Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 6868–6872, September 1986
Cell Biology

Recombinant murine retroviruses containing avian v-*myc* induce a wide spectrum of neoplasms in newborn mice

(*raf*/lymphomas/adenocarcinomas)

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Communicated by George Klein, June 10, 1986

ABSTRACT **NFS/N mice were infected within 48 hr of birth with pseudotypes of recombinant murine leukemia viruses containing avian v-*myc* developed T-cell, pre-B-cell, and B-cell lymphomas and epithelial tumors including pancreatic and mammary adenocarcinomas. Primary hematopoietic and epithelial tumors and continuous *in vitro* cell lines derived from some of these tumors, established in the absence of added growth factors, exhibited clonal integrations of v-*myc* and expressed v-myc RNA. These results show that deregulated expression of the *myc* oncogene in mammalian cells can initiate a wide variety of neoplasms.**

Alterations in the normal patterns of c-*myc* oncogene expression are generally assumed to contribute to the development of a wide variety of neoplasms in vertebrates. The mechanisms responsible for deregulated expression of this gene are multiple, including insertion of proviruses in juxtaposition to c-*myc* (1–3), translocations involving the chromosome containing c-*myc* (4–6), and incorporation of c-*myc* exons in retroviruses (7, 8). Deregulated expression of c-*myc* may be insufficient to induce transformation of normal cells, and alterations in two protooncogenes may be required to induce the transformed phenotype (9, 10). These studies were based on *in vitro* analyses of transformation and their relation to the role of *onc* genes in *in vivo* transformation has yet to be established.

Our attempts to determine if altered *myc* expression is necessary or sufficient for the development of neoplasia *in vivo* have focused on a system in mice using recombinant murine retroviruses that contain avian v-*myc* genes with or without a second oncogene, v-*raf* (11, 12). The results of our earlier studies with these retroviruses demonstrated that the combined effects of the two oncogenes were to greatly accelerate the course of disease over that observed with either oncogene alone and to expand the cell types targeted for transformation by v-*raf* (11, 12), the cellular targets for v-*myc* having not been defined. We concluded that v-*raf* and v-*myc* acted synergistically in the induction of hematopoietic and epithelial neoplasia. However, the observation of synergy between oncogenes *in vivo* does not settle the question of whether more than one virus-transduced oncogene is required for tumor induction.

To address this question, we evaluated the ability of v-*myc*-containing viruses to induce neoplasms in mice infected as newborns. As earlier studies demonstrated that the transforming efficiency of other replication-defective viruses is influenced by the helper viruses used to rescue them (13, 14), we elected to use a variety of helper murine leukemia viruses to produce pseudotypes of the v-*myc* viruses. Our data demonstrate that deregulated expression of the v-*myc* oncogene from a retroviral genome initiated the induction of a wide spectrum of hematopoietic and epithelial tumors.

MATERIALS AND METHODS

Mice. NFS/N mice were obtained from the colonies of the National Institutes of Health. Animals less than 48 hr old were inoculated intraperitoneally with 0.02 ml of tissue culture pools of pseudotyped recombinant J-3 and J-5 viruses (cf. Fig. 1). Moribund mice were sacrificed for examination of their tissues by histopathology and flow microfluorometry (FMF), and samples of the tumors were frozen in ethanol/dry ice for RNA and DNA analyses or for studies of infectious viruses in extracts.

Viruses. Nonproducer NIH 3T3 cells containing the J-3 and J-5 viruses were prepared as described (11). Pseudotype virus stocks were prepared by infection of nonproducer cell lines with helper MuLV. Wild mouse viruses included the ecotropic isolates Cas-Br-M, 1504M (15), and C2S (isolated from the spleen of a normal adult wild mouse trapped near Lake Casitas, CA), and amphotropic 4070A (15). Infected cells were cocultivated with NIH 3T3 cells, transferred once, and harvested for preparation of virus pools 10 days after infection. All preparations were filtered through 45-μm Millipore filters. Transforming activity of ecotropic wild mouse virus pools was measured by titration in SC-1 cells. Titers ranged from $10^{3.7}$ to $10^{4.4}$ focus-forming units/ml.

Histopathology. All diagnoses were made on the basis of gross observations and microscopic examinations of tissues fixed in Tellyesnicky's solution (16), sectioned, and stained with hematoxylin and eosin. Diagnosis of hematopoietic neoplasms were made using published criteria (16, 17).

FMF. Single-cell suspensions of enlarged lymph nodes or spleens were prepared and stained for FMF studies as described (18). FMF analyses were performed on a fluorescence-activated cell sorter (FACS/II, Becton Dickinson) equipped with krypton and argon lasers. Assays were performed on 3×10^4 viable, nucleated cells as gated by narrow forward-angle light scatter and exclusion of propidium iodide. The origins and specificities of the monoclonal and xenoantibodies used in the FMF assays are detailed elsewhere (19–21).

Establishment of Cell Lines *in Vitro*. Long-term cultures of transformed cells from primary tumors of mice infected with Moloney pseudotypes of J-3 were generated using published techniques (19).

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Abbreviations: LL, lymphoblastic lymphoma; FMF, flow microfluorometry; kb, kilobase(s).

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RNA Analysis. Total RNA was prepared from quick-frozen tumors or from the BFS cell line (22), minced, extracted in guanidine isothiocyanate, and pelleted through a CsCl cushion as described (23). The RNA pellet was dissolved, extracted, and selected for poly(A)$^+$ species by oligo(dT)-cellulose chromatography. The RNA (5 μg) was denatured with 50% formamide/5% formaldehyde (vol/vol), separated electrophoretically in 1% agarose, blotted onto nitrocellulose, and hybridized under stringent conditions (24) with ^{32}P-labeled nick-translated DNA probes. The probes were a 1.4-kilobase-pair *Pst* I–*Aha* III v-*myc*-specific fragment of the MC29 provirus (25, 26), a 1.2-kilobase-pair *Eco*RI–*Cla* I fragment of murine c-*myc* (27), and a 2.98-kilobase-pair c-raf-1 cDNA (28).

DNA Analysis. High molecular weight DNA was prepared from homogenized frozen tumors or from isolated nuclei as described (29). Equal quantities of DNA (10 μg) were digested to completion with *Kpn* I or *Bam*HI, separated electrophoretically on 1% agarose, and blotted onto nitrocellulose. Blots were hybridized with the v-myc probe in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0.) at 60°C, and blots were washed extensively with 0.1× SSC at 65°C and exposed to Kodak XAR-5 film.

RESULTS

Time Course of Disease for Mice Inoculated with Different Pseudotypes of Recombinant MuLV-Containing v-*myc*. In this study, we made use of three different recombinant retroviruses (Fig. 1) containing v-*myc*. The J-2 virus, containing v-*raf* and v-*myc*, and the J-3 virus (a derivative of J-2 that contains a deletion and frameshift in v-*raf*) express v-*myc* from a subgenomic RNA (11). The J-5 virus expresses v-*myc* as a *gag-myc* gene fusion protein and contains no v-*raf*-related sequences (11).

Mice were inoculated within 48 hr of birth with recombinant viruses pseudotyped with Moloney, Cas-Br-M, 1504M, or C2S ecotropic MuLV or with 4070A amphotropic virus and observed for development of disease in relation to mice infected with the helper viruses alone (Fig. 2). Mice inoculated with J-3 or J-5 rescued with Moloney MuLV died between 42 and 133 days after infection with time courses of disease that were indistinguishable from that of mice infected with Moloney helper virus alone. By comparison, mortality in mice infected with Cas-Br-M or 4070A pseudotypes of J-3 and J-5 was greatly accelerated over that in mice infected with the helper viruses alone. More than 60% of mice infected with the pseudotyped constructs died before mortality induced by the helper viruses had reached 10%. Only one of the early deaths in mice infected with Cas-Br-M pseudotypes resulted from the development of typical Cas-Br-M-induced neurologic disease (30). Finally, for mice infected with C2S or 1504M pseudotypes of J-5, the time course of disease was accelerated 50–100 days over that induced by the helper viruses alone. These results demonstrate that the time course of disease in mice infected with J-3 and J-5 viruses is influenced by the helper viruses used to rescue them and corresponds well with the relative abilities of the helpers to replicate in tissues of mice infected as newborns (J.W.H., unpublished observations).

Histopathology of Tumors Developing in Mice Infected with Pseudotypes of J-3 and J-5. Gross and histologic examinations of tissues from 133 moribund mice infected with different pseudotypes of J-3 and J-5 revealed that 68% of the animals died with lymphoblastic lymphomas (LL), 18% with pancreatic or mammary adenocarcinomas, 7% with myelogenous or erythroleukemias, and 2% with fibrosarcomas (Table 1). These results differed in several respects from those obtained from 199 mice infected with the helper viruses alone. Perhaps most striking is the high frequency of epithelial tumors. Pancreatic adenocarcinomas are extremely rare spontaneous neoplasms of mice (31) and, in our experience with mice spontaneously producing MuLV at high levels or inoculated with a wide variety of replication-competent and replication-defective MuLV, have been observed previously only in mice infected with pseudotypes of J-2 (11, 12). Pancreatic tumors were first observed at 40 days after infection with J-3 or J-5 and developed with a mean latency of 150 days. Only mice infected with Moloney pseudotypes of the v-*myc* constructs did not develop this type of neoplasm. In general, these lesions and their progression resembled pancreatic tumors induced in rats by azaserine (32).

The three mammary adenocarcinomas developed at 152, 153, and 241 days after infection with Cas-Br-M and C2S pseudotypes of J-5 and are the only examples of this type of neoplasm seen in extensive studies of normal NFS/N mice or mice infected with a variety of retroviruses. Remarkably, the first two tumors developed in male mice and the third in a virgin female.

Over 90% of the J-3- or J-5-inoculated mice with hematopoietic tumors died with LL. The origins of these lymphomas from cells of the T- or B-lymphocyte lineages can be established with certainty by FMF assays employing a panel of antibodies to cell surface antigens (16, 19–21, 33). FMF studies of 35 LL showed that 25 were T-cell lymphomas, 8 were of pre-B- or B-cell origin, and two were mixtures of T and B cells or pre-B and B cells (data not shown).

Several lines of evidence suggest that the J-3 and J-5 constructs, rather than the helper MuLV, were causal in the development of these lymphomas. First, the frequencies of LL in mice infected with Cas-Br-M, C2S, and 1504M pseudotypes of J-3 and J-5 were increased over those for mice infected with the helper viruses alone (Table 1). Second, 4 of 12 LL induced by Moloney pseudotypes of J-3 and J-5 were pre-B- or B-cell lymphomas whereas Moloney MuLV alone induces exclusively T-cell lymphomas (ref. 34; H.C.M. III, unpublished observations). Finally, and most convincingly,

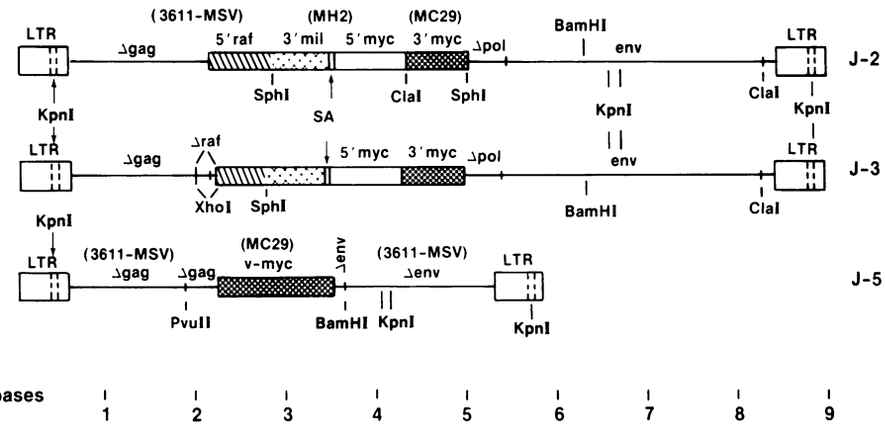

FIG. 1. Genomic organization of the construct viruses J-2, J-3, and J-5. Important restriction enzyme sites are indicated, and the sizes of the various viruses is shown in kilobases (kb). The origin of specific v-*onc* sequences in the constructs is indicated by specific shadings: ▧, 3611MSV v-*raf*; ▩, MH2 v-*mil*; □, MH2 v-*myc*; ▨, MC 29 v-*myc*. Splice acceptor sequences (SA) present in MH2 v-*myc* which allow expression of v-*myc* protein from a subgenomic mRNA (11) are indicated. LTR, long terminal repeat.

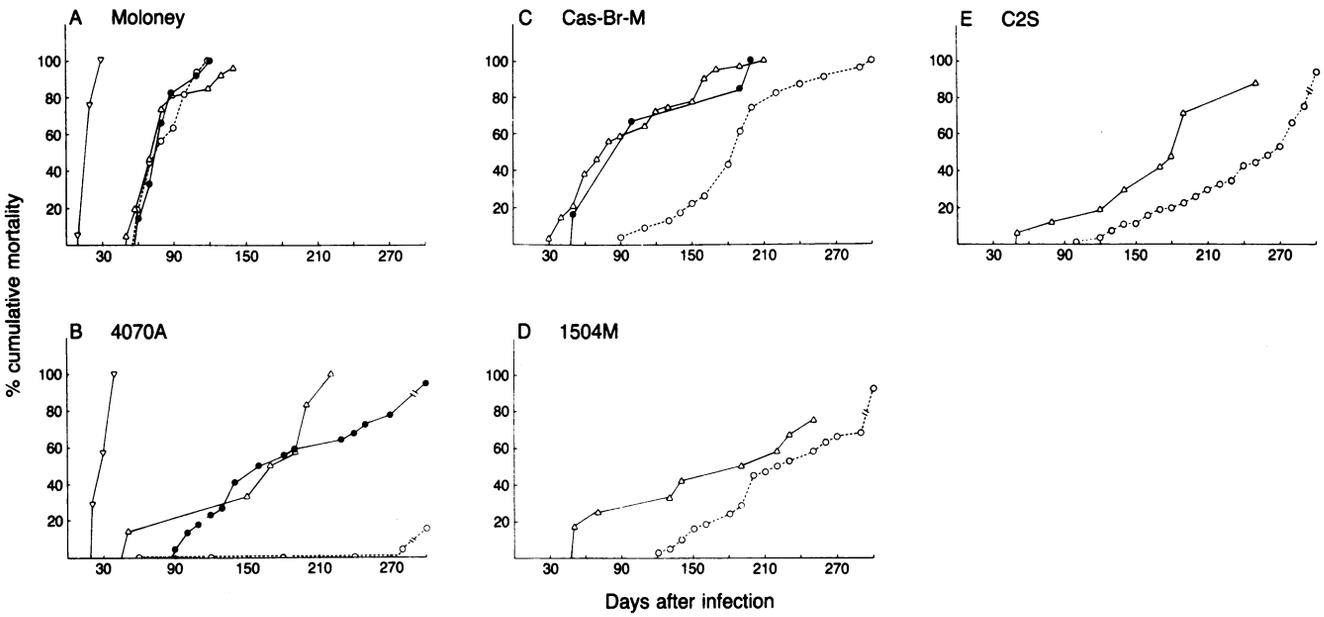

FIG. 2. Mice were inoculated with the following recombinant virus pseudotypes: Moloney (*A*), 4070A amphotropic virus (*B*), Cas-Br-M (*C*), 1504M (*D*), C2S (*E*). Cumulative mortalities of mice infected with helper MuLV (○), or helper pseudotypes of J-2 (▽), J-3 (●), or J-5 (△) MuLV.

molecular genetic studies of 15 T-, B-, and pre-B-cell LL showed that all had clonal integrations of v-*myc* (see below). We conclude that infection with J-3 and J-5 induces LL of T- and B-cell origin.

Ten percent of the mice infected with different pseudotypes of J-3 and J-5 developed myelogenous or erythroleukemias. Since some of the helper viruses induce these diseases at high frequencies and the leukemias have not been examined for v-*myc* integrations, the role of *myc* in transformation of these lineages is not clear.

Autonomous Growth *in Vitro* and *in Vivo* of Cells Transformed by v-*myc*. Spleen or lymph node cells with LL induced by pseudotypes of J-3 were cultured under standard conditions (19) in the absence of added growth factors. Continuous lines were obtained from two pre-B-cell lymphomas, one B-cell lymphoma, and one T-cell lymphoma that, by FMF tests, resembled the original tumors from which they were derived. Cultures from two other primary T-cell tumors yielded mast cell lines, while a macrophage line was recovered from a splenic LL not characterized by FMF. In addition, two pancreatic adenocarcinomas were readily transplanted to suckling syngeneic mice. All the *in vitro* lines were found to contain and express v-*myc* (see below).

Organization and Expression of v-*myc* in Primary Tumors and Continuous Cell Lines. DNA prepared from tumors induced by different pseudotypes of J-3 and J-5 was digested with *Kpn* I and *Bam*HI and examined by Southern blot hybridization with a v-*myc*-specific probe. Digestion with *Kpn* I generates internal v-*myc*-containing fragments of 5.8 kb from J-3 and 4.0 kb from J-5 (Fig. 1). *Bam*HI cuts once within *env* in both constructs such that v-*myc*-hybridizing fragments contain variable amounts of cellular sequences on the 5′ side of the virus (Fig. 1). Representative results of these studies showed that each of the primary tumors of hematopoietic or epithelial origin contained v-*myc*-hybridizing fragments consistent with the size of the virus used in their induction (Fig. 3*A*). In addition, all the primary tumors exhibited from one to four v-*myc* integrations (Fig. 3*B*). Analyses of two cell lines (792C, 793C) obtained from J-3-infected mice showed that they had the same v-*myc*

Table 1. Histologic diagnoses of neoplasms induced by pseudotypes of J-3 and J-5

				Percent of mice with histologic diagnosis*					
Construct	Rescuing virus	Mice inoculated, no.	% mortality	LL	Erythro-leukemia	Myelogenous leukemia	Pancreatic adenocar-cinoma	Mammary adenocar-cinoma	Fibro-sarcoma
None	Moloney	32	100	100 (32)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
J-3		12	100	100 (12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
J-5		26	95	100 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
None	Cas-Br-M	36	100	44 (16)	22 (8)	30 (11)	0 (0)	0 (0)	0 (0)
J-3		6	100	66 (4)	17 (1)	0 (0)	0 (0)	0 (0)	0 (0)
J-5		39	100	69 (27)	8 (3)	0 (0)	23 (9)	5 (2)	0 (0)
None	C2S	73	96	19 (14)	0 (0)	56 (41)	0 (0)	0 (0)	0 (0)
J-5		17	88	47 (7)	0 (0)	13 (2)	13 (2)	7 (1)	0 (0)
None	1504M	38	92	8 (3)	55 (21)	0 (0)	0 (0)	0 (0)	0 (0)
		12	75	44 (4)	11 (1)	0 (0)	11 (1)	0 (0)	0 (0)
None	4070A	20	15	100 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
J-3		22	95	45 (10)	9 (2)	0 (0)	32 (7)	0 (0)	0 (0)
J-5		6	100	33 (2)	0 (0)	16 (1)	33 (2)	0 (0)	16 (1)
Totals for J-3 and J-5		140	95 (133)	68 (91)	5 (7)	2 (3)	16 (21)	2 (3)	2 (2)

*Numbers in parenthesis indicate the number of autopsied mice in each diagnostic category.

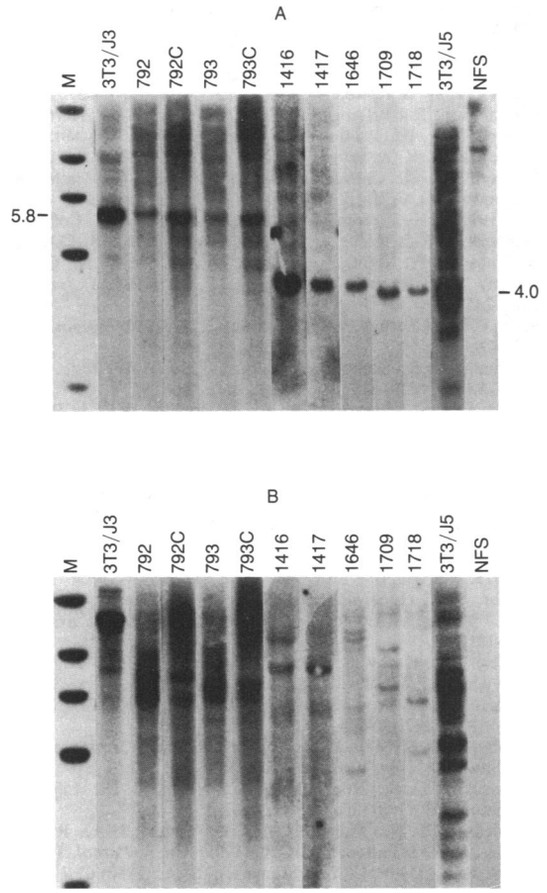

FIG. 3. Southern blot hybridization of cellular DNA digested with *Kpn* I (*A*) or *Bam*HI (*B*) with an avian v-myc probe. Tumors and continuous *in vitro* cell lines indicated by "C" derived from them include two pre-B-cell lymphomas (792, 793) induced by J-3/Moloney, two lymphoblastic lymphomas (1416, 1417), a pancreatic adenocarcinoma (1646), a mammary adenocarcinoma (1718) induced by J-5/Cas-Br-M, and a pancreatic adenocarcinoma (1709) induced by J-5/4070A. Lane M contains molecular size markers. Positions of 5.8 kb and 4.0 kb are indicated.

integrations as the primary tumors from which they were cultured (Fig. 3*B*).

Poly(A)$^+$ RNA prepared from some of the lymphoid and epithelial tumors induced by pseudotypes of J-5 were analyzed by RNA gel blot hybridization with probes specific for v-*myc*, c-*myc*, and c-*raf* (Fig. 4). All the tumors expressed high levels of v-*myc*-containing transcripts of 5.1 and 4.1 kb. In contrast, none of the tumors expressed the normal 2.3 kb c-myc message (Fig. 4*B*). Finally, all the tumors expressed c-raf transcripts to varying extents (Fig. 4*C*). The levels of c-raf messages were significantly higher in the lymphoid than in the epithelial tumors.

DISCUSSION

The data from this report show that deregulated expression of *myc* from integrated murine retroviruses containing avian v-*myc* is critical to the induction of a variety of hematopoietic and epithelial neoplasms. The observation that most of the mice died with LL of T- or B-cell origin is consistent with numerous earlier studies indicating a central role for altered *myc* expression in the development of T and B lineage neoplasms in chickens, mice, humans, and cats (1–8, 35–42). By comparison, there are only single reports of pancreatic tumors developing in chickens infected with the MH2 virus (7) and the development of mammary tumors in transgenic mice carrying a c-*myc* gene under the regulation of mammary tumor virus long terminal repeats (43).

The findings that these tumors developed with relatively long latent periods and were mono- or oligoclonal suggest that altered expression of *myc* is insufficient to cause directly the fully transformed phenotype. This suggestion is consistent with clinical (44) and experimental data (9, 10) indicating that two or more alterations of normal cells are required for transformation. The nature of the second events required to complement *myc* in transformation is not known, but may include the activation of other oncogenes. This possibility is supported by our earlier studies demonstrating that the effect of combining *myc* and *raf* into a single retrovirus was to greatly accelerate the time course of disease over that observed for mice infected with viruses containing *raf* and *myc* alone (ref. 11 and this study). Importantly, the data presented here establishes that the range of cell types targeted for transformation by the *raf/myc* construct represents a simple summation of those affected by each oncogene on its own.

The wide range of murine cells targeted for transformation by v-*myc* contrasts with the more limited spectrum of cells affected by other oncogene-transducing MuLV, most of which induce fibrosarcomas and erythroblastosis (e.g., *raf, ras*) or a limited spectrum of hematopoietic tumors (e.g., *abl*). The basis for these differences is not known but may be related to the position of a particular oncogene in the signal transduction pathway of specific growth factors. For example, an oncogene derived from a ligand gene may be restricted to transformation of cells that normally express the corresponding receptor whereas nuclear oncogenes such as *myc* may act as central information relays that provide many cell

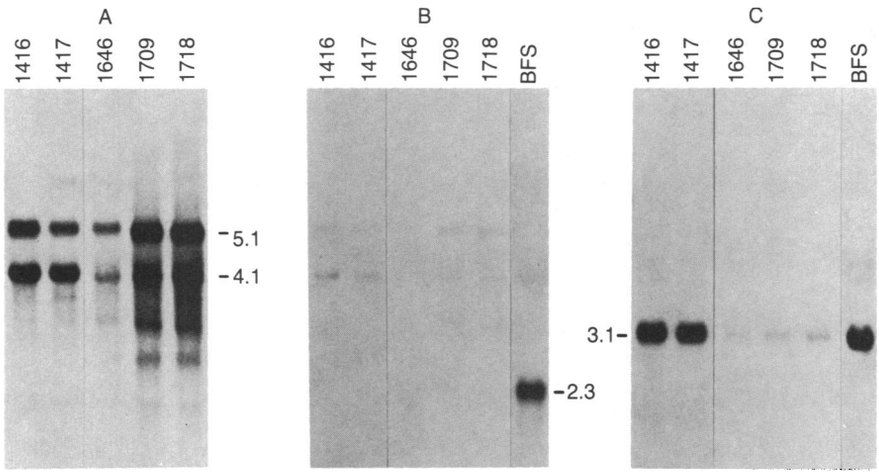

FIG. 4. RNA gel blot hybridizations of poly(A)$^+$ mRNA prepared from some of the primary tumors described in Fig. 3 and a interleukin 2-independent murine T-cell line (BFS) with probes specific for avian v-*myc* (*A*), murine c-*myc* (*B*), and human c-*raf* (*C*). Sizes of mRNAs in kb are indicated.

types with a growth stimulus (43, 44). In the context of our studies, the observations that *raf* induces immortalization of cells that remain factor-dependent whereas cells infected with v-*myc* become factor-independent for growth (45, 46), indicates that these genes differentially affect components of competence and cell cycle progression.

Our data and that of others indicate that the range of cell types susceptible to transformation as a consequence of aberrant *myc* expression is probably substantially greater than that indicated by our studies of primary tumors. We have established mast cell and macrophage cell lines from tissues of mice with no histologic evidence for tumors of these lineages and mice transgenic for c-*myc* have developed mastocytomas and Sertoli cell tumors (43). In addition, Potter *et al.* (personal communication) have shown that pristane-primed BALB/c mice infected with J-3 develop plasmacytomas. Maintenance of normal regulatory controls for the expression of c-*myc* is thus clearly of major importance to the growth of many different cell lineages and for cells at distinct states of differentiation within a single lineage.

It should also be noted that the differences between *raf* and *myc* for target cell specificities cannot be explained by viral regulatory sequences contained within the long terminal repeats as they were identical in all the constructs used in this study and our earlier report (11).

We thank Dr. Kevin Holmes for his assistance in some of the FMF studies, Dr. Fred Muskinski for a critical review of the manuscript, and Patricia Borchet for her expertise in the preparation of virus stocks. R.A.Y. is a Special Fellow of the Leukemia Society of America. This work was supported in part by contract NOI-AI-22673 at Microbiological Associates, Inc., Bethesda, MD.

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