which may be very large, occurring in muscles and spleen (Fig. 3A). Erythroid hyperplasia is observed in the early stages after inoculation, but rarely progresses, being restricted to the splenic red pulp spared by sarcomatous growth and small foci within the hepatic periportal areas where they also occur in J-2 virus-infected mice (Fig. 3C).

Sarcomas induced by either J-1 or J-2 (Fig. 3B) are similar to 3611 MSV-induced sarcomas but are characteristically smaller and spread diffusely through both the red and white pulp. The sarcomas of J-2 virus-inoculated mice which develop sarcomas are comparable in tumor size to age-matched J-1 virus-inoculated mice. These findings suggest that the development of sarcomas induced by v-*raf* in 3611 MSV is not accelerated by the combination with v-*myc* in J-2 virus.

Erythroblastosis is evidenced in J-2-inoculated mice by the uniform population of basophilic erythroblasts and, to a minor extent, more mature erythroid precursors in spleen. Extensive stasis of erythroblasts is observed in the hepatic sinusoids (Fig. 3D), a characteristic of erythroleukemia induced by other MuLV [55]. Usually, the hematocrit is only moderately reduced by the time this lesion has become prominent, indicating a differentiative capacity for these erythroblasts.

Lymphomas occur as uniform enlargements of all lymph nodes and of the splenic periarteriolar sheaths. In nodes, there are fairly uniform sheets of large lymphoid cells characterized by a prominent central nucleus and a thick nuclear membrane (Fig. 3E). These morphological features identify the neoplastic cells as immunoblasts. Within the hepatic parenchyma, foci of immunoblasts are seen (Fig. 3D) and diffuse stasis of these cells is also observed within the alveolar walls of the lung. In advanced cases, immunoblasts are present in peripheral blood in large numbers.

Pancreatic dysplasia is observed as isolated groups of distorted acini formed by very large, palely staining cells containing large vesicular nuclei. Acini surrounding these dysplastic foci are usually unaffected (Fig. 3F). Pancreatic carcinoma caused by the variant J-3 virus is illustrated in Figure 3G and shows extensive dysplasia of the pancreas.

To type the cells that make up the lymphomas, we analyzed J-2 virus-induced tumors by cell sorting and fluorescence assays [42,43] for lineage-specific cell surface markers [40]. Spleens and lymph nodes from neonatally J-2-infected NFS/N mice characteristically contain large populations of blast cells. In spleens, two populations

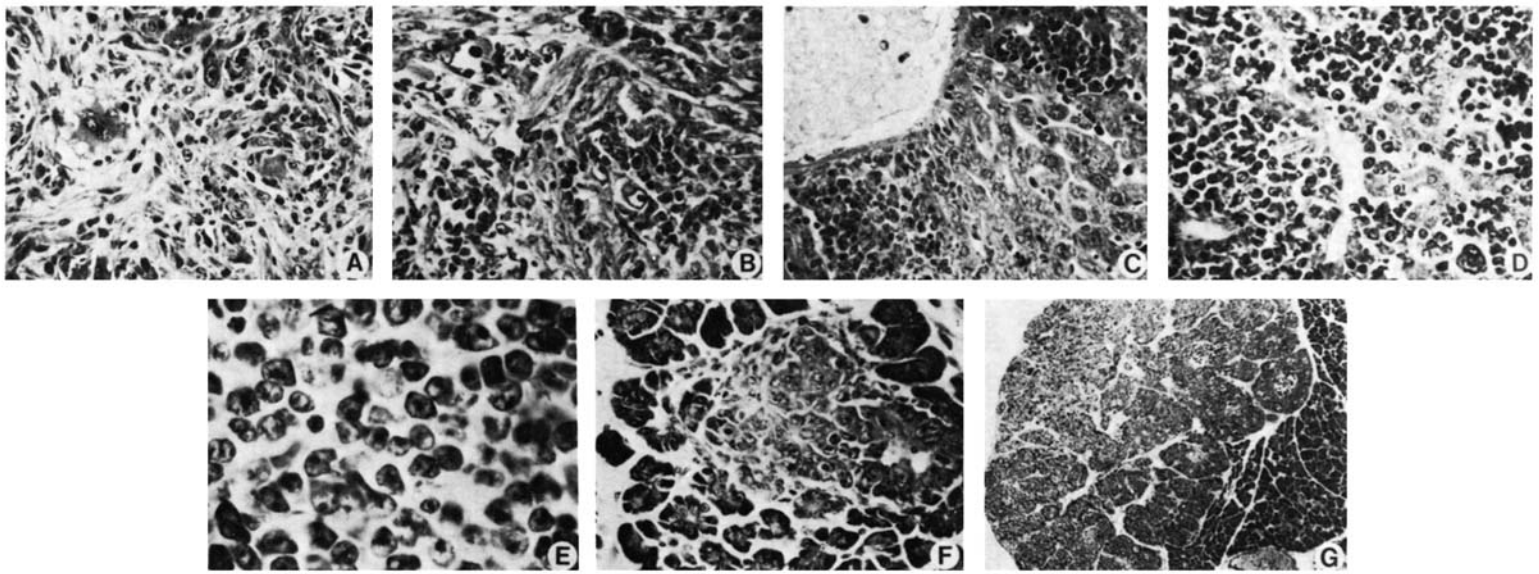

Fig. 3. Lesions of mice inoculated neonatally with J-2, 3611 MSV, or the J-3 variant viruses. Panel A) Sarcomatous lesions in the spleen of a 3611-infected mouse were composed of fusiform cells and irregularly shaped giant cells. These splenic growths were very well vascularized and affected mice sometimes died from hemorrhages due to splenic rupture. Panel B) Sarcomatous lesion in the spleen of a J-2-infected mouse showed a more diffuse type of growth pattern, possibly representative of early stages in the development of this lesion. Erythroblasts, the most prominent feature in spleens of J-2-infected mice, can be seen intermixed with the sarcoma cells. Panel C) A section for the liver of a J-2-infected mouse with a focus of erythroid precursors at the upper right and one of myeloid precursors at the lower left. Panel D) A section from the liver of a J-2-infected mouse showing extensive stasis of erythroblasts within the sinusoids at the upper left and one of immunoblasts at the lower left. Panel E) Immunoblastic lymphoma in the lymph node of a J-2-infected mouse. Panel F) A focus of dysplastic acinar cells in the pancreas of a J-2-infected mouse. Note the presence of mitotic figures and the large vesicular nuclei of the dysplastic cells. Panel G) Adenocarcinoma of the pancreas caused by the J-3 variant showing extensive dysplasia of the pancreas. Modified from previously published figure [40].

of blast cells can be distinguished by their reactivity with antibody to the panlymphocyte antigen, Ly 5. Ly 5^+ blasts comprise 8–56% of spleen cells whereas the remaining blast cells are Ly 5^-, in contrast to a normal NFS spleen with 95% Ly 5^+ cells. As spleen cells from mice with erythroleukemias induced by other MuLV can be either Ly-5^- or Ly-5^+ whereas all lymphomas are Ly-5^+ [55,56], the results of these analyses are consistent with the histologic studies which showed a predominance of erythroblasts over immunoblasts in spleens of mice infected with J-2.

Lymph node cells from J-2-infected mice were always > 95% Ly-5^+ and contained varying proportions of blast cells (40–95%) and residual normal Ly-5^+ lymphocytes; Ly-5^- blasts were never observed. These results support the histologic findings that nodes from mice infected with J-2 contain varying proportions of immunoblasts and normal cells but contain no erythroblasts.

To determine the cellular origins of the Ly 5^+ immunoblasts detected in lymphoid tissues of infected mice, the cells were reacted with antibodies to the T-cell antigen Thy-1 and the B-cell lineage antigens sIg and Ly-5(B220). FMF analysis of these populations demonstrated that they are variably composed of T- and B-lineage immunoblasts. Spleen cells from J-2-infected mice also contain mixtures of Thy-1^+ and Ly-5(B220)$^+$, sIg$^-$ blast cells. We conclude that J-2 virus induces blast cells in several hematopoietic lineages including Ly-5^- erythroblasts and Ly-5^+ immunoblasts of both T- and B-cell origin.

The combined results of histological and FMF studies of mice infected with J-2 at birth show that they die of immunoblastic lymphomas and fulminating erythroblastosis after an average latency of 2 wk. In addition to these advanced neoplasms, all mice had early sarcomas of the spleen (Fig. 3B) and some developed pancreatic acinar dysplasia (Fig. 3F) and frank carcinoma. The rapidity with which the erythroblastosis and lymphomas develop is unparalleled in studies of other rapidly transforming viruses including all of the mammalian single oncogene transducing viruses.

These findings contrast strikingly with the relatively prolonged courses of disease in mice infected with 3611 MSV (6-wk average latency), J-1, J-3, or J-5 virus (> 9 wk latency). Mice inoculated with the first two viruses often died from splenic rupture due to the extensive growth of splenic sarcomas and exhibited numerous sarcomas. The latter animals had no evidence of sarcomas of skeletal and visceral muscles. Mice infected with the J-3 and J-5 constructs rescued with M-MuLV died with T-lineage lymphomas with latency similar to that of the helper virus [40]. However, using several different helper viruses we have recently demonstrated a role for v-*myc* in the development of these lymphomas [Morse et al, submitted].

GROWTH PROPERTIES OF TUMOR-DERIVED CELLS IN CULTURE

Earlier studies have demonstrated that hematopoietic cells from tumors of mice infected as newborns with 3611 MSV and expressing *gag-raf* fusion proteins require interleukin 3 (IL 3) for maintenance of growth in culture (Table II) [Rapp and Ihle, unpublished data, 37,40]. Similarly, infection of fetal liver and bone marrow cells with 3611 MSV in vitro yields permanently growing cultures which retain their dependence on IL 3 [37,40,41]. In contrast to growth requirements of hematopoietic cells from tumors induced by v-*raf*-transducing virus (and v-H-*ras*-transducing virus), cells transformed in vivo by virus carrying both v-*raf* and v-*myc* oncogenes grow independent of IL 3 or any other lymphokine supplement (Table II). In parallel to the

TABLE II. Frequency of Establishing Long-Term Lines*

Virus	Oncogene	In vivo primary tumors		In vitro HPT cultures	
		+IL 3	−IL 3	+IL 3	−IL 3
None	—	0/10	0/10	0/3	0/3
3611	*raf*	10/10	0/10	3/3	0/3
HaSV	*ras*	3/3	0/3	10/10	0/10
J-2	*raf/mil* + *myc*	10/10	10/10	2/2	2/2

*For tumor induction, newborn NSF/N mice were inoculated with 3611 MSV, Harvey SV, and J-2 virus as described previously [37]. Infection of hematopoietic tissue cells (HPT) in culture included fetal liver (3611 MSV, Harvey SV, and J-2) and bone marrow (3611 MSV, J-2). Culture medium was supplemented with IL 3 at 20 μm/ml. IL, interleukin.

synergistic action of *raf* and *myc* in vivo, we have recently demonstrated that co-expression of these two oncogenes induces the selective proliferation of murine bone marrow cells [84] and fetal liver cells [Cleveland, Rapp, Ihle, and Weinstein, in preparation] in the absence of specific growth factor (GF) supplements. Interestingly, in parallel to our results in vivo, in vitro infections of fetal liver cultures with J-2 have given rise to several different lineages of GF-independent, immortalized cells including pre-B cells, mast cells, and myeloid stem cells. In contrast, cells infected with *raf*- or *myc*-only virus did not transform hematopoietic cells in the absence of GF. The high plating efficiency of J-2 tumor-derived hematopoietic cells in regular medium in culture made it possible to use standard radiolabeling techniques for detection of v-*raf*- and v-*myc*-specific protein (see below).

EXPRESSION OF V-*RAF*- AND V-*MYC*-SPECIFIC PRODUCTS IN CELLS TRANSFORMED IN VIVO AND IN VITRO

Expression of the v-*raf* or v-*raf/mil* hybrid oncogene could readily be distinguished from expression of its cellular counterpart because of its linkage to viral *gag* protein. In the case of v-*myc*, expression of the viral oncogene protein was more difficult to establish in tumor cells because of the similarity in size and antigenicity of viral and cellular *myc* protein. We therefore determined the presence of virus-specific RNA as well as the expression of viral oncogene proteins in infected cells.

RNAs from infected fibroblasts and from cell cultures of J-2 virus-induced mouse tumors were analyzed by Northern blot hybridization [40]. RNAs from J-2 virus-transformed fibroblasts and cell cultures derived from J-2 virus-induced tumors hybridized with U3-LTR and *env* probes revealed a genomic species of 7.6 kb and a subgenomic RNA of 5.6 kb. No 3.0-kb *env* subgenomic RNA was detectable. Presumably insertion of additional v-*myc* sequences into this construct abrogates normal splicing of this RNA. These RNAs also hybridized with the v-*myc* and *raf* cDNA probes [40]. Interestingly, hybridization of Northern blots of J-2 virus-infected fibroblast or tumor cells with a c-*myc* probe does not show detectable levels of c-*myc* RNA. In contrast, blots from control fibroblast and 3611 MSV-infected fibroblast cells show readily detectable levels of c-*myc* transcripts [unpublished data].

The translation of v-*raf*- and v-*myc*-specific RNA into v-*raf*- and v-*myc*-specific proteins in infected fibroblast cell lines was established by immunoprecipitations of labeled cell lysates with antibodies directed against MuLV p30, v-*raf*, and v-*myc* (Fig. 4). The first three lanes of Figure 4, panel A, show the *gag-raf* fusion proteins

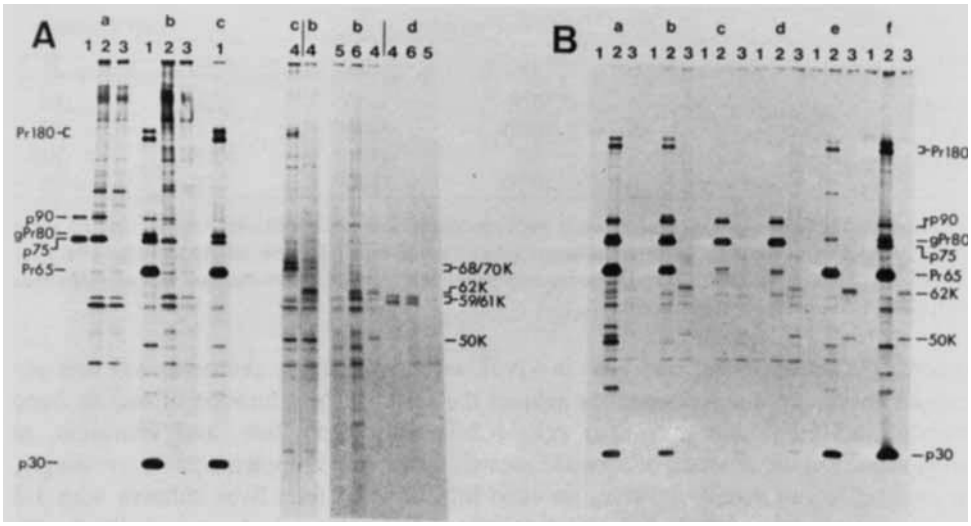

Fig. 4. SDS-PAGE analysis of *gag*-, *raf/mil*-, and *myc*-related proteins in cells transformed in vitro and in vivo by J-2 construct virus. Boiled SDS lysates of cells labeled with ^{35}S-methionine were prepared [51,52] and immunoprecipitation was performed by using aliquots of lysates containing identical amounts of radioactivity [30]. A) Comparison of proteins precipitated from the following cell lines: (a) 3611B3, (b) NIH/J-2 (M-MuLV), (c) NIH/J-1 (M-MuLV), (d) MH2-A103NP. Precipitation was done with the following antisera: lanes 1) anti-p30*gag*, lanes 2) anti-*raf*(SP63), lanes 3) anti-*raf*(SP63) preadsorbed to SP63 peptide, lanes 4) anti-*myc* [53], lanes 5) anti-*myc*C (IgG) preadsorbed to *myc*C peptide, lanes 6) anti-*myc*C (IgG) without peptide block. Fluorographs were exposed for 3–10 days. The positions of the *gag-raf/mil* products p75 and p90, and those of the helper-virus-encoded proteins Pr180 *gag-pol*, gPr80*gag*, and Pr65*gag* are indicated to the left. Proteins precipitated by anti-*myc* sera are indicated in kilodaltons to the right. B) Analysis of proteins from cells derived from mouse tumors induced by J-2. Precipitation was performed with normal goat serum (lanes 1), goat-anti-p30*gag* (lanes 2), and anti-*myc* (lanes 3) from the following tumor cell lines: (a) spleen A, (b) spleen C, (c) spleen D, (d) thymus C, (e) submaxillary lymph node A, (f) NIH/J-2 (M-MuLV). The exposure time of the fluorograph was 5 days. Reproduced from previously published figure [40].

p75 and p90 in fibroblasts transformed by 3611 MSV. These proteins are also expressed in fibroblasts transformed with J-2 and J-1 viruses carrying the *raf/mil* hybrid gene (panel A, lanes b 1–3 and lane c 1, respectively). These lanes also show additional virus structural proteins precipitated by anti-p30 as expected for helper-virus-producing cells. Expression of v-*myc* was determined by immunoprecipitation with two different anti-*myc* sera, a synthetic peptide serum [51] and a serum raised against *myc* protein produced in *Escherichia coli* [53]. v-*myc*-specific bands corresponding to a 62K protein were detected only in J-2 virus-transformed cells (panel A, lanes b 4–6). The 62-kD protein is considered to be the product of v-*myc* since (1) it is detected by both sera, (2) precipitation with v-*myc* peptide serum was competed by synthetic peptide, and (3) its presence was restricted to J-2 virus-infected cells. The v-*myc*-specific proteins in MH2-transformed chicken cells are slightly smaller at 59/61K (panel A, d 4–6).

Cells from tumors induced by J-2 virus all express p75/90 v-*raf/mil*-specific proteins [38,39] with the exception of cells from submaxillary lymph node (panel B, lane e 2). In agreement with this observation, we found by infectious center assays

that only 0.04% of these cells released focus-forming virus, as compared to 60% for spleen A (lane a), 20% for spleen C (lane b), 100% for thymus C (lane d), and 100% for spleen D (lane C). Spleen D and thymus C show the lowest levels of helper-virus-specific proteins. Variable levels of a 62K *myc* protein band were detected in all the tumors presented in Figure 4B. We conclude that v-*raf* and v-*myc* are expressed in J-2 virus-infected fibroblast lines and the majority of J-2-induced tumors.

MECHANISMS OF *RAF* AND *MYC* SYNERGISM: ROLE OF *MYC* IN ABROGATING SPECIFIC GROWTH FACTOR REQUIREMENTS

The majority of neoplasms caused by the dual oncogene virus J-2 were of hematopoietic/lymphoid lineage. To assess the contribution of *raf* and *myc* in these neoplasms, we examined the effects of these recombinant retroviruses on two growth-factor-dependent lymphoid and hematopoietic cell lines requiring either interleukin 2 (IL 2) or interleukin 3 (IL 3) for viability.

IL 2 is required for the proliferation of antigen-activated mature T cells [reviewed in 58] while IL 3 induces the proliferation and differentiation of early hematopoietic/lymphoid stem cells [reviewed in 59,60]. A number of IL 2-dependent cytotoxic T cell lines have been established. Although the frequency of establishing lines suggests that an immortalizing event is required [61], once established, the cell lines continue to depend on IL 2 for growth. For our experiments we used an SV 40-specific, H-2Kb-restricted, cytotoxic T cell line (CTB6) from C57BL/6J mice [36] (provided by Dr. Barbara Knowles, Wistar Institute, Philadelphia, Pennsylvania).

IL 3-dependent cell lines have been isolated from primary retrovirus-induced lymphomas [54,62] and from long-term bone marrow cultures [63,64]. As above, the frequencies for establishing lines suggest that an immortalizing event may be required. For our studies we used the FDC-P1 cell line, which has properties of early myeloid lineage cells [63,65]. These cells differ from normal IL 3-responsive cells in their limited capacity to differentiate, but have a normal diploid karyotype.

To evaluate the effects of v-*myc* expression, we used the constructs shown in Figure 1 and, in addition, another recombinant (HF) containing the complete MC29 v-*myc* cloned into the *gag* gene of Moloney leukemia virus [85]. In the absence of IL 3, FDC-P1 cells rapidly lose viability and factor-independent variants have not been obtained [63,65,66]. However, when the cells which had been exposed to the J-2 virus were cultured in the absence of IL 3, factor-independent cells were obtained. Factor-independent cell lines were not obtained with FDC-P1 cells alone, with cells exposed to 3611 MSV [*raf*, 37,41], HaSV [H-*ras*, 37], FBR [*fos*, unpublished data] (see Table III), or to helper virus (data not shown). When the factor-independent cells were assayed, transforming virus was detectable. Similarly when CTB6 cells were exposed to the J-2 or HF viruses and cultured in the absence of IL 2, factor-independent cell lines were obtained which replicated transforming virus. To evaluate the contribution of the v-*raf*/v-*mil* oncogene, FDC-P1 cells were exposed to the J-3 virus or the HF virus. In both cases, factor-independent FDC-P1 cell lines were obtained when cells were cultured in the absence of IL 3.

To characterize the cells, we examined representative lines for the expression of the v-*raf*/*mil* and/or v-*myc* oncogenes by Northern blot analysis (Fig. 8). In uninfected NIH 3T3, FDC-P1, or CTB6 cells there are no detectable v-*myc* hybridizing RNAs (Fig. 5A), whereas there was a 3.1-kb RNA detected with the *raf* probe

TABLE III. Isolation of Factor Independent Lines*

Virus	Oncogene	CTB6 (T)		FDPC-1 (myeloid)	
		+IL 2	−IL 2	+IL 3	−IL 3
None	−	+	−	+	−
3611	*raf*	+	−	+	−
HaSV	*ras*	NT	NT	+	−
J-2	*raf* + *myc*	+	+	+	+
J-3	*myc*	+	+	+	+
HF(J-5)	*myc*	+	+	+	+
FBR	*fos*	+	−	+	−

*The indicated cell lines were infected with the various viruses in the presence of polybrene (25 μM) in IL 3 (FDC-Pl cells [35]) or IL 2 (CTB6 cells [36]). Following infection, the FDC-Pl or CTB6 cells were maintained in media supplemented with IL 3 or IL 2 [37] for 4 days and cultured in media with or without factors for 4 wk. During this period the majority of the cells died out and factor-independent cells emerged. NT, not tested.

(Fig. 5B). This RNA has the expected size of the endogenous c-*raf*-1 gene mRNA and was observed at comparable levels in all infected cell lines. The 7.6- and 7.4-kb genomic RNAs of the J-2 and J-3 viruses, respectively, were readily detectable using v-*myc* and *raf* probes in infected NIH 3T3 cells, in FDC-P1 cells infected with the J-2 or J-3 viruses, or CTB6 cells infected with the J-2 virus. With the v-*myc* probe, major subgenomic RNAs of 6.3 and 6.1 kb for the J-2 and J-3 viruses, respectively, were also observed. In FDC-P1 cells infected with the HF virus there was a major 6.4-kb RNA which hybridized with the v-*myc* probe but not with the *raf* probe, consistent with the structure of the viral genome. In addition, there was a major subgenomic RNA of 5.1 kb whose origin is not known. Similar anomalous transcripts have been observed with other recombinant retroviruses [67].

The Northern blot data suggested differences in the levels of viral RNA. To quantitatively evaluate differences, dot blot analysis was done. As shown in Figure 5C, FDC-P1 cells infected with either the J-2 or J-3 virus had approximately a ten-fold higher level of v-*myc* hybridizing RNA than that observed with fibroblasts. In CTB6 cells infected with the J-2 virus, there was approximately a 100-fold higher level of RNA. The basis for these differences is not known although Southern blot analysis suggests that it is not due to multiple integrated proviruses (data not shown) and therefore may be due to lineage-specific effects on the enhancer sequences of the virus [68].

We also examined the cell lines for expression of the v-*myc* and v-*raf/mil* proteins. By immunoprecipitation all the lines examined expressed the v-myc protein of 62 kD comparable to that seen in infected fibroblasts and at levels similar to that of c-*myc* in FDC-P1 cells grown in the presence of IL 3 (data not shown). Because of the cross reactivity of the anti-*myc* sera with mouse c-*myc* protein and the similarity in the sizes in c-*myc* and v-*myc* proteins [69,70], the identification of *myc* protein as viral relies on the observation that none of the infected lines contains c-*myc* RNA (see below). With either FDC-P1 or CTB6 cells infected with the J-2 virus the expected *gag*-(v-*raf/mil*) fusion protein of 75 kD [38] was detectable by immunoprecipitation (data not shown).

To evaluate the factor dependence, ^{3}H-thymidine incorporation assays were used. As shown in Figure 6A, parental FDC-P1 cells required approximately 0.2 ng/ml of IL 3 for half-maximal activity. In contrast, J-2, J-3, or the HF virus-infected

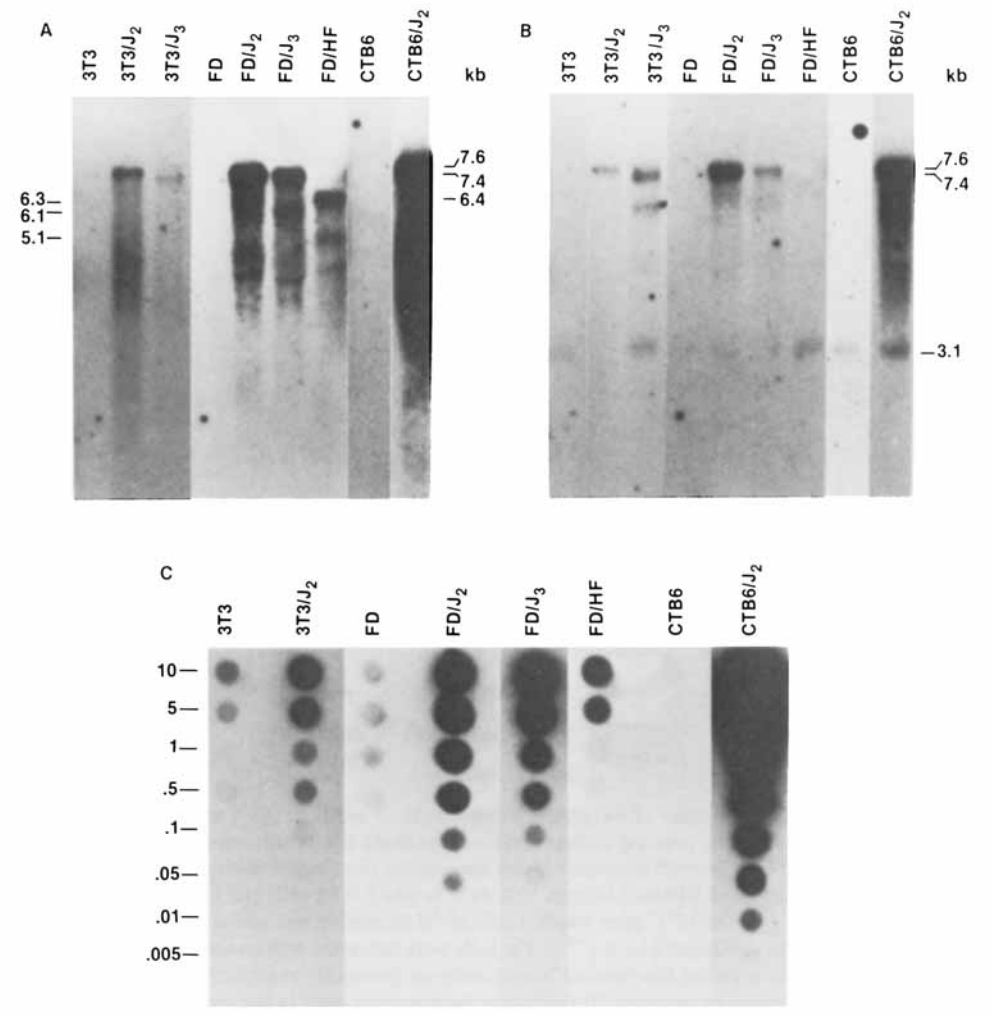

Fig. 5. Northern analysis of recombinant retroviruses in factor-independent cell lines. Polysome-associated RNA was prepared as described [45] and poly A RNA was selected by two cycles of oligodeoxythymidilic cellulose chromatography. The RNA (10 μg) was denatured by glyoxalation [46], separated electrophoretically in 1.2% agarose, blotted onto nitrocellulose (Schleicher & Schuell), and hybridized under stringent conditions [44] with ^{32}P-labeled nick-translated [47] DNA probes. A) V-*myc* hybridizations. For a v-*myc* probe, the 1.4-kbp PstI/AhaIII fragment (Oncor, Incorporated, Gaithersburg, MD) of the MC29 provirus was used [48,49]. The intensity of hybridization between the various samples varies considerably; lanes 1-3 and 8 were exposed for 3 days whereas lanes 4–7 and lane 9 were exposed for 20 hr. Recombinant retroviral genome-sized RNAs are indicated at the right; subgenomic RNAs are indicated in the left margin. Sizes are given in kilobases and were determined from ^{32}P-labeled, denatured Hind III fragments of bacteriophage lambda DNA. B) *Raf* hybridizations. The blot shown in (A) was stripped [44] and hybridized as above with 2.9-kbp human *raf* cDNA probe [Bonner et al, in press]. The exposure of all the samples was 3 days. The 3.1-kb transcript is the endogenous *raf* mRNA. C) Quantitative analysis of v-*myc* RNA levels in factor-independent cell lines. Poly A RNA, isolated from the indicated cell lines, was denatured by treatment with formamide/formaldehyde [44] and loaded onto wells of a dot blot apparatus (Schleicher & Schuell). The amount of poly A RNA (μg) in each well is indicated at the left margin. Hybridizations were done with the v-*myc* probe as in (A) and blots were exposed for 20 hr.

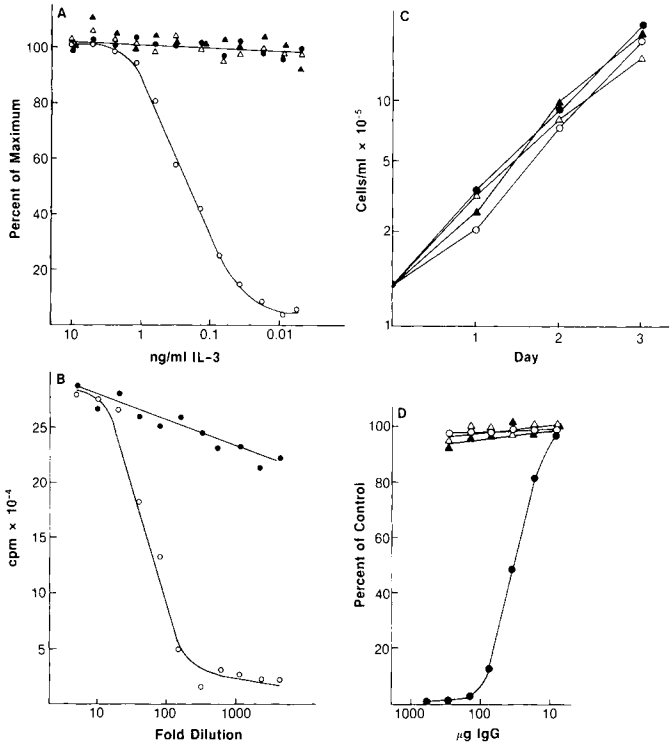

Fig. 6. Proliferative responses of various cell lines to IL 3 or IL 2. A) The indicated cells were obtained from exponentially growing cultures and were pelleted and resuspended twice in RPMI-1640 containing 10% FCS. In 96-well microtiter plates appropriate dilutions of purified IL 3 were made in wells containing 0.05 ml of RPMI-1640 with 10% FCS to which 0.05 cells (10^5) were added. The cells were incubated for 24 hr at 37°C after which 1 μCi of ^{3}H-thymidine was added per well and the cells were incubated for an additional 6 hr at 37°C. The cells were harvested with an automated cell harvester. IL 3 was purified to apparent biochemical homogeneity as previously described [82]. The results are plotted as the percentage of maximal ^{3}H-thymidine incorporation seen in the presence of excess IL 3. The cell lines examined included the parental FDC-P1 cells (○—○) and FDC-P1 cells infected with the J-2 (●—●), J-3 (△—△), or HF (▲—▲) viruses. B) The experiments were as in (A) except that the cells were incubated for 48 hr prior to pulsing with ^{3}H-thymidine. The IL 2 was partially purified from conditioned media from PMA-induced EL-4 cells as previously described [83]. The cells examined included the parental CTB6 cells (○—○) and CTB6 cells infected with the J-2 virus (●—●). C) Growth curves for control and infected FDC-P1 cells. The growth curves were obtained by seeding the cells at 5 × 10^5 cells/ml in RPMI-1640 containing 10% FCS in the presence or absence of IL 3. Viable cell numbers were determined at the indicated times. The cells examined included the parental FDC-P1 cells (○—○) which were grown in the presence of IL 3 (20 units/ml) or J-2 (●—●), J-3 (△—△) or HF (▲—▲) infected FDC-P1 cells which were grown in the absence of IL 3. D) Lack of inhibition of growth by antisera against IL 3. The indicated cells were obtained from exponentially growing cultures and were pelleted and resuspended twice in RPMI-1640 containing 10% FCS. In 96-well microtiter plates, twofold dilutions of immune or control protein A Sepharose-purified IgG were made in 0.05 ml of RPMI-1640 containing 10% FCS. For the parental FDC-P1 cells 0.01 ml of IL 3 (2 units) was added to each well. The samples were incubated for 2 hr at 37°C and the cells (10^5) in 0.05 ml were added. The cells were incubated for 24 hr at 37°C and pulsed with ^{3}H-thymidine as in Figure 4. The cell line examined included the parental FDC-P1 cells (○—○) and J-2 (●—●), J-3 (△—△) and HF (▲—▲) infected FDC-P1 cells. The preparation and characteristics of the immune IgG have been previously described [71].

cells showed no dependency on IL 3. Similarly the J-2-infected CTB6 cells showed a vastly decreased requirement for IL 2 (Fig. 6B). As shown in Figure 6C, the growth curves and the doubling times were comparable for each of the virus-infected FDC-P1 cell lines in the absence of IL 3 and for the parental cell line grown in the presence of IL 3.

One mechanism for the abrogation of factor dependence is the production of a requisite growth factor. However, with none of the infected cell lines did we detect mitogenic activity for the parental cell lines in conditioned media (data not shown). In the assay for IL 3, the lack of detectable activity indicates concentrations of less than approximately 0.02 ng/ml. Another approach was to examine the effect of an antiserum [71] against IL 3. As shown in Figure 6D, the immune IgG inhibited the IL 3-dependent proliferation of the parental FDC-P1 cells but had no effect on J-2, J-3, or HF virus-infected cells. In agreement with these results, Northern hybridization with IL 3 cDNA as probe demonstrated that none of the FDC-P1 factor-independent cell lines produced IL 3 mRNA (data not shown).

The absence of a requirement for IL 3 suggests that the expression of IL 3 receptors might be altered. To evaluate this, the ability to bind ^{125}I-labeled IL 3 was determined. The FDC-P1 cells infected with the J-2, J-3, or the HF virus bound iodinated IL 3 at levels comparable to the uninfected parental cells (data not shown).

The retention of receptors for IL 3 raised the question of whether IL 3 could alter the expression of c-*myc*. This was of interest since the rapid loss of viability in the absence of IL 3 has not allowed a direct analysis of IL 3-induced expression of c-*myc*. As shown in Figure 7A, c-*myc* RNA was detectable in the parental FDC-P1 cells grown in IL 3. However, no c-*myc* was detectable in the virus-infected cell lines grown in the presence or absence of IL 3. To determine whether the absence of c-*myc* RNA was due to low levels under steady-state conditions, cells were examined at various times after exposure to IL 3. As shown in Figure 7B, no c-*myc* RNA was detected at 30 min, 2 hr, or 72 hr in culture. To determine whether the absence of c-*myc* RNA was a general property of cells infected with the recombinant viruses, we examined the cloned, producer-fibroblast cell lines. As shown in Figure 7C, c-*myc* RNA was readily detectable in the control fibroblasts but was not detected in the infected cells.

To determine whether the abrogation of a requirement for IL 3 affected tumorigenicity, the J-3- and HF-infected cells were tested for tumor formation in *nu/nu* mice, since neither of these viruses induces tumors over the time period examined [40]. As shown in Table IV, the parental FDC-P1 cells were not tumorigenic as previously described [35]. In contrast, FDC-P1 cells infected with the J-3 or the HF virus did induce tumors.

The ability of v-*myc* expression to abrogate the requirements for IL 3 or IL 2 supports the hypothesis that these growth factors regulate *myc* expression. This is consistent with the observation that IL 2 can be shown to increase c-*myc* transcription in normal, lymphoid cells [K. Kelly, personal communication]. Thus it appears that c-*myc* acts as a central relay for the transmission of signals from several distinct (competence-inducing) growth factors including PDGF, IL 3, and IL 2. Consistent with our speculation on the induction of autonomous growth by a combination of progression (v-*raf*)- and competence (v-*myc*)-inducing factors is our finding that lymphoid cells from J-2 virus-induced tumors could be readily grown in culture without addition of specific lymphokines.

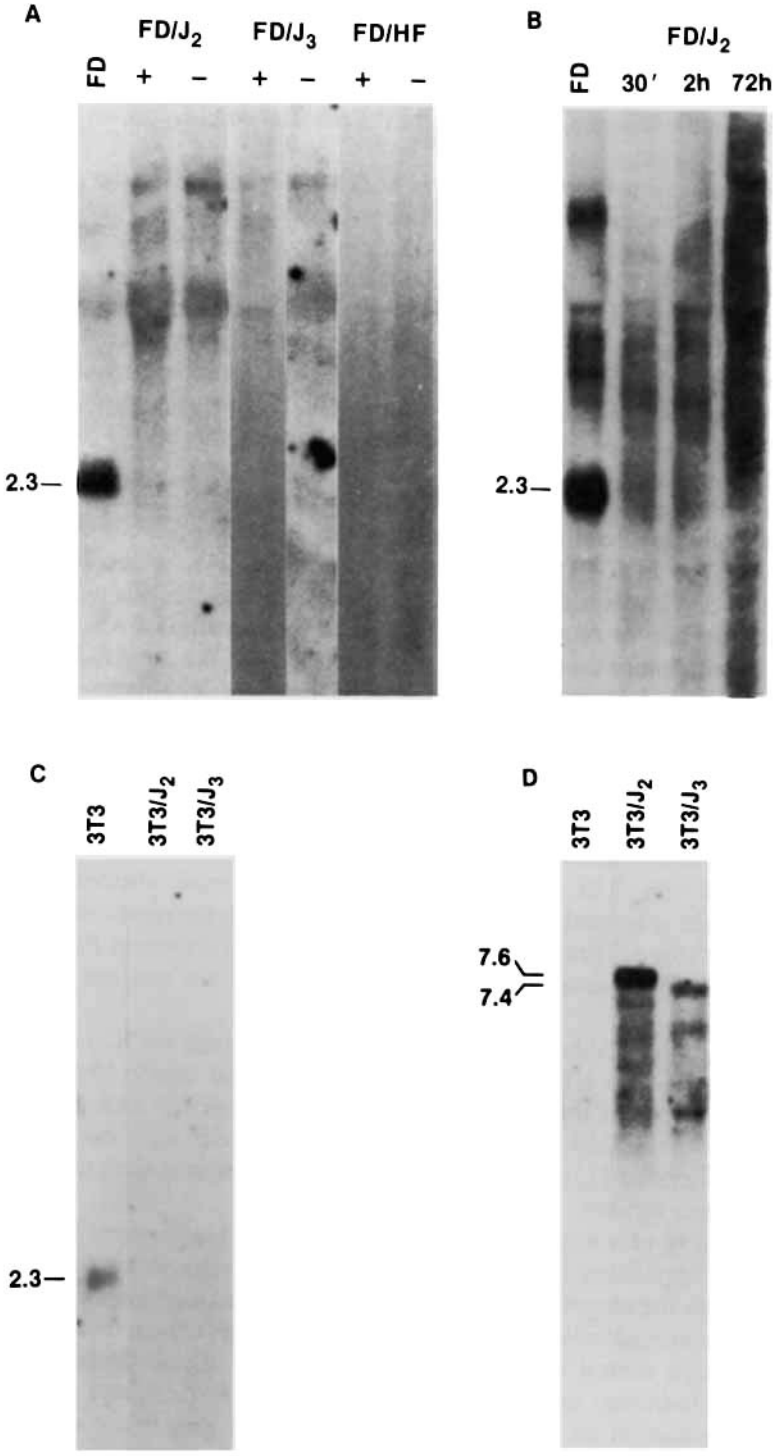
A
FD/J2
FD/J3
FD/HF
FD
+
−
+
−
+
−
2.3
B
FD/J2
FD
30′
2h
72h
2.3
C
3T3
3T3/J2
3T3/J3
2.3
D
3T3
3T3/J2
3T3/J3
7.6
7.4

Fig. 7

It was surprising that we were unable to induce c-*myc* expression in the infected FDC-P1 cells with IL 3 even though these cells retained receptors for this ligand. The absence of c-*myc* in these cells seems likely due to a regulatory effect of v-*myc* on c-*myc* expression as suggested by the observation that infected fibroblasts, J-2-transformed pre-B cells, and myeloid cells from fetal liver (data not shown) also have no detectable c-*myc* RNA. The specific suppression of c-*myc* expression is suggested by the lack of comparable effect on c-*raf* (and c-*abl* and c-*myb*, not shown) expression. The inability to induce c-*myc* expression is not due to alteration of the endogenous loci as determined by Southern blot analysis of EcoRI-restricted DNAs (data not shown). The ability of an activated c-*myc* locus to negatively regulate the normal allelle has been suggested by other studies [75–77]. The mechanisms for this effect are not known but may involve the interaction of the *myc* protein or a *myc*-induced protein with potential regulatory sequences defined by sensitivity to DNAase I [77]. Experiments are currently in progress to assess the DNAase I sensitivity of these sites in the c-*myc* loci of infected FDC-P1, CTB6, and NIH 3T3 cells.

Recent studies have demonstrated that tumorigenesis requires multiple events. In hematopoietic tumors such as myeloid tumors, stages of tumor progression have been described [78,79]. For example, murine myelogenous leukemias appear blocked in differentiation but often require IL 3 for growth in vitro [54,62] and some HTLV-1-induced cutaneous T cell lymphomas require IL 2 for growth [80,81]. In these cases, the activation of c-*myc* can be envisioned as one potential mechanism for the progression to a factor-independent phenotype. This type of role for c-*myc* activation may also occur in other lineages including B-cells in which there is a high incidence of altered c-*myc* expression associated with transformation.

TABLE IV. Tumorigenicity of Recombinant Retrovirus Infected FDC-Pl Cells in NU/NU Mice*

Cell line	Tumors/total	Average latency (days)
FDC-Pl	0/5	>60
FDC-Pl (J-3)	5/5	28
FDC-Pl (HF)	5/5	30

*Nu/nu BALB/c mice of 4–6 wk of age were inoculated intraperitonealy with 2×10^6 cells. Morbid mice were killed and examined microscopically for evidence of tumors.

Fig. 7. A) C-*myc* expression in FDC-P1 lines in the presence (+) and absence (−) of IL 3. Total RNA was prepared and polyA-containing RNA was selected by oligo d[T] cellulose chromatography. The RNA (15 μg) was denatured, electrophoresed, blotted, and hybridized as in Figure 2. The c-*myc* probe was the 5.6-kbp BamHI fragment of the mouse genomic c-*myc* [75]. B) Time course examining c-*myc* expression. Total RNA was prepared from FDC-Pl (J-2) cells at the indicated times after the addition of IL 3 and polyA RNA was analyzed as above. The autoradiographs were overexposed to detect c-*myc* transcripts. C,D) *Myc* expression in NIH/3T3 lines. PolyA RNA (15 μg) was prepared from the indicated cell lines and analyzed by Northern blot hybridization. C-*myc* hybridization is shown in C in which exposure of the autoradiographs was for 1 wk. V-*myc* hybridization is shown in D and the exposure of the autoradiograph was for 3 days.

CONTRIBUTIONS OF *RAF* TO SYNERGISM

The role of *raf* in the synergy between *myc* and *raf* has been more difficult to define. The properties of the *raf* oncogene group, a multigene family, are summarized in Table V. The viral oncogene v-*raf* was originally isolated by our group from mouse cells by retrovirus transduction [38,39] into the defective virus 3611 MSV. Subsequently, we have identified the putative oncogene v-*mil*, of the avian carcinoma virus MH_2, as being homologous to v-*raf* [32]. The normal c-*raf*-1 polypeptide has a MW of 74 kD (Fig. 8E,F) and is a phosphoprotein (Fig. 8C,D) with associated in vitro autophophorylating protein kinase activity (Fig. 8A,B).

The function of the cellular homolog of v-*raf*, c-*raf*-1, in this synergism is unknown although we do know c-*raf*-1 is a cytosolic protein and we have detected Ser/Thr-specific protein kinase activity for the transforming versions of c-*raf*-1 [72]. All of the transforming forms of c-*raf*-1, ie, v-*raf* containing 37 kD of *raf*, v-*mil* containing 40 kD of *raf*, and an LTR-activated version of c-*raf*-1 containing 50 kD of

TABLE V. The *Raf* Oncogene Family

A: Genes homologous to v-*raf*	
Transduced as the transforming gene, v-*raf*, of 3611 MSV	Isolated from a mouse with lung and peritoneal tumors induces fibrosarcoma/histiocy toma in newborn mice
Avian homolog of v-*raf* (v-*mil*) is part of MH2	An avian carcinoma virus which also contains v-*myc*
Member of the *src* family	Has associated *ser/thr* specific protein kinase activity
Two genes in man, an active gene c-*raf*-1 and a pseudogene c-*raf*-2	The active gene maps on chromosome 3 at p25; site is specifically altered in small cell lung carcinoma, ovarian carcinoma, familial renal carcinoma, and mixed salivary gland tumors
Active gene has 17 exons	11 exons homologous to v-*mil* and 9 exons to v-*raf*; 5 additional coding exons at 5′ end spread over another 15 kb
	A 3.4-kb mRNA in human lymphocytes, fetal liver, and placenta is colinear with v-*raf*
One of the gene products is 74 kD	Liver and placental mRNAs code for a 648AA protein which contains no transmembrane region
The 74 kD c-*raf*-1 protein from NIH 3T3 is phosphorylated in *ser/thr*	
B: Genes related to v-*raf*	
There are at least two different active *raf*-related genes which are closely related to each other	These are designated δ-*raf*-1 and δ-*raf*-2 and are distinguishable by in situ hybridization to human metaphase chromosomes
δ-*raf*-1	δ-*raf* is located on mouse and human chromosome X, in man at Xp12
Isolated from spleen cDNA library using v-*raf*	
Expression is tissue specific	Is transcribed as a 2.4 kb mRNA
Homology between δ-*raf* and c-*raf*-1 is 69% for DNA and 74% for amino acid sequences	Homology is highest in the kinase domain
δ-*raf* recombinant retrovirus transforms NIH 3T3 cells	Induces sarcomas in newborn NFS/N mice
δ-*raf*-2	Located on human chromosome 7 near the centromere

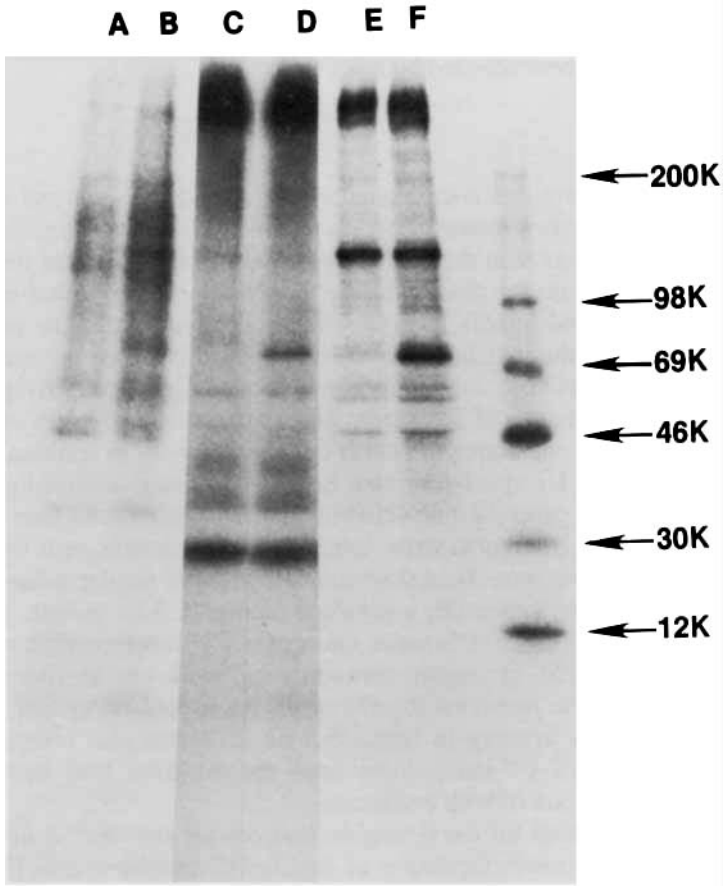

Fig. 8. SDS-PAGE analysis of c-*raf*-1 proteins in NIH/3T3 fibroblasts. Boiled SDS lysates of unlabeled cells (A,B) and of cells labeled with ^{35}S-orthophosphate (C,D) or with ^{35}S-methionine (E,F) were prepared and immunoprecipitation carried out as previously described [38]. In vitro kinase assay of immunoprecipitated c-*raf*-1 (A,B) was performed as published [38]. Precipitation was done with anti-c-*raf*-1 (SP63) antisera with (A,C,E) or without (B,D,F) peptide block. The exposure time of the fluorograph was 2 days.

raf, are truncated at their amino terminus. Therefore, the truncated portion of the c-*raf* protein may correspond to a binding site for a regulatory ligand which may normally activate the protein.

Whatever its exact function in the cell it is clear that transforming versions of c-*raf*-1 can immortalize hematopoietic cells without affecting specific growth factor requirements (see Table II) and that, in addition to transforming NIH/3T3 fibroblasts, v-*raf* is capable of morphologically transforming primary rat and human embryo fibroblasts [25]. Furthermore, v-*raf*-transformed fibroblasts share with cells transformed by other acute transforming retroviruses the loss of EGF binding activity and the release of transforming growth factor (TGF) α and TGFβ into the culture medium [unpublished data]. Moreover, secretion of TGFs can be demonstrated without concentration of culture fluid by seeding a single transformed nonproducer cell together

with 10^5 untransformed FRE rat fibroblasts cells into soft agar where recruitment of soft agar growth can be observed in the vicinity of the large colony of virus-transformed cells (Fig. 9). These data suggest that *raf* affects different components of growth regulation than those affected by *myc*.

CONCLUSIONS

We have shown here that combination of the oncogenes v-*raf* and v-*myc* in a murine retrovirus accelerates tumor induction relative to v-*raf*- or v-*myc*-transducing viruses by a factor greater than three. The spectrum of cell types that make up the tumor mass is shifted from the predominantly fibroblastic and erythroid populations observed in v-*raf*-induced tumors to erythroblasts and lymphoid cells of T and B lineages. In addition to the lymphomas and erythroblastosis which generally are the cause of deaths of inoculated mice, fibrosarcomas and pancreatic dysplasia also develop. However, carcinoma of the ovary, which appears to be typical of the avian retrovirus MH2 [33,34] containing the avian homolog of v-*raf* in addition to v-*myc*, have not been observed. Using a J-3 variant, however, we have observed carcinomas of the liver, lung, and pancreas [unpublished results]. This is the first molecular analysis of a mammalian carcinoma virus. Lymphoid/hematopoietic cells from tumors produced by the dual-oncogene-containing virus J-2 grow in regular culture medium whereas cells from tumors induced by v-*raf* alone require IL 3 for growth. In contrast to the complementation observed between oncogenes for transformation of primary fibroblasts in vitro [24,25], synergism between v-*raf* and v-*myc* in vivo appears to exclude fibroblasts and be restricted to cells of the hematopoietic system and endodermal epithelium. This synergy in hematopoietic cells was also evident in vitro where immortalization of GF-independent lines derived from fetal liver or bone marrow required expression of both oncogenes.

What may be the basis for the synergism between *raf* and *myc*? A dissection of factors involved in the growth regulation of BALB/3T3 fibroblast cells [78,79] has led to the identification of two categories of signals, competence signals (acting early in G1 phase) and progression signals (which act later on G1 phase of the cell cycle), both of which are required for cell population to proceed. Analogous combinations of factors may be required for growth regulation of hematopoietic cells as well. In the fibroblast system, progression factors include epidermal growth factor (EGF) and presumably also transforming growth factor α (TGFα), which acts through the same receptor [11]. Fibroblast cells transformed by v-*raf* share with cells transformed by v-*mos*, v-*fes*, v-*abl*, and v-*ras* the loss of EGF binding activity and the induction of growth factor secretion including TGF α [9,10]. We therefore suggest that v-*raf*, directly or indirectly, provides progression signals to these cells. Interestingly, constitutive expression of the EGF-specific progression signal is associated with development of erythroleukemia in birds infected with the v-*erb*B-transducing virus AEV [6] and may therefore be the basis for the erythroid hyperplasia induced by 3611 MSV. Does v-*raf* also provide competence signals? A hallmark of the competence-inducing factor PDGF is its ability to induce transient transcription of the cellular protooncogenes *myc* [5] and *fos* [20] but not *raf* or *ras*. Moreover, *myc* can partially substitute for PDGF [16] and thus may act as a central transmitter of its competence signal. Transformation of fibroblast cells with v-*raf* is not generally associated with increased expression of *myc* [Rapp, unpublished]. It is therefore tempting to speculate that the

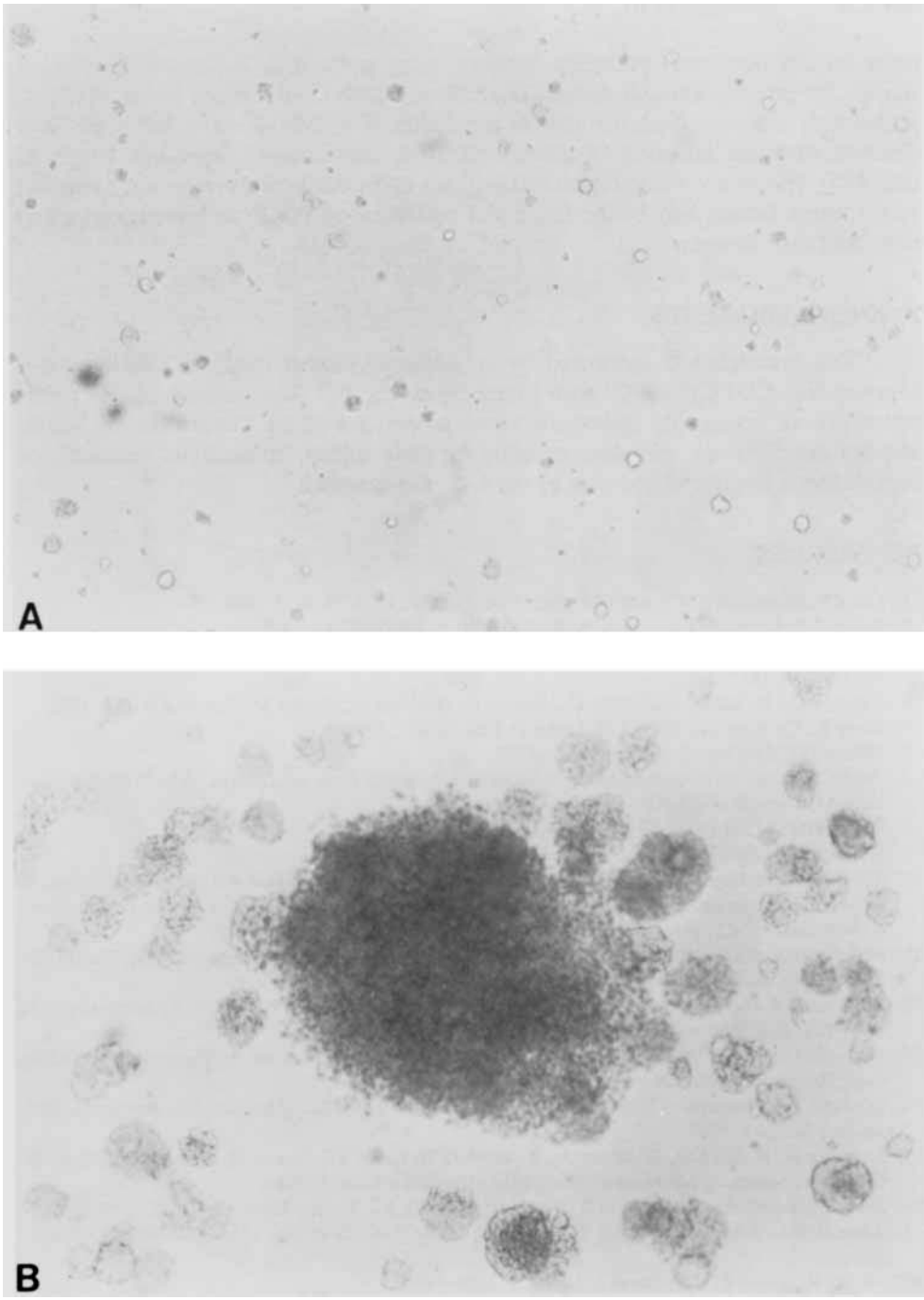

Fig. 9. Release of transforming growth factors by 3611 MSV FRE nonproducer cells. Transformed nonproducer cells were mixed (panel B) with untransformed FRE cells at a ratio of $1/10^5$. Cells were suspended in 0.3% noble agar in DMEM substituted with 10% FCS and seeded on top of 5 ml 0.5% agar base in 60-mm petri dishes. Colonies were scored after 10 days. Growth of untransformed FRE cells is shown in panel A.

basis for the observed synergism between v-*raf* and v-*myc* is the combination of signals for progression and competence. However, the competence factor PDGF is presumably not important for growth regulation of lymphoid cells. Since we have demonstrated that infection of interleukin 3- or interleukin 2-dependent lymphoid cells [85] with v-*myc* recombinant retroviruses abrogates both growth factor requirements, these factors may be the functional equivalent of PDGF in hematopoietic and lymphoid cell lineages.

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