JOURNAL OF VIROLOGY, Jan. 1989, p. 205–215
0022-538X/89/010205-11$02.00/0

Vol. 63, No. 1

Properties of a Murine Retroviral Recombinant of Avian Acute Leukemia Virus E26: a Murine Fibroblast Assay for v-*ets* Function

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Received 14 July 1988/Accepted 30 September 1988

A replication-defective murine retroviral construct, termed pME26, was generated by inserting avian *gag-myb-ets* sequences derived from the cloned avian acute leukemia virus E26 into an Abelson murine leukemia virus-derived retroviral vector. ME26 virus can be rescued efficiently from transfected NIH 3T3 cells by replicating murine leukemia viruses. Either pME26-transfected nonproducers or ME26 virus-infected NIH 3T3 cells expressed a 135-kilodalton fusion protein (p135) which was detectable by immunoprecipitation with antiserum directed against avian leukemia virus $p27^{gag}$, *myb* or *ets* oncogene protein, or murine leukemia virus $p15^{gag}$ and was principally localized in the nucleus. NIH 3T3 cells infected with ME26 exhibited morphological alterations and increased proliferation in reduced serum and formed small colonies in agar suspension. Discrete foci could be readily recognized in cells maintained in a defined medium containing 0.03 to 0.1% calf serum. In newborn NFS/N mice, ME26 induced a significantly higher mortality and incidence of erythroid and myeloid leukemias. Analysis of a series of mutants affecting the expression of various portions of p135 indicated that the v-*ets* gene acts to mitogenically stimulate the proliferation of NIH 3T3 fibroblasts and reduces or abolishes their serum dependence. These properties provide an assay system to study functions of the *ets* gene family.

The avian acute leukemia virus E26 is a defective avian retrovirus which principally induces erythroleukemia, with some myeloid leukemia, in infected birds (30, 37). The virus can transform cells of both erythroid and myeloid lineages in vitro (31, 37), and it has been suggested that the virus acts to transform an initially uncommitted hematopoietic precursor stem cell (31). E26 virus has also been shown to morphologically transform primary quail fibroblasts (20) and to morphologically alter and mitogenically stimulate chicken primary fibroblasts in tissue culture (25).

Molecular analysis of the cloned E26 provirus (16, 27, 33, 46) has shown that the virus contains truncated versions of two cellular genes, *myb* and *ets*, fused to each other and to a portion of the *gag* sequences of avian leukemia virus. This gene fusion encodes a 135-kilodalton (kDa) fusion protein made up of portions of avian $p27^{gag}$ and *myb* and *ets* oncogene proteins (3, 6, 9), which has been localized in the nuclei of infected avian myeloblasts (9, 26). An overlapping subset of c-*myb* sequences has been independently captured by the avian myeloblastosis virus (19), which induces only myeloblastosis in infected birds (29). The *ets* sequences, however, are unique to E26, and this fact, coupled with studies of conditional lethal mutants of E26, has suggested that v-*ets* sequences are responsible for both the fibroblast and erythroid transforming potential of the virus (4).

Analysis of the cellular homologs of v-*ets* has identified a family of well-conserved, related genes (33, 38, 40, 49; D. Watson, manuscript in preparation), of which the gene designated *ets*-1 (49) is the likely cellular precursor of the viral gene (15). However, *ets*-1 and v-*ets* share extensive sequence and amino acid homology with both *ets*-2 (8, 48, 49) and *erg* (38, 40), particularly at the carboxy terminus of the proposed c-*ets* gene products (15, 47). The *ets* gene family appears to represent a group of related genes whose expression is independently regulated in both a developmental and tissue-specific fashion (5, 10, 17, 18, 35) and whose chromosomal localization has suggested possible involvement in both human leukemia (12, 41) and other nonmalignant diseases (N. Sacchi, J. Nalbantoglu, F. R. Sergovich, and T. S. Papas, Proc. Natl. Acad. Sci. USA, in press).

The high degree of evolutionary conservation of the *ets* genes (11, 36) suggested that the oncogenic potential of v-*ets* was probably not confined to avian species. Therefore, we constructed a murine viral recombinant of the avian E26 virus to measure its oncogenic potential in murine cells and mice and to enable us to take advantage of the well-characterized aspects of murine hematopoiesis and development in order to study the role of v-*ets* and the other portions of the E26 genome in oncogenesis. This report describes the construction and initial characterization of the virus, which we have termed ME26, and of several mutant derivatives of that virus. Our results demonstrated that ME26 behaves as a stable murine retrovirus and is able to induce leukemia when it is injected into newborn mice; the virus also alters the growth properties of infected or transfected NIH 3T3 mouse fibroblasts and induces foci of morphologically altered cells with increased ability to proliferate in the absence of serum growth factors. Our data indicated that the v-*ets* product is responsible for this mitogenic effect, and is the first direct demonstration of a biological assay for an *ets*-specific function.

MATERIALS AND METHODS

Cells and viruses. The NIH 3T3 cells used in this study have been described previously (7). The cells were grown in Dulbecco modified minimum essential medium with 5% calf serum or, in low-serum experiments, in the defined medium QBSF-51 which, with the addition of epidermal growth factor, will support growth of normal NIH 3T3 cells (R. Brown, Quality Biological). The amphotropic murine leukemia viruses, 4070A and 1504A, were kindly provided by T. Robins and were described by Hartley et al. (22), and the ecotropic murine Moloney leukemia virus (Mo-MuLV) was derived from NIH 3T3 cells transfected with pMOV3, a

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cloned Mo-MuLV provirus, which has also been described previously (24).

DNA transfection of NIH 3T3 cells was performed by the calcium phosphate precipitation technique (7). The plasmid pSV2neo (44) was cotransfected at a ratio of 0.1 μg of pSV2neo to 2 μg of recombinant pME26 (see below), and colonies resistant to G418 (Geneticin, 400 μg/ml; GIBCO Laboratories) colonies were isolated after 14 days of incubation and grown as individual cultures. For virus infection, 10^5 cells were seeded in 60-mm-diameter plates and infected with 0.1 ml of viral supernatant in growth medium containing 4 μg of Polybrene per ml. After overnight incubation, the medium was changed to QBSF-51 containing low concentrations of serum. Plates were incubated at 37°C for 14 to 21 days with changes of medium every 2 to 4 days, then fixed in 70% methanol, and stained with Giemsa stain. Foci of densely growing cells were enumerated with a microscope. ME26 virus was rescued from nonproducer cells which had been transfected with pSV2neo and pME26. Cells were trypsinized, reseeded at a 1:10 dilution, and split once when they reached confluence; the virus-containing supernatants were harvested 72 h later. To measure the colony-forming ability of transfected or infected NIH 3T3 cells in semisolid medium, cells were grown in medium containing 0.24% agarose and 10% calf serum and overlaid with agarose once a week, and colonies were observed after a period of 30 days.

Construction of ME26 provirus. The pE26 plasmid DNA containing the entire avian acute leukemia retrovirus E26 proviral genome was kindly provided by P. Duesberg (32). The vector, K29, was constructed and kindly provided by E. P. Reddy. It is derived from the proviral genome of Abelson murine leukemia virus cloned in plasmid pBR322 (39) and contains a single *Eco*RI site located 34 amino acids downstream from the murine *gag* ATG initiation codon. To construct ME26 DNA, the 3.4-kilobase (kb) *Bam*HI-*Stu*I fragment of pE26 (Fig. 1) that contains the v-*myb* and v-*ets* sequences and portions of avian *gag* and *env* derived from E26 was purified and the *Bam*HI site was converted to a blunt end with appropriate nucleotides and *Escherichia coli* polymerase I. To protect the *Eco*RI site in the v-*myb* region, this DNA fragment was methylated by *Eco*RI methylase (New England BioLabs, Inc.) prior to the addition of a 12-mer *Eco*RI linker (Collaborative Research, Inc.) by using T4 ligase. To create cohesive termini, this fragment was then digested with *Eco*RI and inserted into the *Eco*RI site of plasmid K29. Recombinant clones were selected by in situ colony hybridization using a v-*ets*-specific probe (*Bam*HI-*Stu*I fragment) derived from pE26. A positive colony containing the *Bam*HI-*Stu*I fragment inserted in the proper orientation was selected for further characterization. The junction between the murine *gag* of K29 and the avian *gag* of pE26 was sequenced by the chain terminator method (42) with double-stranded DNA and a synthetic oligonucleotide as described by Zagursky et al. (50). DNA sequence in this region demonstrated that the open reading frame of the murine *gag* p15 was contiguous with that of the *Bam*HI-*Stu*I fragment of pE26 (data not shown). This clone, termed pME26, was subsequently used for the generation of ME26 virus.

Construction of ME26 mutants. To construct mutant A, pME26 was partially digested with *Eco*RI and treated with mung bean nuclease to restore the reading frame prior to ligation. This deleted a 1-kb fragment containing the entire avian Δ*gag* and about 300 base pairs of 5′ *myb* gene. B mutant was constructed by deleting a 300-base-pair (bp) *Hpa*I-to-*Bgl*II fragment within the v-*ets* gene. The open reading frames of both these two mutants were confirmed by sequence analysis. Mutant C was constructed by filling in the *Bgl*II site in the v-*ets* gene with *E. coli* polymerase Klenow fragment to generate a 4-base insertion resulting in a frameshift.

Southern blot analysis. DNA was isolated from cell lines and tissues as previously described (7). High-molecular-weight DNA (10 μg) was digested with restriction enzymes, electrophoresed in a 0.8% horizontal agarose gel, and transferred to a nitrocellulose filter by the technique of Southern (43). Filters were hybridized with nick-translated ^{32}P-labeled DNA (10^7 cpm/ml) under stringent conditions (50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1× Denhardt, 20 mM $NaPO_4$ [pH 6.5], 0.1% sodium dodecyl sulfate [SDS]) at 42°C for 40 to 48 h and washed in 0.2× SSC–0.1% SDS at 65°C for 1 h. The hybridizing fragments were visualized by autoradiography with XAR-5 film (Eastman Kodak Co.).

Metabolic labeling, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis. Subconfluent NIH 3T3 cells were starved in methionine-free minimum essential medium (GIBCO) for 30 min and then labeled with [^{35}S]methionine (Dupont, NEN Research Products) for 1 h at 200 μCi/ml. Labeled cells were scraped from flasks and washed four times in ice-cold phosphate-buffered saline. Cells were lysed in RIPA buffer (50 mM Tris [pH 7.5], 0.5 M NaCl, 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS), sonicated for 10 s, and centrifuged at 40,000 rpm on a TY50 rotor (Beckman Instruments, Inc.) at 4°C for 1 h. Supernatant samples containing equal trichloroacetic acid-precipitable counts were used for immunoprecipitation assay. Antisera used for immunoprecipitation were swine anti-avian myeloblastosis virus p27 (Microbiological Associates), goat anti-AKR leukemia virus p15 (Microbiological Associates), mouse anti-*myb* oncogene protein (13), and rabbit anti-*ets* oncogene protein polypeptide ZC50 (34). Competition experiments were performed by preincubating anti-ZC50 with the ZC50 peptide for 2 h at 4°C prior to the addition of the labeled extract. Immunoprecipitates were recovered with the aid of protein A-Sepharose CL4B (Sigma Chemical Co.) and analyzed by 10% SDS-polyacrylamide gel electrophoresis.

Subcellular fractionation. The procedures used for subcellular localization are basically the same as described previously (26); all operations were performed at 0°C. Briefly, the [^{35}S]methionine- or [^{35}H]myristic acid-labeled cells were gently lysed by suspending them in a low-salt buffer containing 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 6.8], 5 mM KCl, and 1 mM $MgCl_2$ for 15 min and disrupting them by 20 to 30 strokes in a tightly fitting Dounce homogenizer. Nuclei were separated from the cytoplasmic and membrane fractions by sedimentation at 1,000 × *g* for 15 min. Nuclei were washed twice in low-salt buffer containing 0.5% Nonidet P-40 and 0.25% sodium deoxycholate, and the supernatants were combined and designated the nuclear wash fraction. The washed nuclei were lysed in RIPA buffer by 10 s of sonication, centrifuged at 40,000 rpm for 30 min, and the supernatant was designated the nuclear fraction. The cytoplasmic fraction was separated from the membrane fraction by centrifugation at 40,000 rpm for 1 h, and the pellet of the membrane fraction was resuspended in RIPA buffer. The cytoplasmic and nuclear wash fractions were adjusted to the same salt and detergent concentrations as those of RIPA buffer. All the subcellular fractions were analyzed by immunoprecipitation of equal radioactive

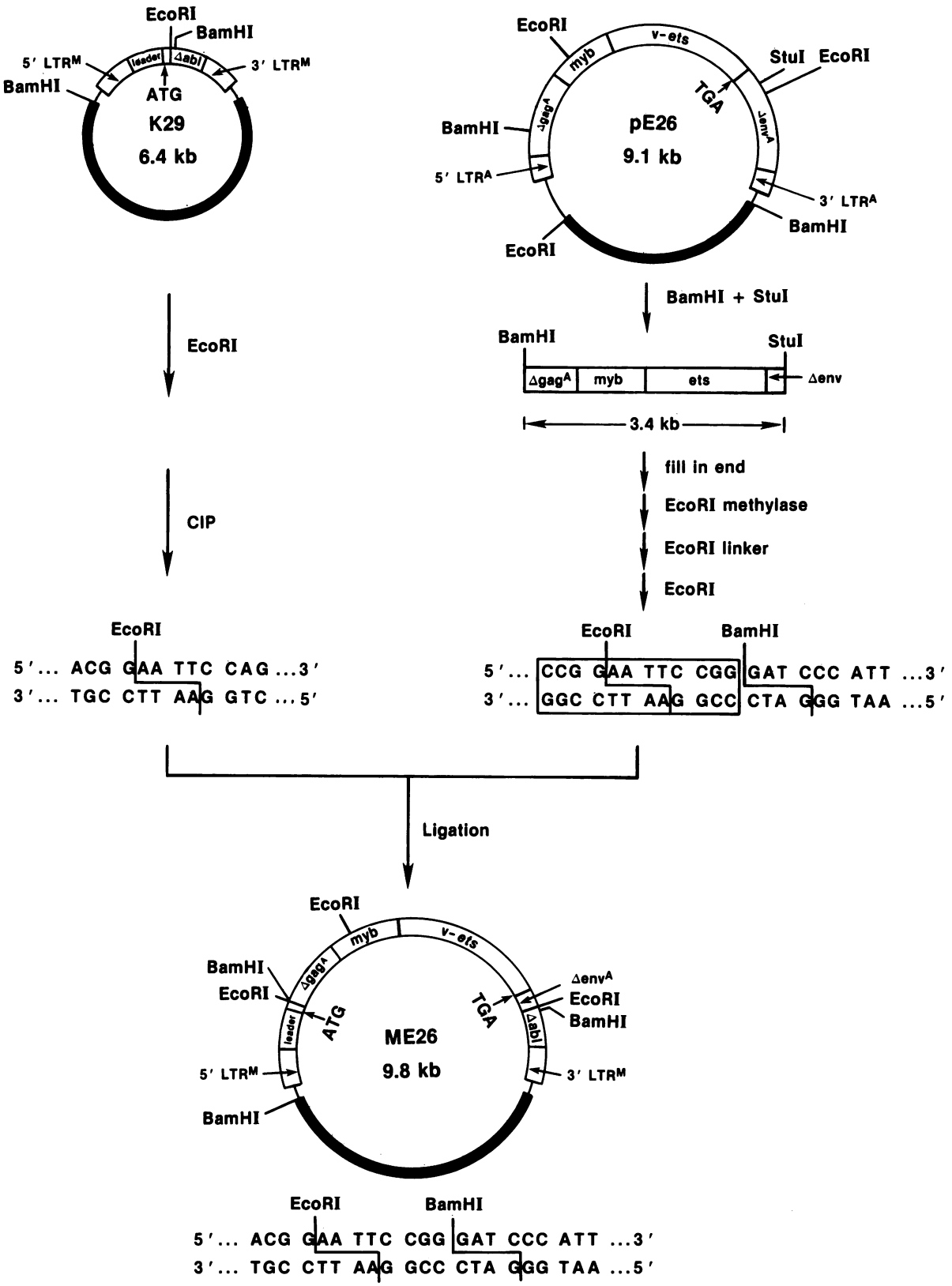

FIG. 1. Schematic of construction of the ME26 retroviral recombinant. A 3.4-kb *Bam*HI-*Stu*I fragment from the avian E26 virus was inserted in-frame into a vector (K29) derived from Abelson MuLV. Sequences of junctions are shown. The solid bars indicate the pBR vector.

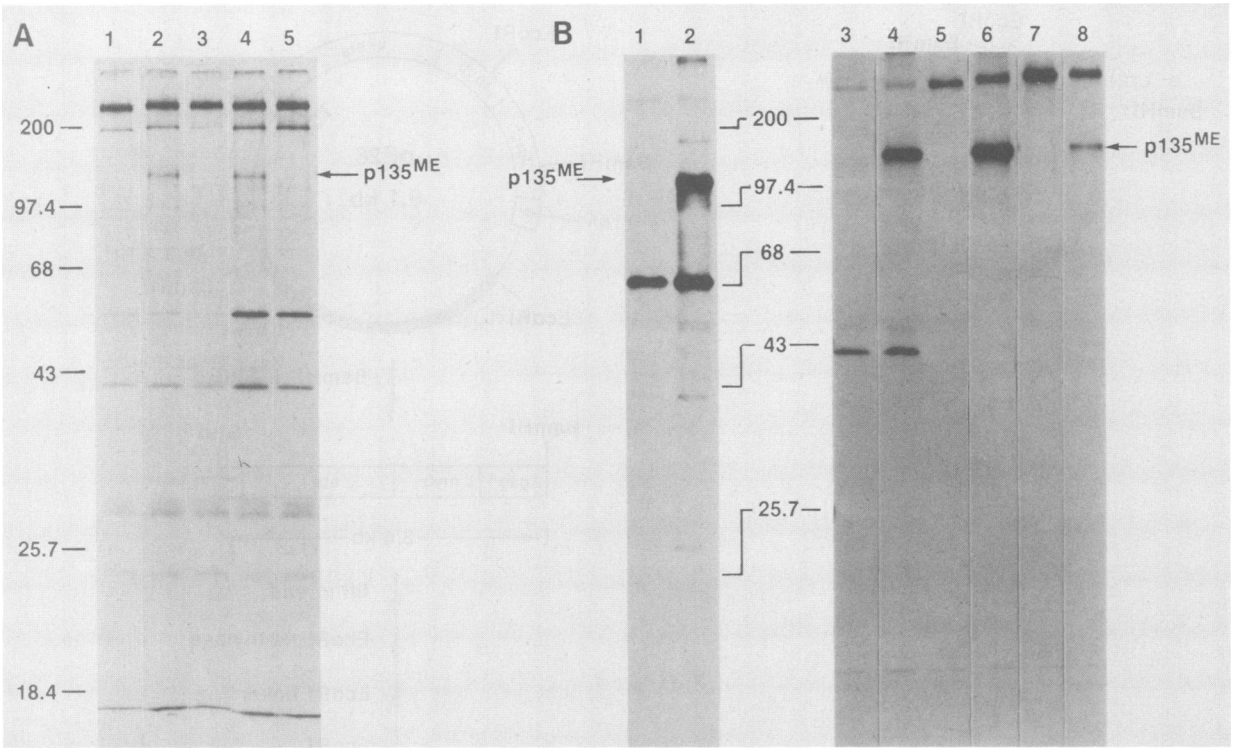

FIG. 2. Immunoprecipitation analysis of ME26-encoded p135^{ME}. (A) [^{35}S]methionine-labeled proteins from two single G418-resistant colonies transfected with pME26 (lanes 2 through 5) or pSV2neo alone (lane 1) were immunoprecipitated with anti-*ets* peptide antibody. In lanes 3 and 5, immunoprecipitation of antigen was inhibited by using the homologous ZC50 peptide. (B) [^{35}S]methionine-labeled proteins from NIH 3T3 cells infected with ME26 (lanes 2, 4, 6, and 8) or with 4070A helper alone (lanes 1, 3, 5, and 7) were immunoprecipitated with antisera against murine p15 (lanes 1 and 2), avian p27 (lanes 3 and 4), *myb* oncogene proteins (lanes 5 and 6), and *ets* oncogene ZC50 peptides (lanes 7 and 8). The positions of molecular size markers (in kilodaltons) are shown at the sides of the gels.

counts and polyacrylamide gel electrophoresis described above.

Animal inoculation. NFS/N mice were obtained from the animal production facility of the Frederick Cancer Research Facility. Newborns (72 h) were injected intraperitoneally with 0.1 ml of virus supernatants. Weanlings were injected intraperitoneally, intravenously, or subcutaneously. Animals showing any sign of distress or poor health were sacrificed for examination. Multiple organs, including lung, thymus, spleen, liver, kidney, and a short section of spinal vertebra, were fixed in Formalin for histopathological diagnosis (R. M. Kovatch, Laboratory of Pathology and Histology, Frederick Cancer Research Facility), and abnormal tissue samples were frozen for DNA analysis.

RESULTS

Construction and generation of recombinant ME26 retrovirus. The construction of the pME26 plasmid recombinant is shown schematically in Fig. 1. This construct was introduced into NIH 3T3 cells along with the plasmid pSV2neo, and G418-resistant colonies were selected. DNA analysis revealed that 13 of 18 G418-resistant colonies contained multiple integrated copies of pME26 (data not shown). Three pME26-transfected colonies and one pSV2neo-transfected colony were selected for further characterization. pME26-transfected colonies contained the predicted 3.4-kb *Bam*HI fragment (data not shown) that hybridized to a v-*ets*-specific probe. The colony-derived cell lines were subsequently infected with Mo-MuLV, 4070A, or 1504A murine leukemia virus (MuLV). Rescued virus present in the cell supernatants was harvested and viral RNA was extracted as described in Materials and Methods. Northern blot analysis of this RNA demonstrated the presence of a 5.4-kb *ets*-containing viral genome (data not shown). This size is consistent with that expected from the pME26 construct and suggests that no gross alterations of the genome of ME26 had occurred during DNA transfection and virus rescue.

Analysis of ME26-specific protein. Avian retrovirus E26 encodes a single transforming protein of 135 kDa (p135) with the structure p27^{gag}-*myb* oncogene protein-*ets* oncogene protein. The pME26 construct shown in Fig. 1 contains an open reading frame which can encode a protein product of similar size (designated p135^{ME}) containing four retroviral and oncogenic domains. The N terminus consists of 34 amino acids of murine p15^{gag}, followed by avian p19^{gag}, p10^{gag}, Δp27^{gag}, and the entire v-*myb* and v-*ets* oncogene proteins derived from E26. The carboxy terminus is the same as that of E26 p135. When ^{35}S-labeled extracts of pME26-transfected cell lines were immunoprecipitated with an anti-*ets* peptide antibody (ZC50; antibody B [34]), a 135-kDa protein was detected (Fig. 2A, lanes 2 and 4). This protein was not observed in pSV2neo-transfected control cells (Fig. 2A, lane 1) or in cells infected with amphotropic helper virus alone (Fig. 2B, lanes 1, 3, 5, and 7). Precipitation of p135^{ME} was blocked by the synthetic peptide used to induce ZC50 (Fig. 2A, lanes 3 and 5). A 135-kDa protein

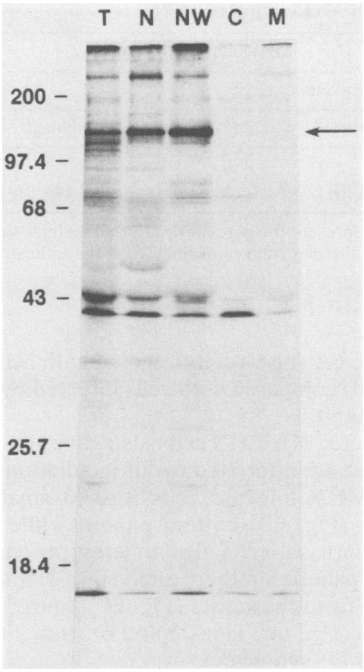

FIG. 3. Subcellular localization of p135^{ME}. Subcellular fractions of [^{35}S]methionine-labeled proteins of ME26-transfected NIH 3T3 cells were immunoprecipitated with swine anti-avian p27 serum. Abbreviations: T, total fraction; N, nuclear fraction; NW, nuclear wash fraction; C, cytoplasmic fraction; M, membrane fraction. The position of p135^{ME} is indicated by the arrow. The positions of molecular size markers (in kilodaltons) are shown at the sides of the gels.

could also be immunoprecipitated with antibodies against murine p15^{gag} (Fig. 2B, lane 2), avian p27^{gag} (Fig. 2B, lane 4), and *myb* oncogene protein (Fig. 2B, lane 6). Subcellular fractionation experiments (Materials and Methods) indicated that p135^{ME} was predominantly localized within or associated with the nucleus (Fig. 3). p135^{ME} could be washed from the nuclear fraction with a low-salt buffer containing 0.5% Nonidet P-40 and 0.25% sodium deoxycholate (Fig. 3, lane NW), and a small amount of protein was also present in the membrane fraction (lane M).

Biological function of ME26 in vivo. ME26 did not induce either solid tumors or acute leukemia when introduced either intraperitoneally, intravenously, or subcutaneously into adult mice (data not shown), while newborn NFS/N mice injected intraperitoneally with ME26 pseudotypes showed a high mortality and incidence of various leukemias. Mice injected with ME26 rescued by Mo-MuLV died between 63 and 112 days after infection, with disease time courses that were indistinguishable from those of mice injected with Mo-MuLV alone (Fig. 4). However, time courses of disease were accelerated almost 100 days in ME26-1504A-injected mice (compared with mice injected with equal titers of the poorly leukemogenic 1504A virus alone). Figure 4 also indicates that about 50% of mice injected with the amphotropic pseudotype of ME26 died within 200 days, while only a single mouse injected with the helper virus alone died within the same time frame. Gross necropsy observation of ME26-inoculated animals showed enlarged spleens and livers in 83 and 66% of the cases, with abnormal thymus glands, lungs, or kidneys observed in many animals.

Pathological diagnoses of diseased mice from several other experiments are shown in Table 1. Of 158 mice, 76 (48%) injected with the 4070A amphotropic pseudotype of ME26 died or developed symptoms of acute disease within 180 days of injection. Of the 46 animals subject to pathological analysis, 55% were diagnosed as having myeloid or erythroid leukemia, while 34% showed lymphoblastic lymphomas. The leukemias were seen most frequently in animals exhibiting the shortest disease latency. In contrast, only 6 of 53 mice (11%) injected with the 4070A helper alone developed disease symptoms and all of the mice analyzed exhibited lymphoblastic lymphoma.

DNA was isolated from spleens of animals which had been injected with amphotropic pseudotypes of ME26. All sam-

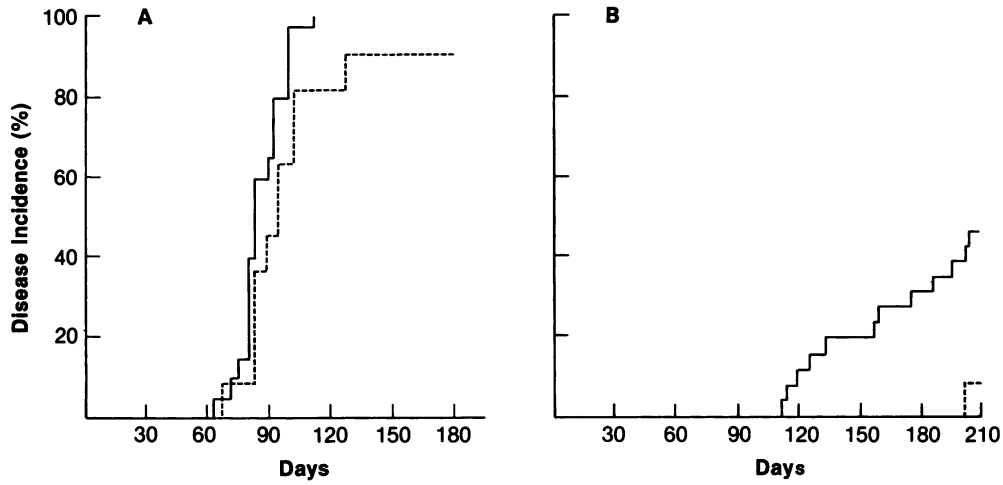

FIG. 4. Disease incidence in ME26-infected newborn NFS/N mice. NFS/N mice were injected within 72 h of birth with 0.1 ml of supernatant from ME26-transfected cells infected with either ecotropic Mo-MuLV (A) or amphotropic 1504A MuLV (B). The titers of the inoculated ME26 and helpers are approximately 10^3 and 10^6 focus-forming units, respectively. ME26 was assayed on NIH 3T3 cells as described in Materials and Methods. Helper virus was assayed on FG10 as previously described (2). (A) A total of 20 mice were infected with ME26 and Mo-MuLV (———), and 11 mice were infected with Mo-MuLV alone (- - - - -). (B) A total of 26 mice were infected with ME26 and 1504A (———), and 11 mice were infected with 1504A alone (- - - - -).

TABLE 1. Histopathological diagnoses of neoplasms in ME26-injected mice

Virus	Total no. of animals injected	Animal mortality[a]	No. of animals subjected to pathological diagnosis	Pathological diagnosis[b]				
				Leukemia			Lymphoma	
				Myeloid	Erythroid	Undesignated	Malignant	Lymphoblastic
4070A	53	6 (11)	3	0	0	0	0	3 (100)
ME26 + 4070A	158	76 (48)	46	16 (35)	9 (20)	1 (2)	2 (4)	18 (39)

[a] The numbers of animals sacrificed or found dead within 180 days. The values in parentheses represent the percent dead of all animals injected.
[b] Data shown are the numbers of mice with specific diagnoses. The values in parentheses are the percentages of mice with the indicated diagnosis of all diagnosed animals.

ples (8 of 8) from leukemic animals showed integrated v-*ets* sequences, as did 2 of 3 samples from animals which had developed lymphoblastic lymphomas. This observation supported the hypothesis that ME26 is causally related to the diseases observed in ME26-infected mice. Southern analysis revealed the expected 3.4-kb *Bam*HI fragment which hybridized to an *ets*-specific probe (Fig. 5A). Analysis of DNA digested with *Bst*EII (Fig. 5B), which cut once within the ME26 viral sequences, indicated that multiple integrated copies of the v-*ets*-containing viral sequences were present in three of the four samples. In one mouse (Fig. 5, lanes 5 and 6), the same hybridization pattern was clearly observed in both the spleen and liver samples, suggesting that the progeny of a single infected cell had spread to multiple sites. However, in the tissue samples from the second mouse (Fig. 5B, lanes 2 and 3), the integration pattern suggests the occurrence of multiple events.

Biological effects of ME26 in vitro. Infection of NIH 3T3 cells with ME26 resulted in the appearance of discrete foci of cells densely overgrowing the monolayers. These foci were poorly defined in cells growing in 5 to 10% calf serum but could be readily recognized in cultures growing in low serum. Figure 6A shows cells infected with ME26 and 4070A (bottom panel) compared with cells infected by 4070A helper alone (top panel).

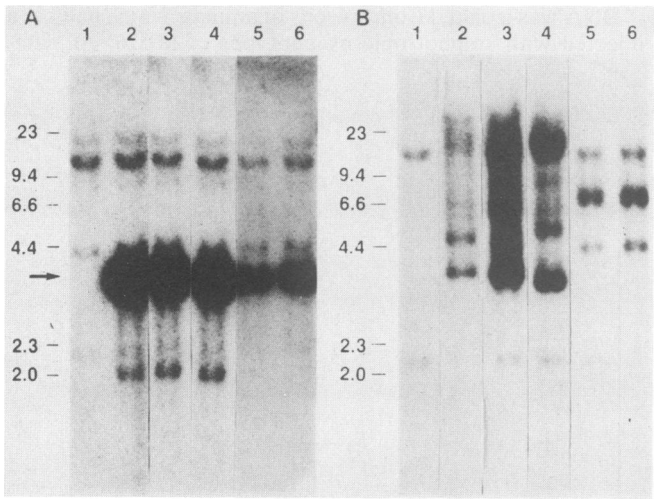

FIG. 5. Southern analysis of spleens and livers from leukemic mice infected with ME26. Spleen DNA (lanes 1, 2, 4, and 5) and liver DNA (lanes 3 and 6) were extracted from four leukemic mice injected with amphotropic pseudotype ME26. In two cases, lanes 2 and 3 and lanes 5 and 6, spleen and liver samples were obtained from the same mouse. DNA was digested with *Bam*HI (A) or *Bst*EII (B) and hybridized with an *ets*-specific probe (E1.28 [46]). The arrow indicates the 3.4-kb *ets*-specific fragment of *Bam*HI digestion. The positions of molecular size markers (in kilobases) are shown to the left of the gels.

ME26-infected NIH 3T3 cells also gave rise to colonies in semisolid medium after 30 days of incubation with periodic refeedings. ME26-infected cells formed small or medium-size colonies (Fig. 6B, bottom panel), while cells infected with helper virus alone failed to grow in agar suspension (Fig. 6B, top panel). Southern analysis of DNA isolated from cells derived from these foci (Fig. 6C, lanes 1 through 4) or agar colonies (Fig. 6C, lanes 5 and 6) show the presence of virus-derived *ets* sequences.

We observed that focus formation of ME26 on NIH 3T3 cells was dependent on serum concentration and that ME26-infected NIH 3T3 cells exhibited a decreased serum requirement. ME26-infected cells proliferated and formed foci in serum concentrations from 0.01 to 0.2%, with an optimum between 0.03 to 0.1% (Fig. 7). Focus formation was reduced, however, at serum concentrations higher than 0.2%. Table 2 shows the focus formation by cells infected with serial dilutions of ME26-4070A and grown in QBSF-51 medium with 0.1% calf serum. The foci number was approximately proportional to the virus dose and indicated that the titer of this stock was about 4×10^4 focus-inducing units per ml. The level of helper virus in this stock was measured at 10^6 in S^+L^- assays (2). The addition of helper virus at a multiplicity of infection of 0.1 to 0.2 did not enhance the efficiency or the rate of focus formation, while a reduction in focus titers (data not shown) was observed at higher multiplicities of helper virus, presumably as a result of viral interference.

Intact v-*ets* expression is required for NIH 3T3 focus formation. In order to determine the regions of the ME26 genome responsible for the induction of foci on NIH 3T3 cells, we constructed a series of mutants (Fig. 8) and tested their biological activity. Each construct was transfected into NIH 3T3 cells together with pSV2neo, and portions of the resultant pools of G418-resistant colonies were replated in low serum to assay for focus formation. In addition, cells from each pool were infected with amphotropic MuLV to rescue the mutant virus, and the viral supernatants were then tested for their ability to induce foci on NIH 3T3 cells. Both transfected cells and cells infected with rescued viruses gave qualitatively identical results. Cells transfected with the K29 vector (Materials and Methods) or pSV2neo alone failed to form foci in low serum, and no focus-inducing virus could be rescued from these cells. Foci of densely growing cells were detected after transfection by the wild-type ME26 and the deletion mutants designated ME26A and ME26B. Mutant A was constructed by deleting ~1 kb of the sequence encoding avian *gag* and the first 314 bp of E26-derived *myb* sequence. Mutant B is an in-frame deletion mutant, which has lost 300 bp of *ets*-specific sequences between the *Hpa*I

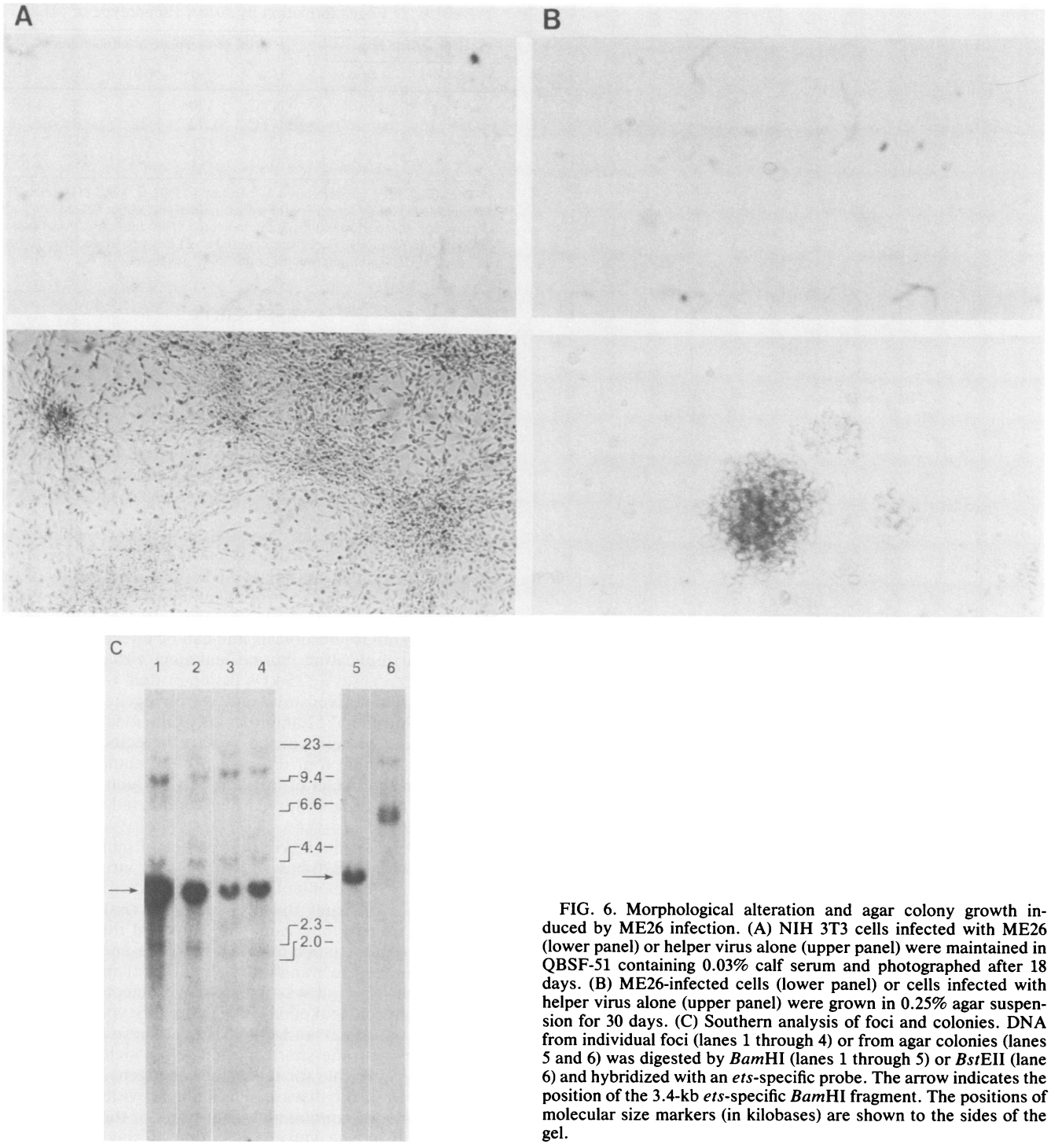

FIG. 6. Morphological alteration and agar colony growth induced by ME26 infection. (A) NIH 3T3 cells infected with ME26 (lower panel) or helper virus alone (upper panel) were maintained in QBSF-51 containing 0.03% calf serum and photographed after 18 days. (B) ME26-infected cells (lower panel) or cells infected with helper virus alone (upper panel) were grown in 0.25% agar suspension for 30 days. (C) Southern analysis of foci and colonies. DNA from individual foci (lanes 1 through 4) or from agar colonies (lanes 5 and 6) was digested by *Bam*HI (lanes 1 through 5) or *Bst*EII (lane 6) and hybridized with an *ets*-specific probe. The arrow indicates the position of the 3.4-kb *ets*-specific *Bam*HI fragment. The positions of molecular size markers (in kilobases) are shown to the sides of the gel.

and *Bgl*II sites (Fig. 8A). These results indicate that neither of these two regions was essential for NIH 3T3 focus-forming activity. Our results indicate that neither the avian *gag*- nor the 5′ *myb*-containing regions of ME26 were required for the focus-inducing activity of the viral construct. In addition, a murine viral construct obtained from E. P. Reddy and expressing *myb* sequences derived from avian myeloblastosis virus failed to induce foci in NIH 3T3 cells (data not shown).

Furthermore, cells transfected with mutant C (Fig. 8) failed to form foci when grown under limiting serum concentrations, and supernatants from these cells infected with replicating helper virus were also unable to induce focus formation on uninfected NIH 3T3 cells. Mutant C contains a 4-base insertion at the *Bgl*II site 530 bp downstream from the start of the v-*ets*-specific sequence (32). This introduces a frameshift and should result in termination of the ME26 fusion protein 30 codons beyond this site. A protein of 97 kDa could be detected in ME26C-transfected cells by immunoprecipitation analysis of labeled cell proteins using antise-

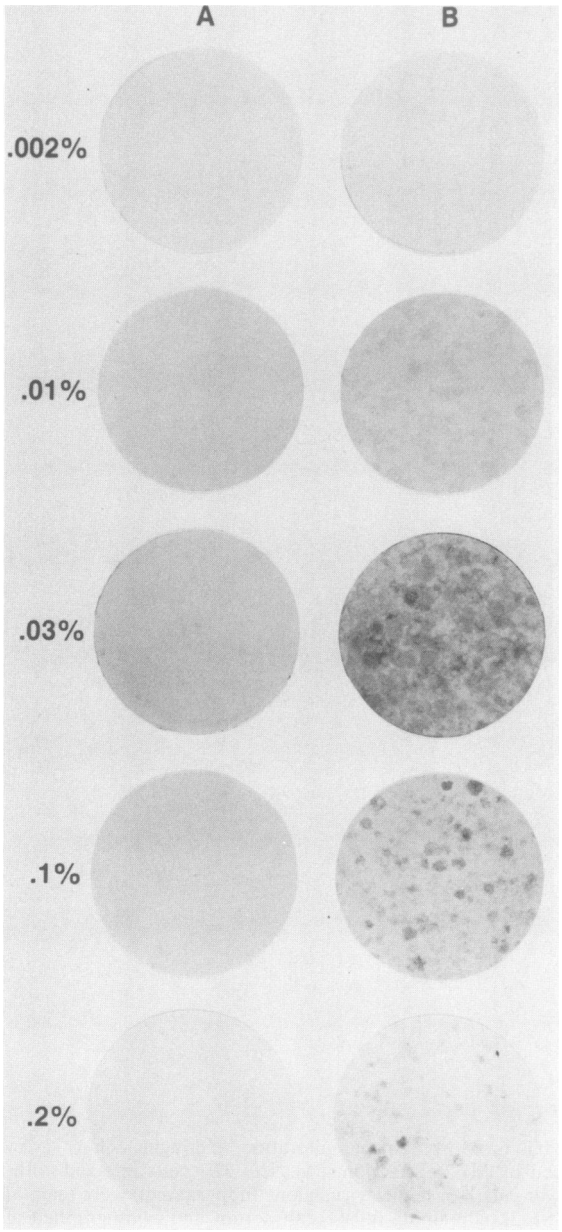

FIG. 7. Appearance of infected NIH 3T3 cells grown in defined medium. ME26-infected NIH 3T3 cells (column B) or cells infected by helper virus alone (column A) were grown in QBSF-51 medium containing the indicated levels of calf serum for 21 days, fixed, and Giemsa stained.

rum against murine leukemia virus p15^{gag} (Fig. 8B, lane 6), and this size is consistent with that predicted for an ME26C-encoded protein. Similarly, proteins of sizes consistent with predictions based on the E26 sequence have been detected in cells transfected with wild-type ME26 and ME26A and ME26B mutants (Fig. 8B, lanes 2, 5, and 3). These results indicate that the inability of ME26C to induce foci in NIH 3T3 cells was not due to a failure to express the viral fusion protein and suggests that the expression of a protein containing at least the C-terminal 284 amino acids of v-*ets* was required for focus induction and growth enhancement in low serum.

TABLE 2. Focus formation by 4070A Pseudotype of ME26

Cells infected by		No. of foci/plate at virus dilution[b]				
ME26 stock	4070A[a]	1/10	1/10^2	1/10^3	1/10^4	1/10^5
+	−	TNTC	120	9	4	1
+	+	TNTC	72	12	3	1
−	−	1	NT	NT	NT	NT
−	+	1	NT	NT	NT	NT

[a] The 4070A titer was measured by titration in FG10 (2) and PG4 cells (21). The multiplicity of infection for the added helper was about 0.1.

[b] Infection was performed using the indicated virus dilutions as desribed in Materials and Methods. Foci were counted at 21 days postinfection. Abbreviations: TNTC, Too numerous to count; NT, not tested.

DISCUSSION

Oncogenes represent a class of highly conserved cellular sequences whose functions are also believed to be conserved across species. Oncogenes identified in one species by virtue of their incorporation into oncogenic retroviruses are usually capable of transforming the appropriate target cells or cell line of another species. We have tested this functional conservation observed in other oncogene systems and have made use of it to study the properties of the fusion oncogene of avian leukemia virus E26.

We have described the construction of a functional murine replication-defective retrovirus in which the E26 *gag-myb-ets* coding region was fused to the first 34 amino acids of the murine p15^{gag} derived from Abelson murine leukemia virus. ME26 virus, as our results have demonstrated, replicates to high titer in mouse fibroblasts and can be effectively rescued by different replicating murine leukemia viruses. The viral construct expresses a 135-kDa fusion protein which includes the entire E26-derived *myb* and *ets* oncogene peptides, as well as all but the 5′ 51 amino acids of the avian *gag* region. The resulting protein can be readily detected by antisera directed against all four different protein components. It is localized in the nuclei of infected cells, which is consistent with what has been observed in E26-infected avian myeloblasts (9, 26).

Our analysis of the in vivo properties of murine recombinant retrovirus ME26 has shown that the virus induces an increased incidence of leukemia when injected into newborn mice. This establishes that the E26 retroviral oncogene is capable of transforming nonavian cells and thus, like many of the oncogenes studied to date (e.g., v-*src* and *erbB*) (1, 14, 45), can function to transform cells other than those of the species from which it was isolated. We detected both myeloid and erythroid leukemias, which are the types induced by E26 in the natural avian hosts (31, 37). These arose in most of the cases with the shortest latencies, which is consistent with a causative role for the ME26 oncogene sequences in the induction of the disease. Preliminary evidence indicates that virus stocks containing higher titers of the ME26 pseudotypes will induce leukemias within 40 days of injection into newborn NFS/N mice.

We also observed other types of hematopoietic neoplasms in our ME26-infected mice, including a significant number of lymphoid leukemias, although in most instances, these arose with longer latencies. It is interesting to note that the earliest reports of the properties of E26 described a longer latency (10 to 64 days [23]) than the 14 to 21 days described in later studies (24). The changes in the properties of E26 could have been a property of increased virus titers or adapted virus, which could be similar to the changes we have observed with higher-titer, later stocks of our ME26 viral construct. Our

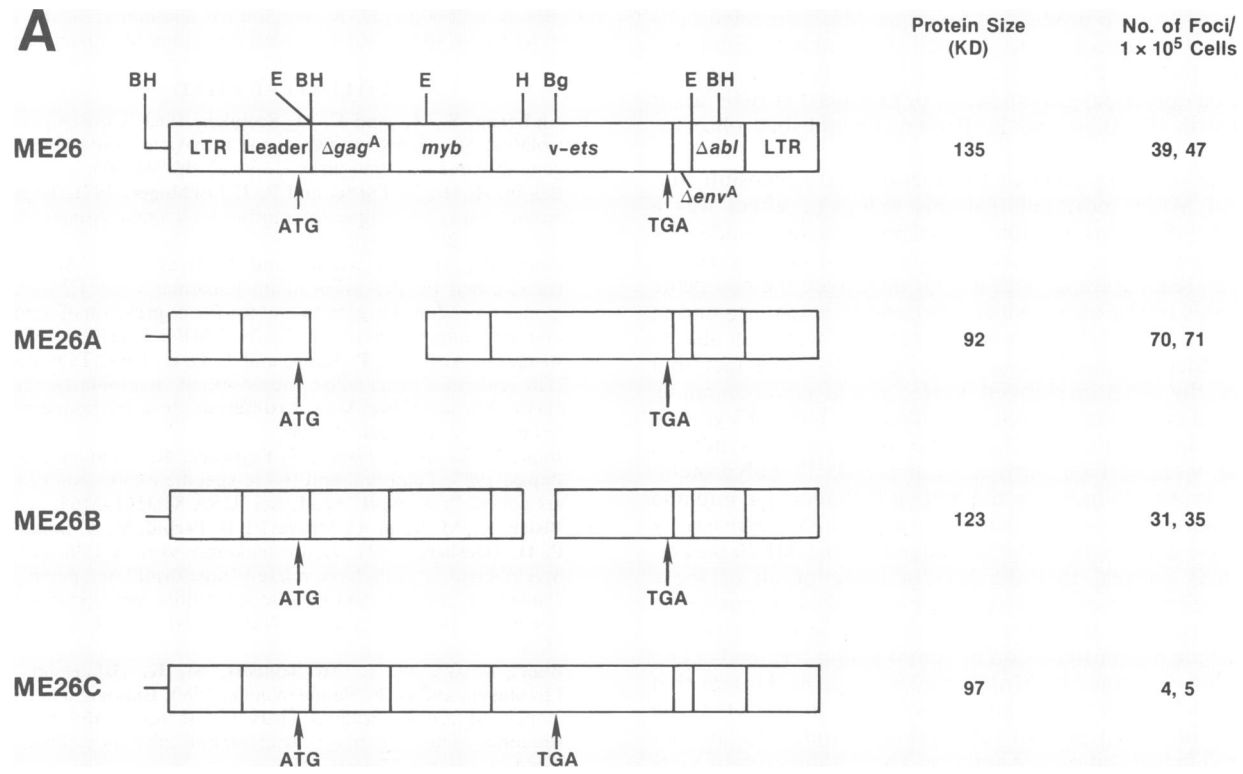

FIG. 8. Structure and properties of ME26 mutants. (A) The deleted portions and the sites of the initiation and termination codons are shown schematically. The viral protein sizes (in kilodaltons) were calculated from the known viral sequence (27, 32) and verified by immunoprecipitation. DNA of the ME26 construct or of the mutants was transfected into NIH 3T3 cells along with pSV2neo. G418-resistant colonies were pooled and replated at 10^5 cells per plate and maintained in QBSF-51 containing 0.1% serum for 21 days. The foci on duplicate plates were counted after fixing and staining. The number of foci observed on plates transfected with vector (K29) alone were 2 and 3. Restriction site abbreviations: BH, *Bam*HI; E, *Eco*RI; H, *Hpa*I; Bg, *Bgl*II. (B) Polyacrylamide gel electrophoretic analysis of ^{35}S-labeled lysates of cells transfected with the K29 vector, wild-type ME26, and mutant ME26 constructs. Immunoprecipitation was performed with an anti-murine $p15^{gag}$ antibody. Arrows indicate the positions (in kilodaltons) of the encoded proteins. Lanes: 1, vector; 2, ME26 wild type; 3, mutant ME26B; 4, vector; 5, mutant ME26A; 6, mutant ME26C.

stocks of amphotropic murine helper virus also induced lymphoid leukemia, as well as a low incidence of myeloid leukemias more than 6 months after infection. However, since ME26-infected mice developed lymphoid leukemia with a shorter latency than those infected with helper virus alone, and two of three tested spleen DNAs from the lymphoid leukemic animals contained an *ets* sequence, we cannot eliminate the possibility that ME26 viral sequences play a role in the induction or accelerate the development of this and other types of leukemia in infected mice. We have observed that in mice infected with the Mo-MuLV pseudotype of ME26 the latency of leukemia was not reduced relative to Mo-MuLV alone. Of 11 animals injected with Mo-MuLV pseudotypes, 9 developed lymphoid leukemia (the other 2 were follicular cell lymphoma) and only 1 of 9 tissue samples of leukemic mice contained ME26 sequences (data not shown). Although E26 specifically induces erythroid and myeloid neoplasms in vivo in birds, an expanded spectrum of tumor types in mice has been observed for a murine recombinant of the avian-*myb* oncogene (28).

We also demonstrated that ME26 induced an in vitro mitogenic response in NIH 3T3 mouse fibroblasts. Our data indicated that ME26-infected NIH 3T3 cells grew to higher density in low serum and formed foci of densely growing cells on top of the flat, slowly growing uninfected monolayer. These foci were only detectable at low serum levels (0.05 to 0.1%), which could reflect the fact that in higher serum concentrations growth is too rapid and efficient for the stimulation induced by the ME26 oncogene to be detectable. It is also possible that serum factors inhibit and interfere with the process of mitogenic stimulation by this oncogene and that these inhibitors must be diluted to allow the

gag-myb-ets gene product to function. ME26-infected cells are also able to grow in the absence of serum in a serum-free medium formulation in which NIH 3T3 cells show a dependence on epidermal growth factor or fibroblast growth factor for growth (D. Blair and R. Brown, unpublished observations).

Our preliminary analysis of deletion and frameshift mutants of ME26 indicated that this mitogenic effect was a property of the *ets* portion of the polyprotein encoded by p135 *gag-myb-ets*, since an insertion which interrupted the *ets* open reading frame within v-*ets* destroyed the mitogenic properties of the virus, while deletions of avian *gag* and at least the 5′ end of the *myb*-related peptides were still able to induce foci of proliferating NIH 3T3 cells. The fact that neither the Abelson-derived vector nor a *myb*-containing retroviral construct showed any effects on NIH 3T3 growth further supports the hypothesis that this property is induced by the v-*ets*-derived portion of the $p135^{ME}$ polyprotein. However, foci induced by the mutant lacking avian *gag* and the 5′ portion of *myb* appeared to grow more aggressively and more densely than those induced by the ME26 parent. This may suggest that the loss of some or all of these sequences increases the mitogenic potential of the remaining v-*ets* portion of the gene product. Although our results cannot rule out participation by the 34 amino-terminal amino acids derived from the MuLV $p15^{gag}$ protein, the fact that E26, which lacks these sequences, has a similar effect on chicken fibroblast growth (25), indicates that they are not required for this mitogenic activity. The involvement of v-*ets* in the mitogenic pathway is consistent with the proposed functions of both c-*ets*-1 (35) and c-*ets*-2 (5).

Our results suggest that ME26 could induce a partially transformed state in NIH 3T3 cells but was unable to fully transform mouse fibroblasts. Infected NIH 3T3 cells formed small, slowly growing colonies in soft agar, but transfected or infected NIH 3T3 cells did not efficiently form tumors when injected into athymic nude mice. We have seen tumors in some experiments, but these arose with a relatively long latency (≥40 days), which is consistent with the occasional spontaneous tumors which arose when 1×10^6 to 3×10^6 NIH 3T3 cells were injected into nude mice. Furthermore, we have never observed solid tumors in either nude mice or newborn or adult immunocompetent mice injected with various virus preparations. Although we cannot rule out the possibility that under certain conditions (such as high levels of $p135^{ME}$ expression), ME26 may transform mouse fibroblasts, our results suggest that in this lineage the virus is capable of inducing only a mitogenic stimulation, rather than a complete transformed state. Additional studies to more completely characterize the transforming potential of this virus in nonhematopoietic cells are in progress.

In summary, we have described the properties of a murine recombinant retrovirus carrying the *gag-myb-ets* oncogene of E26 which induces leukemia in mice in vivo and which induces a titratable mitogenic response in mouse fibroblasts. The mitogenic effects appear to be *ets* induced, and thus our results represent the first easily quantifiable assay for one of the possible functions for this oncogene sequence. It should provide a usable system in which to further characterize the properties of this family of oncogenes, and experiments utilizing this assay to evaluate the functions of c-*ets* and other *ets*-related genes are in progress.

ACKNOWLEDGMENTS

We thank R. Brown, who developed the defined medium used in this study; M. A. Baluda, G. I. Evan, S. Fujiwara, and S. Showalter for gifts of antibodies; D. K. Watson for comments on the manuscript; and K. Cannon and C. Nolan for typing the manuscript.

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