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Two Nuclear Oncogenic Proteins, P135$^{gag\text{-}myb\text{-}ets}$ and p61/63^{myc}, Cooperate To Induce Transformation of Chicken Neuroretina Cells

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Several studies have shown that full transformation of primary rodent fibroblasts can be achieved in vitro through the cooperation of two oncogenes (usually one nuclear and one cytoplasmic) classified on the basis of different complementation groups. We have shown previously that cooperation between v-*mil* (cytoplasmic, serine-threonine kinase product), and v-*myc* (nuclear, DNA-binding product) is required to transform 7-day-old chicken neuroretina cells, which in usual culture medium do not rapidly proliferate. v-*mil* induces sustained growth of chicken neuroretina cells without transformation; v-*myc* fails to stimulate the proliferation of chicken neuroretina cells but is required to achieve transformation of the proliferating cells. Here, we present results indicating that the P135$^{gag\text{-}myb\text{-}ets}$ nuclear protein of avian erythroblastosis virus E26 is able to induce proliferation but not transformation of chicken neuroretina cells. v-*myc* is required in addition to P135$^{gag\text{-}myb\text{-}ets}$ to achieve chicken neuroretina cell transformation. In contrast, we found that the P135$^{gag\text{-}myb\text{-}ets}$ and P100$^{gag\text{-}mil}$ proteins are not able to cooperate in this system.

Several lines of evidence suggest that cell proliferation and differentiation are controlled by an intricate network of biochemical pathways extending from the cell membrane to the nucleus (4). Oncogene products control key positions along these pathways; they are able to act as growth factors, growth factor receptors, transducing agents, or nuclear products. Nuclear oncogene products may have the power to substantially increase the replication potential of infected cells in vitro (3, 34).

The E26 retrovirus contains the v-*myb-ets* sequence fused in the same reading frame with the residual *gag* sequence (19, 26). A unique 5.7-kilobase genomic RNA encodes a 135-kilodalton *gag-myb-ets* protein located in the nucleus (17). After infection with E26, chickens develop mixed leukemias involving both myeloid and erythroid lineages (23, 30). *In vitro*, this virus may transform bipotent uncommitted hematopoietic cells, as well as myeloid and erythroid progenitor cells (24, 30). E26 also increases the life span of infected chicken fibroblasts but is unable to transform these cells (16).

The v-*myc* oncogene is the major transforming gene found in MH2. This viral oncogene is encoded by a subgenomic RNA as a nuclear protein (6). v-*myc* is also found in three other independent virus isolates (MC29, OK10, and CMII) (32). These four viruses transform macrophages and fibroblasts in culture (15). The other cell-derived sequence contained in the MH2 virus, v-*mil*, is responsible for an increase of the oncogenic potential of MH2 when compared with that of MC29. v-*mil* also induces the proliferation of chicken neuroretina (CNR) cells (2, 21). The protein encoded by v-*mil* from a genomic size RNA contains an associated serine-threonine kinase activity and is located in the cytoplasm (22).

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We have constructed a recombinant virus, MHE226, expressing the E26 P135$^{gag\text{-}myb\text{-}ets}$ nuclear protein from a genomic RNA and the MH2 v-*myc*-encoded nuclear protein (p61/63^{myc}) from a subgenomic RNA. In this study, we present evidence that after infection with E26, CNR cells are induced to proliferate but are not transformed. Following infection with MHE226, CNR cells proliferate and become tumorigenic in young chickens. In addition, we demonstrate that the P135$^{gag\text{-}myb\text{-}ets}$ and the P100$^{gag\text{-}mil}$ proteins are not able to cooperate in CNR cell transformation.

MATERIALS AND METHODS

Viruses and molecular clones. The XSK provirus results from a *Xho*II-*Sph*I deletion leading to the inactivation of the *mil* oncogene in the MH2 provirus (8, 9). MHE226 results from the substitution of the *Bam*HI-*Hpa*I *gag-mil* fragment of the pMH2-Hd DNA by the *Bam*HI-*Stu*I *gag-myb-ets* fragment from the λE26 Q1 provirus (19). This virus should express P135$^{gag\text{-}myb\text{-}ets}$ through a genomic mRNA and p61/63^{myc} from a subgenomic spliced mRNA. MHE226ΔETS results from the substitution of the *Bam*HI-*Hpa*I *gag-mil* fragment of the pMH2-Hd DNA by the *Bam*HI-*Hpa*I *gag-myb-*Δ*ets* fragment from the λE26 Q1 provirus.

The viruses used were obtained by cotransfection of DNA from the molecular clones depicted in Fig. 1 with Rous-associated virus type 1 (RAV-1) DNA into quail embryo cells, as previously reported (2), for MHE226, XSK, and MC29. E26 pseudotyped with E26AV was a gift from P. Jurdic.

Cell culture, growth assay, and soft-agar cloning. Neuroretinas were dissected from 7-day-old chicken embryos (2). Dishes (diameter, 60 mm) containing 5×10^6 dissociated cells were infected with various viruses in Eagle basal medium supplemented with 10% fetal calf serum at 37°C. The infected and uninfected cells were passaged four times to allow virus spreading. Cells were then seeded on collagen-coated dishes. CNR E26 cells were superinfected by MH2-PA200 (ring-necked pheasant virus) after 15 passages. These

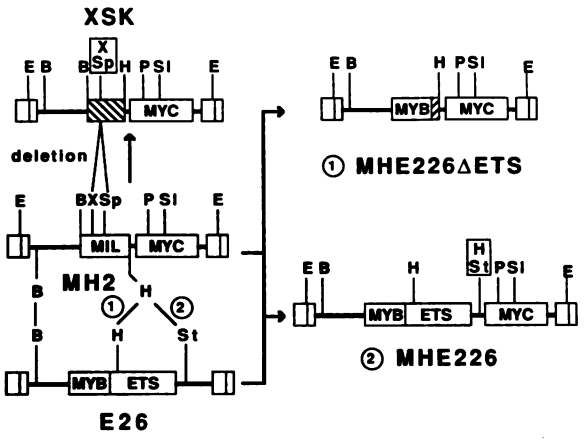

FIG. 1. Molecular clones used. B, *Bam*HI; E, *Eco*RI; H, *Hpa*I; P, *Pvu*II; Sl, *Sal*I; Sp, *Sph*I; St, *Stu*I; X, *Xho*II. □, Long terminal repeat; ▭, functional proviral oncogenes; ▨, nonfunctional oncogenes.

cells were then passaged three times more to allow virus spreading.

Some cells grown without collagen were fixed and stained with Giemsa blue, and representative areas were photographed at a magnification of ×40.

To study the growth kinetics of virus-infected CNR cells, 1×10^5 cells were seeded in 35-mm-diameter collagen-coated dishes in 10% fetal calf serum and 2×10^5 cells were seeded in medium containing 1% fetal calf serum. Medium was renewed daily, and cells from two dishes were counted. Then, 10^5 cells of each type were seeded in 4 ml of soft-agar-containing medium (Eagle basal medium, 10% fetal calf serum, 0.7% agar). Colonies were observed 14 days later and were only found in MHE226-infected cells.

In vivo tumorigenicity assay. Infected cells were suspended at the desired concentration in 100 μl of complete medium and injected in the wing webs of 1-day-old outbred chickens (Barrez Poultry). In some cases, chickens were similarly injected intraperitoneally.

For the chorioallantoid membrane (CAM) assay, eggs were prepared by the method of Poste and Flood (29). Briefly, after the host eggs had been incubated for 10 days, the blunt end was pierced and a small window was opened in the shell overlaying the CAM; removing the air from the air chamber created a pseudo-chamber, and 10^6 cells were layered in 100 μl of complete medium onto the surface of the CAM. The window was sealed with Micropore tape, and the eggs were horizontally incubated for an additional week. At that time, the shell was opened and the CAM was removed and rinsed in phosphate-buffered saline (PBS). Tumors were detected by macroscopic examination, and samples were dissected out, fixed with 3.7% paraformaldehyde in PBS, and processed for histological examination.

Cell labeling and immunoprecipitation analyses. Infected CNR cells were incubated for 45 min in the presence of 30 μCi of [L-^{35}S]methionine (specific activity, 1,000 Ci/mmol) per ml., lysed, and immunoprecipitated as described previously (2). Sera used included preimmune rabbit serum, rabbit anti-*ets* serum (14), rabbit anti-*myc* serum (13), and rabbit anti-*gag* serum. Immunoprecipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by fluorography.

Immunofluorescence study. Uninfected or virus-infected CNR cells cultured on collagen-coated 12-mm microscope cover slips were fixed for 20 min with 3.7% paraformaldehyde in PBS and then treated 20 min at 4°C with 150 mM ethanolamine (pH 7.5), rinsed, and permeabilized with 0.15% Triton X-100 in PHEM {60 mM piperazine diethylsulfonic acid, 25 mM hydroxyethylpiperazine-*N*-1,2-ethanesulfonic acid, 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 2 mM $MgCl_2$ [pH 6.9]} for 1 min. To stain actin cables, cells were incubated with methanol-reconstituted nitrobenzoxadiazole-labeled phallacidin (Molecular Probes, Inc.) (1/20) in PBS for 30 min and then washed in PBS. With 3A7 (1/200), anti-vimentin (1/200), or anti-*mil* (1/100) antibodies (2), cells were incubated for 1 h, washed in PBS, and stained for 30 min with fluorescein isothiocyanate-labeled second antibody. The same procedure was used with undiluted supernatant of monoclonal A2B5 (hybridoma cell line from the American Type Culture Collection) or anti-fibronectin antibodies (1/100) (Bethesda Research Laboratories, Gaithersburg, Md.) but without Triton X-100 treatment of the cells.

Fluorescein isothiocyanate-labeled antibodies (Biosys) were as follows: fluorescein-labeled sheep anti-mouse antibodies were used with 3A7 and A2B5 antibodies; goat anti-rabbit antibody was used with anti-vimentin anti-fibronectin and anti-*mil* antibodies. After washing in PBS, cells were mounted in glycerol and examined with a Zeiss IM 35 fluorescence microscope at ×416 magnification.

RESULTS

Dissociated CNR cells from 7-day-old chicken embryos contain neuroepithelial cells from glial and neuronal lineages, as well as postmitotic neurons (10, 18). CNR cells were plated in Eagle basal medium containing 10% fetal calf serum and were infected with retroviruses produced from molecular clones (Fig. 1 and MC29) pseudotyped with RAV-1 helper virus or with E26 pseudotyped with its natural helper E26AV. We have also used the supernatant from myeloid cells infected with the virus produced from a molecular clone of E26 with similar results. Infection of CNR with E26 resulted in the appearance of actively dividing elongated cells (doubling time of ca. 26 h) after four passages (within 20 days). The infected cells could be subcultured for at least 20 generations (Fig. 2A, panel d, and Fig. 2B). These cells were morphologically normal and did not display anchorage-independent growth properties (Table 1). When CNR cells were infected with XSK (MH2 mutant deleted in the *mil* gene; Fig. 1) or MC29 viruses and cultured by using similar conditions, numerous dense foci of epithelium-like cells were observed. These foci were isolated in the culture dish and were clearly distinct from normal cells (Fig. 2A, panels b and c). The cells of the foci displayed no proliferating properties or cloning capacity when tested in soft agar (Table 1). These cells could not be propagated through more than five passages, even on collagen-coated dishes.

Striking results were obtained when CNR cells were infected with MHE226 virus which contains the *myc* oncogene of MH2 in addition to the *gag-myb-ets* oncogene of E26 (Fig. 1). Infected cultures displayed foci of morphologically transformed cells (Fig. 2A, panel e) after only two passages. These cells exhibited a strong proliferative capacity (Fig. 2B; doubling time of ca. 13 h) and induced numerous large colonies in soft agar (more than 200 cells per colony; Fig. 2A, panel f, and Table 1). In addition, MHE226 CNR cells showed marked morphological changes; they were much smaller and markedly less adherent than E26-, XSK-, or

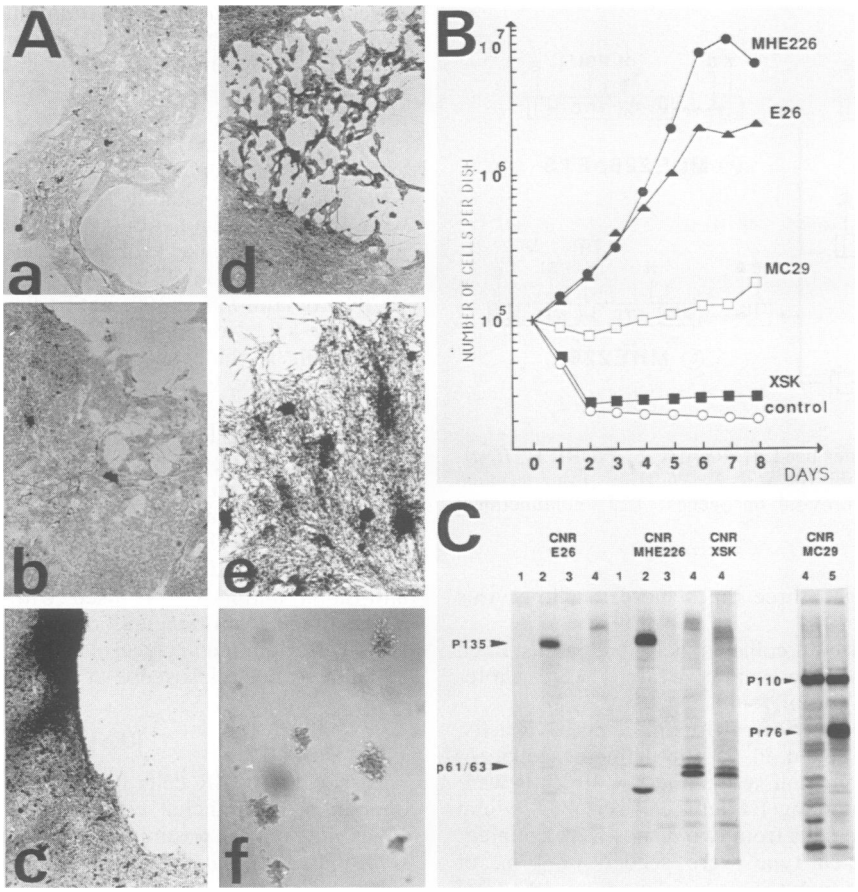

FIG. 2. (A) Microphotographs of virus-infected neuroretina cells after four passages: normal CNR cells (a); XSK- (b), MC29- (c), E26- (d), and MHE226- (e) infected CNR cells; and soft-agar colonies induced by MHE226-infected CNR (f). Magnification, ×40. (B) Growth kinetics of virus-infected CNR cells. Results for XSK- and MC29-infected and control (uninfected) cells are after 4 passages, and results for E26- and MHE226-infected cells are after 11 passages. (C) Analysis of viral proteins in CNR cells. Sera used were as follows: lanes 1, *ets* preimmune rabbit serum; lanes 2, rabbit anti-*ets* serum (14); lanes 3, *myc* preimmune rabbit serum; lanes 4, rabbit anti-*myc* serum; lane 5, rabbit anti-*gag* serum (13). Cell types used are listed across the top.

MC29-infected cells. After four passages, MHE226 CNR cells were cultured on collagen-coated dishes. Only in these conditions could MHE226 CNR cells be subcultured for more than 20 generations.

To determine whether the P135$^{gag\text{-}myb\text{-}ets}$ product was expressed in proliferating CNR cells infected with E26 or MHE226, cells were labeled with [L-^{35}S]methionine and immunoprecipitated with anti-v-*ets* antibodies (Fig. 2C, lanes 2) (14). These lysates, as well as lysates of CNR cells infected with XSK or MC29 viruses, were immunoprecipitated with anti-v-*myc* antibodies (Fig. 2C, lanes 4) (13). The levels of P135$^{gag\text{-}myb\text{-}ets}$ (Fig. 2C) expressed by E26 and MHE226 CNR cells were comparable to those of E26-transformed hematopoietic cells and much higher than those of E26-infected fibroblasts (16). The amounts of p61/63^{myc} and P110$^{gag\text{-}myc}$ present in XSK- and MC29-infected CNR cells were comparable to the amount present in infected fibroblasts. In addition, we localized p61/63^{myc} and P135$^{gag\text{-}myb\text{-}ets}$ in MHE226-infected cells by immunofluorescence experiments and subcellular fractionation (data not shown). Antisera against v-*myc* proteins stained nuclei. The p61/63^{myc} proteins were also found in the nuclear compartment by using immunoprecipitation techniques. P135$^{gag\text{-}myb\text{-}ets}$ could not be detected by immunofluorescence with our anti-v-*ets* antibodies, but this protein was immunoprecipitated by subcellular fractionation in the nucleus of MHE226 CNR cells (data not shown).

TABLE 1. Transformation-related changes in CNR cells

Cell treatment	Induction of cell proliferation[a]	Colony formation in semi-solid medium for 10^5 cells	Loss of fibronectin network[b]	% of cells without actin cables
None	−	0	−	0
MC29	−	0	−	36
E26	+	0	−	25
MHE226	+	3,050	+	100

[a] −, No induction; +, induction.
[b] +, Network was lost; −, network remained intact.

Since only MHE226 CNR cells appeared transformed, we tested MHE226-, E26-, and MC29-infected CNR cells for several transformation parameters. The alterations in cell morphology and in potential for cell proliferation correlate with changes in the extracellular matrix and cytoskeletal proteins. A loss of ordered actin-containing microfilaments and a reduction in the amount of cell surface fibronectin are frequently observed in virus-transformed cells (11, 27).

Actin, vimentin, and glial intermediate filaments are found in neuroretina cells (18). Immunocytochemical techniques were used to determine whether changes in cytoskeletal proteins occur in E26-, MHE226-, and MC29-infected CNR cells. Cultures infected with E26 (Fig. 3g) or MC29 (Fig. 3d) contained few cells without actin cables (25 and 36%, respectively) relative to normal cells (Table 1); however, MHE226 cultures exhibited no staining with actin antibodies (data not shown) or with NBD-labeled phallacidin (Fig. 3j). Furthermore, MHE226-transformed CNR cells completely lacked the fibronectin network (Fig. 3k) yet exhibited an almost normal pattern of vimentin and glial intermediate filaments (Fig. 3o and p). Thus, MHE226-infected CNR cells exhibited a transformed phenotype while CNR cells infected with E26 or MC29 appeared closer to normal.

To test whether MHE226-infected cells could be tumorigenic in chickens, we transfected MHE226 without RAV-1 DNA into CNR cells. Nonproducing transformed cells were propagated for 18 passages and then injected (1.8×10^7 cells) in the wing web or in the peritoneal cavity of 1-day-old chickens. After 1 week, tumors were found at the injection site, and animals were sacrificed 1 week later. Four out of five animals injected in the wing web developed large tumors, and three out of five injected intraperitoneally developed tumors. MHE226 provirus was found by Southern blot analysis in the DNA of the three different tumors tested (data not shown). No tumor was found outside of the injection areas, and the liver, lungs, and kidneys were found normal by macroscopic examination. Additional experiments were also performed with CNR cells infected with E26 RAV-1, MHE226 RAV-1, and MHE226ΔETS RAV-1, an in vitro deletion mutant lacking most of the *ets* sequences. This last virus is able to induce CNR cell proliferation and direct the synthesis of the expected $P90^{gag\text{-}myb\text{-}\Delta ets}$ fusion protein in transfected or infected cells (Fig. 4A). All 12 animals injected with 3×10^6 MHE226 RAV-1 CNR cells produced tumors at the injection site within 15 days. All birds injected with the same amount of E26 CNR cells developed leukemias without solid tumors in 1 month or longer. In addition, no bird injected with MHE226ΔETS developed solid tumors. Since immunological rejection could occur in animals, we performed additional experiments to compare the ability of these infected CNR cells to induce tumors in the chorioallantoic membrane from 11-day-old chicken embryos. Again, only MHE226 CNR cells (productive or nonproductive cells) were found able to induce tumors in this assay (Fig. 4B and C).

Since E26 CNR cells proliferate, we analyzed whether the E26 nuclear product, $P135^{gag\text{-}myb\text{-}ets}$, could at least in part overcome growth factor dependence (Fig. 5). E26 CNR cells, seeded in low-serum medium containing 1% fetal calf serum, exhibited active proliferation with a doubling time similar to that observed when a medium containing 10% fetal calf serum was used. MHE226-transformed cells plated in low-serum medium adhered to collagen-coated dishes and grew efficiently but with a doubling time of ca. 26 h compared with ca. 13 h in medium containing 10% fetal calf serum. Thus, $P135^{gag\text{-}myb\text{-}ets}$ can lower the requirement for growth factors in rapidly dividing MHE226 CNR cells. MC29 CNR cells died a few hours after seeding in low-serum medium.

Because seemingly normal CNR cells could be induced to proliferate by the v-*mil* oncogene, it was of particular interest to know whether the $P100^{gag\text{-}mil}$ and $P135^{gag\text{-}myb\text{-}ets}$ could cooperate in CNR cell transformation. Superinfection of E26 CNR cells with MH2-PA200 (21), pseudotyped with RPV, induced slight changes in cell morphology after three passages; cells appeared more fusiform than E26 CNR cells or E26 CNR cells superinfected with RPV alone. However, the growth rate and the inability to grow in soft agar were not modified whereas most, if not all, of the superinfected cells exhibited significant amounts of $P100^{gag\text{-}mil}$, as shown by immunoprecipitation and immunofluorescence experiments using anti v-*mil* antibodies (Fig. 5A through D). To exclude the possibility that E26 CNR cells were selected from cells insensitive to $P100^{gag\text{-}mil}$, we reversed the infection superinfection protocol; superinfection of MH2-PA200 CNR cells (RAV-1) with E26 pseudotyped with RPV induced the same slight changes in cell morphology (data not shown). In all these experiments, effective infection was controlled by immunoprecipitation assay. Thus, these two oncogene products, each able to induce CNR cell proliferation and located in different subcellular compartments, were not able at least in these cells to achieve CNR cell transformation together.

We partially characterized the lineage of E26 and MHE226 CNR cells. Cells from 7-day-old chicken embryos can give rise to neuronal, glial, and progenitor cells of both lineages (10, 18). Virus-infected cells were characterized for the expression of tissue-specific antigens, including a tetrasialoganglioside present on neural cells recognized by monoclonal A2B5 antibodies (12), intermediate filament of glial cells recognized by monoclonal 3A7 antibodies (18), and synthesis of acetylcholine (33). CNR cells transformed with MHE226 expressed A2B5 antigen similarly to MC29-infected CNR cells (Fig. 3l and f). E26-infected CNR cells expressed this antigen only faintly; 1% of the cells reacted strongly with this monoclonal antibody (Fig. 3i). However, normal CNR cells expressed this marker only faintly after four passages (Fig. 3c), suggesting that A2B5-positive cells were maintained in the culture by the viral oncogene product. All infected or normal CNR cells expressed 3A7 glial intermediate filaments (Fig. 3n and p and data not shown). E26-infected CNR cells synthesized greater amounts of acetylcholine than normal cells, but only low levels were detected in MHE226 CNR cells (M. Fauquet, unpublished data). These observations suggest that cells of both neuronal and glial lineages were induced to proliferate after infection with these viruses.

DISCUSSION

In the study presented here, we investigated the effect of E26 $P135^{gag\text{-}myb\text{-}ets}$ nuclear protein in CNR cells, alone or together with the $p61/63^{myc}$ product from MH2. As reported for chicken embryo fibroblasts (16), E26 does not induce the expression of the usual features associated with transformation (Table 1). E26-infected cells did not show anchorage-independent growth, disorganized actin microfilaments, or loss of fibronectin network. However, in contrast to normal or control RAV-1-infected CNR cells, E26 CNR cells were able to grow both for at least 20 generations in culture and at low cell density in low-serum medium. Similar results have been obtained in chicken embryo fibroblasts infected with E26, which exhibited increased proliferation in vitro and maintained long-term growth in low-serum medium (16). In contrast, in other cell systems, chicken myeloid cells transformed by E26 are still dependent on chicken myelomonocytic growth factor (20) to proliferate. Thus, the effect of $P135^{gag\text{-}myb\text{-}ets}$ on growth factor dependence is probably governed by genetic differences between the cell systems used.

MC29-transformed chicken fibroblasts have been shown to lack the ability to proliferate when cultured in medium

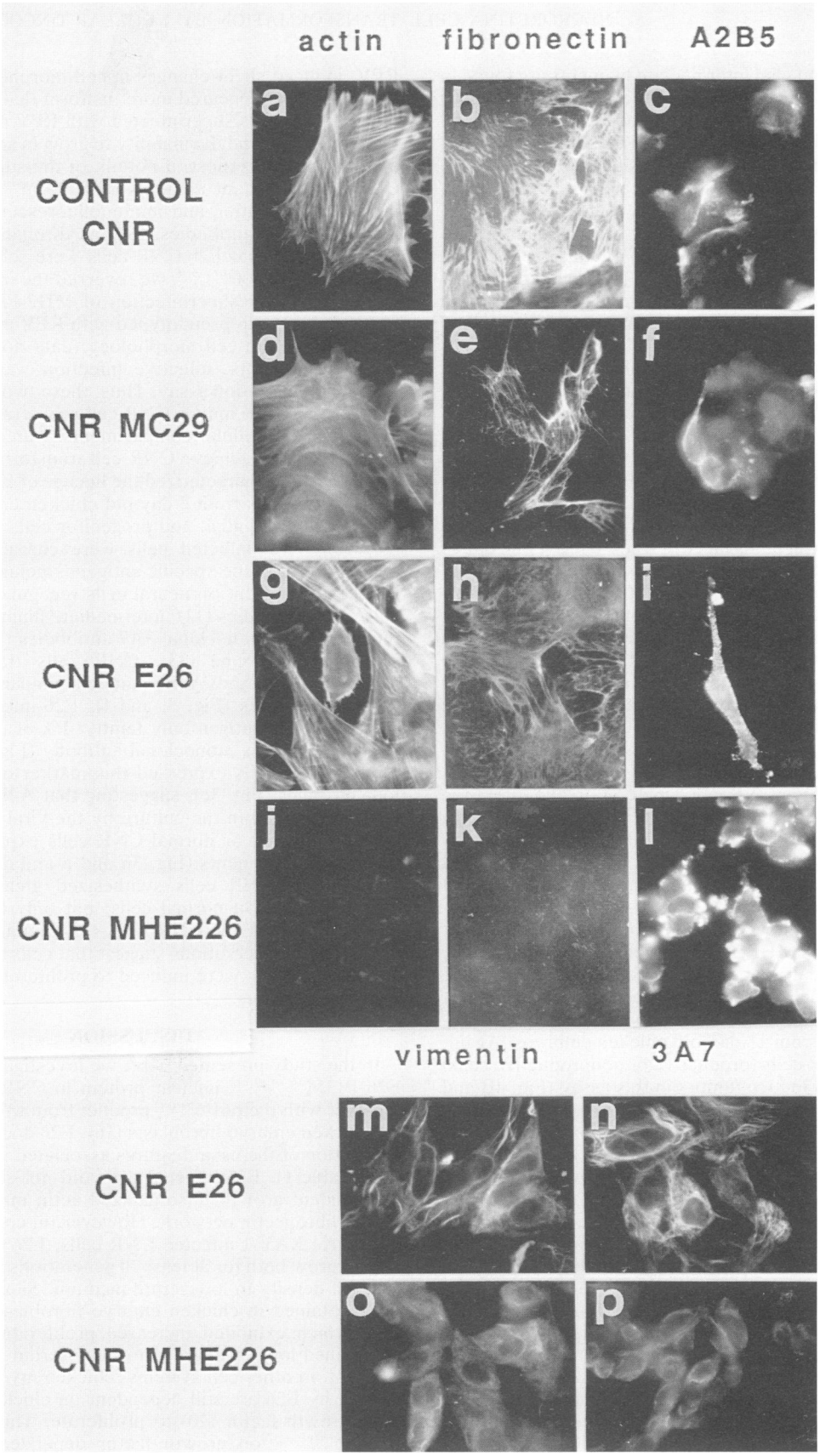

FIG. 3. Immunofluorescent visualization of actin, fibronectin, neural antigen A2B5, vimentin, and 3A7 glial intermediate fragments in normal and virus-infected CNR cells. Cells were stained after 4 (a through f) or 15 (g through p) passages. Staining was done with NBD-labeled phallacidin (actin), with an anti-fibronectin antibody (fibronectin), with a monoclonal A2B5 antibody (A2B5), with a polyclonal anti-vimentin antibody (vimentin), or with a monoclonal 3A7 antibody (3A7). Magnification, ×416.

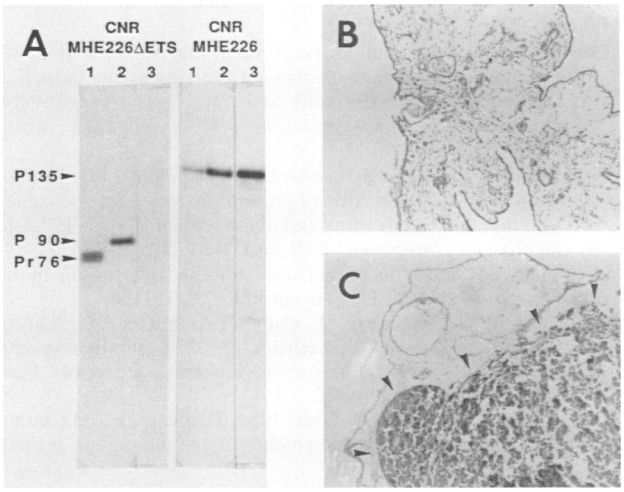

FIG. 4. Histological analysis of tumors developed on CAM. (A) Analysis of viral proteins in MHE226ΔETS and CNR MHE226NP. Sera used were as follows: lanes 1, rabbit anti-*gag* serum; lanes 2, rabbit anti-*myb* serum (5); lanes 3, rabbit anti-*ets* serum. (B and C) Inoculation of CAM with CNR cells infected with MHE226ΔETS RAV-1 (panel B) and CNR cells transfected with MHE226 (panel C). Arrowheads in panel C delineate the tumor found. The slides were stained with May Grünwald-Giemsa (RAL 555 kit). Magnification, ×40.

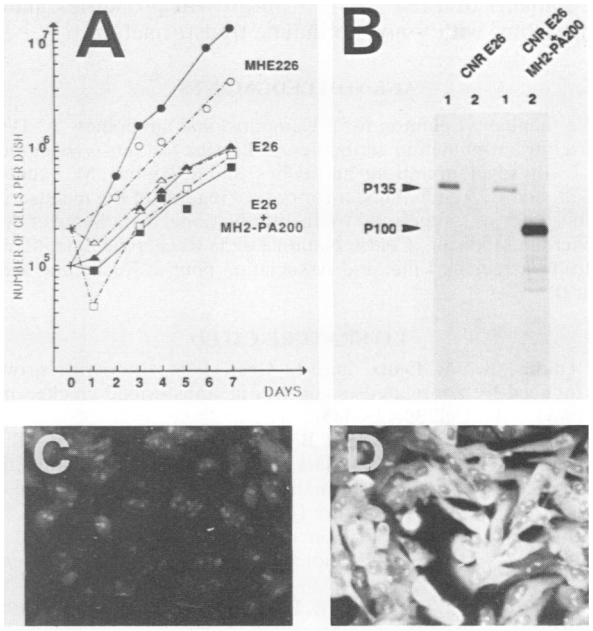

FIG. 5. (A) Growth kinetics of virus-infected CNR cells in medium containing 10% (closed symbols) or 1% (open symbols) fetal calf serum. ●, ○, MHE226; ▲, △, E26; ■, □, E26+MH2-PA200. (B) Analysis of viral proteins in CNR E26RAV-1 superinfected by MH2-PA200RPV virus. Lane 1, rabbit-anti *ets* serum (14); lane 2; rabbit-anti *mil* serum (2). (C and D) Analysis of viral proteins in CNR E26 RAV-1 superinfected by MH2-PA200 RPV virus by immunofluorescence studies. Panel C, CNR E26 RAV-1; panel D, CNR E26 superinfected by MH2-PA200 RPV; cells were stained with anti-*mil* antibodies as described in Materials and Methods. Magnification, ×150.

containing a low concentration of serum (31). Chicken myeloid cells transformed by v-*myc* require growth factors to proliferate (1). The dependence of v-*myc*-infected myeloid cells could be abolished by several oncogene products, such as v-*src*-related tyrosine kinases and v-*mil* (but neither v-*myb* nor the v-*gag-myb-ets*) (1). However, the effect of oncogenes on growth factor dependence varies with the cell systems used, since chicken embryo fibroblasts infected by MH2 decrease in number and die in medium containing 1% serum, whereas v-*mil* containing MH2-PA200-infected fibroblasts are able to grow in such culture conditions (28). These results suggest that v-*myc*-infected chicken cells are dependent on serum factors for survival and that in MHE226 CNR cells, $P135^{gag\text{-}myb\text{-}ets}$ overcomes this requirement.

Jurdic et al. (16) proposed that E26 $P135^{gag\text{-}myb\text{-}ets}$ could induce either cell growth activation or cell transformation, according to its level of expression. Our results are not in agreement with this proposal, since high levels of $P135^{gag\text{-}myb\text{-}ets}$ only induced CNR cell proliferation, not transformation. Transformation was achieved by addition of the v-*myc* oncogene. Therefore, cooperation of two retrovirus-encoded oncogenic proteins, both located in the nucleus, was found to operate in CNR cells, in contrast to the previously reported systems (3, 34). Since the v-*myc* oncogene alone was found able to induce CNR cell proliferation and transformation in complex culture conditions (7), $P135^{gag\text{-}myb\text{-}ets}$ could mimic such culture conditions, allowing transformation through v-*myc*.

The $P100^{gag\text{-}mil}$ product induces CNR cell proliferation. However, the $P135^{gag\text{-}myb\text{-}ets}$ and $P100^{gag\text{-}mil}$ proteins do not cooperate in CNR cell transformation. This could suggest that *gag-myb-ets* and *mil* oncogene products may act on the same cellular pathway to induce CNR cell proliferation.

Although the mechanisms through which the nuclear $p61/63^{myc}$ and $P135^{gag\text{-}myb\text{-}ets}$ proteins operate are unknown, the observation that these two proteins are able to cooperate in the conversion of CNR cells into tumorigenic cells suggests that the proteins work through different mechanisms. This was also suggested from experiments conducted with E26 and MC29 in the hematopoietic system (25) which suggested a v-*myb* dominance over v-*myc* in doubly transformed chick myelomonocytic cells. A portion of $P135^{gag\text{-}myb\text{-}ets}$ encoded by the *ets* oncogene is essential to allow the cooperation between this oncogenic protein and the *myc* product, since MHE226 deleted in most of the *ets* sequences, MHE226ΔETS, is not able to transform CNR cells. However, this virus is still able to induce both CNR cell proliferation in vitro and hematopoietic disorders in infected birds. Results obtained on the cooperation between *myc* and several mutants of $P135^{gag\text{-}myb\text{-}ets}$ will be presented elsewhere.

The effect of the two nuclear proteins on CNR cell differentiation is not clear; A2B5 antigen, which quickly disappears from cultured normal CNR cells, is maintained in MC29- and MHE226-infected cells. Only 1% of the E26-infected cells retained the A2B5 antigen. Thus, the *myc* gene product could block the maturation of neuroretinal cells expressing A2B5 antigen. Alternatively, the *myc* gene product could induce the survival of a cell type which is not maintained in the culture conditions; such a cell type would be induced to proliferate in MHE226-transformed cells by the addition of the $P135^{gag\text{-}myb\text{-}ets}$ protein.

These, and previous, results demonstrate that CNR cells constitute a powerful system to detect v-*myc* cotransforming genes. This biological system could also reveal previously unexpected targets for oncogenes. In addition, CNR cells should be useful to define the relative importance of *myb* and

ets domains of P135$^{gag\text{-}myb\text{-}ets}$ in growth properties and in cooperation with v-*myc* to induce transformation.

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