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# Transforming and Mitogenic Effects of Avian Leukemia Virus E26 on Chicken Hematopoietic Cells and Fibroblasts, Respectively, Correlate with Level of Expression of the Provirus

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**We have investigated the effect of E26, an avian leukemia retrovirus, on the growth properties of chicken embryo fibroblasts (CEFs). E26-infected CEFs were not transformed, according to several transformation parameters, but exhibited an activated growth in vitro. They started to grow without latency in serum-supplemented medium, maintained long-term growth in regular or low-serum medium, and could grow when seeded at low cell density in low-serum medium. We compared the integration and the level of expression of the proviral DNA in E26-infected CEFs and E26-transformed hematopoietic cells. An average of two provirus copies were found in each kind of cells. However, whereas high contents of both viral mRNA and E26-specific protein products were found in transformed hematopoietic cells, we detected only low amounts of viral mRNA and no E26 protein in infected CEFs. These data show that the level of expression of the E26 provirus is lower in CEFs than in hematopoietic cells. They suggest that transformation efficiency of the virus depends on its level of expression.**

Several lines of evidence suggest that proto-oncogenes are involved in the intracellular events that govern cell proliferation. Some of their viral counterparts, namely, viral oncogenes, could act in the infected cells by altering regulation of cell proliferation. It has been proposed that the oncogenes whose protein products share nuclear localization might have in common the power to convert a cell of limited replication potential in vitro into one that can be passaged without limit in culture (2, 30).

The E26 retrovirus genome includes a fused gene made, from 5′ to 3′, of residual *gag* nucleotides associated to v-*myb* and v-*ets* sequences (14, 19). A unique 5.7-kilobase (kb) genomic mRNA encodes a 135-kilodalton *gag-myb-ets* protein (the $p135^{gag\text{-}myb\text{-}ets}$) (14, 19) that is localized in the nucleus (13).

After infection with E26, chickens develop mixed leukemias involving both myeloid and erythroid lineages (17). In vitro, E26 may transform bipotent uncommitted hematopoietic cells as well as myeloid and erythroid committed progenitor cells (18, 21).

Moreover, E26 retrovirus is, with avian myeloblastosis virus, the only avian defective leukemia virus which does not give any solid tumors in chickens and is unable to transform chicken embryo fibroblasts (CEFs) in vitro (3, 16). In contrast, avian erythroblastosis virus (AEV) can transform both CEFs and erythroid progenitor cells (10, 23). However, Graf et al. (11) claimed that E26 is able to transform quail embryo fibroblasts in vitro. In view of this latter result and the nuclear localization of the $p135^{gag\text{-}myb\text{-}ets}$, we decided to reinvestigate the effect of E26 on CEFs.

In this work, we present evidence that upon infection with E26, CEFs are not fully transformed, but acquire increased growth potential in vitro. Moreover, in contrast to normal CEFs, the E26-infected CEFs can grow in medium containing a reduced amount of serum. We show that there is a weak expression of the provirus in the E26-infected CEFs, compared with its high expression in E26-transformed hematopoietic cells.

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## MATERIALS AND METHODS

**Viruses and cells.** E26 leukemia virus, pseudotyped with its natural helper E26-associated virus (E26AV), and E26AV were obtained from C. Moscovici (University of Florida, Gainesville). A virus stock of E26 was prepared from a single myeloid colony obtained from in vitro infection of chicken bone marrow cells. AEV (RAV-1) was collected from CEF cultures cotransfected with the pAEV-11 and pRAV-1 plasmid DNAs (6a).

Primary CEFs were prepared as described previously (Gandrillon et al., in press) from 10-day-old C/E Spafas chicken embryos provided by Rhône-Mérieux (Lyon, France). The cells were grown in regular CEF medium containing 6% newborn calf serum or in low-serum medium containing 0.5% fetal calf serum. For infection, $10^6$ secondary CEFs were seeded in a 60-mm petri dish. After 4 to 12 h, 0.5 ml of virus stock suspension was added. Two days later, the CEFs were passaged. E26-transformed myeloblasts were obtained by infecting chick bone marrow cells with E26 virus as described earlier (18).

The release of transforming particles from AEV- or E26-infected CEFs was tested by incubating 0.1 ml of these supernatants with $2 \times 10^5$ chicken bone marrow cells. The infected cells were then incubated in semisolid methylcellulose medium containing appropriate growth factors. Details of this procedure have been described elsewhere (18).

**Long-term growth kinetics.** Cells growing in regular CEF medium were seeded at $5 \times 10^5$ cells per 60-mm dish. Medium was changed every other day. Every 3 to 4 days, the cultures were trypsinized and the cells were reseeded at the same initial density. The cumulative number of cells at each passage was calculated, taking into account the fact that at each passage only a fraction of the culture was reseeded. Cells grown in low-serum medium were seeded at $7.5 \times 10^5$

cells per 60-mm dish, and the medium was changed every day. These cultures were passaged once a week, and the cumulative growth curve was calculated as described above.

**Colony assay in semisolid medium.** The fibroblast colony assay was performed according to the procedure described by Dodge and Moscovici (6), except we used Ham's F-10 medium without additional folic acid, vitamins, or conditioned medium. Cell suspensions were diluted to either $5 \times 10^3$ or $5 \times 10^4$ cells per 35-mm dish. Colonies were scored 12 to 16 days later.

**Growth at low cell density under low-serum agar overlay.** Infected or uninfected CEFs ($5 \times 10^4$) were seeded in 0.5% serum medium in 35-mm dishes. After 18 to 24 h the medium was removed and a soft agar layer was added over the cells. This contained Ham's F-10 medium supplemented with 10% tryptose phosphate broth (Difco), 0.5% fetal bovine serum (Seralab), and 0.36% agar (Difco). One week later, the agar layer was removed, cells were rinsed once with 0.5% serum medium, and a fresh soft agar layer was added. After a further week, agar was removed and the dishes were stained with Wright-Giemsa.

**Hexose uptake.** Cells were seeded at $2 \times 10^5$ cells per 35-mm dish. After 24 h the cell layer was washed three times with phosphate-buffered saline without calcium or magnesium and was then incubated for 15 min at 37°C in 2 ml of phosphate-buffered saline containing 4 μCi of [$^3$H] deoxyglucose (Amersham; 5.7 Ci/mmol). Thereafter, cells were washed three times with phosphate-buffered saline and covered with 1 ml of distilled water. After a few minutes, the supernatants containing the cell lysate were recovered, and duplicate samples were then processed for scintillation counting. Cells from dishes seeded in parallel were trypsinized and numbered. The radioactive counts were standardized for an equal number of cells, and the incorporation in infected CEFs was expressed by reference to that in uninfected control CEFs.

**Detection of actin cables.** CEFs were seeded in Labtek slides. After 24 h the cells were fixed for 30 min in 4% paraformaldehyde and then permeated for 30 min with 0.2% Triton X-100. The slides were incubated with antiactin antibody (Bio-Yeda) for 45 min, washed with phosphate-buffered saline, and then incubated with a fluorescein isothiocyanate-conjugated $F(ab')_2$ goat anti-rabbit immunoglobulin G (heavy plus light chain; Zymed Laboratories Inc.) for 30 min at room temperature. After washing, the slides were observed under UV light through a Nikon Labophot microscope.

**Protein labeling and immunoprecipitation.** For protein labeling and immunoprecipitation, we used procedures previously described by Ghysdael et al. (7, 8). Cells in 100-mm dishes were rinsed once with 5 ml of methionine-free minimal essential medium (GIBCO) supplemented with 5% dialyzed calf serum (GIBCO). Cells were incubated for 1 to 6 h in this medium with 0.2 mCi of [$^{35}$S]-methionine (1,000 Ci/mmol; Amersham). Cells were then washed in TNE buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA) and lysed with 3 ml of ice-cold RIPA buffer (10% TNE, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% Trasylol [Sigma]). After centrifugation of the lysate at $100,000 \times g$ for 1 h, the supernatant was collected, and total incorporation of [$^{35}$S]methionine into cellular proteins was determined by trichloroacetic acid precipitation and scintillation counting.

An amount of each lysate equivalent to $2 \times 10^6$ cpm was incubated for 1 h with 4 μl of antibody. The antibody complexes were precipitated by the addition of 50 μl of protein A-Sepharose (Pharmacia). Immunoprecipitates were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel. Radiolabeled protein standards (Amersham) were used as size markers.

Antibody against viral *gag* proteins, anti-*ets* antibody, and anti-*ets* antibody adsorbed by preincubation with an excess of the bacterially expressed *ets* peptide (8) were generously provided by J. Ghysdael (Lille, France).

**DNA probes.** The v-*ets* probe was a 0.7-kb *Pst*I fragment kindly provided by D. Leprince (Lille, France). The *myb* probe HAX-4, obtained from M. Baluda (University of California, Los Angeles), was a 1-kb *Bam*HI fragment. Probes were amplified after subcloning into plasmids. They were released from the plasmids by cutting with the corresponding enzymes and purified by electroelution from agarose gels.

**DNA analysis.** Trypsinized uninfected or E26-infected CEFs and E26-transformed chicken myeloblasts were lysed overnight in a solution containing 0.6% SDS, 10 mM EDTA, and 150 μg of proteinase K (Boehringer) per ml. DNAs were precipitated in ethanol at −20°C in the presence of 200 mM NaCl and then pelleted, dried, and suspended in water. They were then extracted with phenol-chloroform and chloroform–isoamyl alcohol successively. Dried DNAs were suspended in TE buffer (10 mM Tris, pH 7.5, and 1 mM EDTA).

Purified cellular DNAs were digested by either *Eco*RI, *Hin*dIII, or a mixture of *Bam*HI and *Hin*dIII. Samples of 15 μg of digested DNA were electrophoresed on agarose gels and transferred to cellulose nitrate filters according to Southern's procedure (25).

**RNA analysis.** Total RNAs were extracted from either uninfected CEFs, E26-infected CEFs, or E26-transformed chicken myeloblasts by homogenization in a solution containing 1% SDS, 200 μg of proteinase K per ml, 20 mM Tris hydrochloride (pH 7.4), 150 mM NaCl, and 200 mM EDTA. The homogenate was centrifuged overnight through a CsCl gradient at 20°C at 25,000 rpm in an SW27 rotor (Beckman) as described by Glisin et al. (9). For Northern blot (RNA blot) analysis, 10 μg of total RNA was electrophoresed on a 1.2% agarose gel containing 1× MOPS (morpholinepropanesulfonic acid) buffer and 3% formaldehyde and then transferred to a nitrocellulose filter pre-equilibrated in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as described by Maniatis et al. (15). rRNAs electrophoresed in parallel were used as size markers.

**DNA probe labeling and hybridization.** The DNA probes were labeled by nick translation with [$^{32}$P]-dTTP according to Maniatis et al. (15). Hybridizations of Southern and Northern blots were performed for 48 h at 42°C with $1 \times 10^6$ to $2 \times 10^6$ cpm of labeled probe per ml in a final hybridization solution containing 50% formamide, 3× SSC, 50 mM Tris hydrochloride (pH 7.5), 20 μg of tRNA per ml, 20 μg of denatured salmon sperm DNA per ml, 1 mM EDTA, and 1× Denhardt reagent. After hybridization, the nitrocellulose blots were washed for 1 h in 2× SSC at 42°C and then twice for 30 min each in 0.1× SSC–0.1% SDS at 50°C; they were then rinsed with 0.1× SSC, dried, and exposed to Kodak X-ray film.

## RESULTS

**In vitro growth kinetics of E26-infected CEFs.** Secondary CEFs, growing in regular medium containing 6% newborn calf serum, were infected with various viruses. The infected cells were passaged twice to allow virus spreading. Cells were finally seeded at $5 \times 10^5$ cells per 60-mm dish. This time

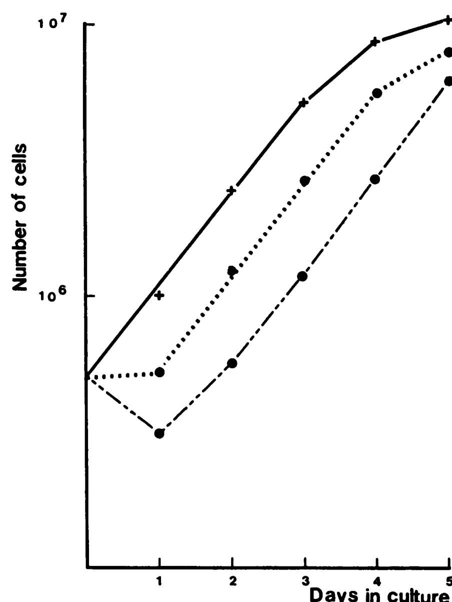


FIG. 1. Growth kinetics of CEFs infected with E26 (+——+) or E26AV (●– – – –●), or uninfected CEFs (●·····●).

was considered as day 0 for the growth analysis. At the times indicated in Fig. 1, two culture plates within each series were trypsinized and the cells were counted. It can be seen from the growth curves that the E26-infected CEFs started to multiply immediately after seeding whereas the control uninfected or E26AV-infected CEFs displayed some growth latency. The same curves were obtained whether the cells were subcultured from confluent or exponentially growing cultures (data not shown). The lag in the growth of normal CEFs was regularly seen when the cells were seeded at this density (6a). The difference in the onset of growth between control uninfected and E26-infected CEFs was not the result of different efficiencies of attachment. Six hours after seeding, the same number of CEFs, i.e., $4.5 \times 10^5$, attached in both cultures. In contrast, in the cultures of CEFs infected by E26AV alone, nearly 40% of the cells did not attach during day 1. This effect probably results from a cytotoxic effect produced by E26AV on CEFs, which has been observed with several other helper viruses (not shown). During the exponential growth period, all the cultures exhibited the same doubling time (22 h) and reached the same saturation density.

Infection of the CEFs by E26 seems therefore to activate the onset of growth.

**Long-term growth kinetics of E26-infected CEFs.** Since E26 virus seems to activate the growth of CEF cultures, we examined whether it had any effect on the long-term growth potential of CEFs.

Cultures were prepared as described above, but were then passaged every 3 to 4 days before reaching confluence. A culture of CEFs transformed by AEV was included in this experiment as a reference. All the cultures were passaged at the same time. The cumulative growth curves are presented in Fig. 2. The uninfected and E26AV-infected CEFs continued to multiply for 6 weeks, corresponding to 14 passages after infection. At this point their growth rate was reduced, and the cells became unhealthy and entered senescence. From the time the cells were explanted in primary cultures, these cells had undergone about 20 cell generations. In contrast, the AEV- or E26-infected CEFs showed a faster growth rate; after 6 to 7 weeks, the total numbers of cells produced were 10,000- and 1,000-fold higher, respectively, than in uninfected or E26AV-infected cultures. It was determined that the AEV- and E26-infected CEFs had undergone, respectively, 33 and 30 generations. After this passage level, the cells could still be maintained as growing cells for 2 to 3 weeks, after which their growth slowed down and finally stopped. The AEV-transformed CEFs then detached from the dishes, and the cultures could not be maintained. In contrast, the E26-infected CEFs remained attached, and although they grew slowly, if at all, they looked healthy and could be kept for a further 3 to 4 months in these conditions. These results show that, upon infection with E26, CEFs acquire enhanced growth potential and increased life span.

**Long-term growth kinetics of E26-infected CEFs in low-serum medium.** Since E26 seemed to activate the growth of infected CEFs, we studied these cells' dependence upon serum growth factors.

Uninfected CEFs or CEFs infected by different viruses were passaged four times in regular medium and then seeded in low-serum medium containing 0.5% fetal calf serum as the only source of serum. The cells were then passaged once a week. The growth curves are shown in Fig. 3. Zero time in the curves is taken as the time when the cells were passaged from regular medium to low-serum medium. After 1 week, no significant changes were observed in cell numbers in any culture. However, upon subsequent passages, not all the CEFs from the uninfected cultures readhered to the dishes, and the cells that attached did not show any sign of growth.

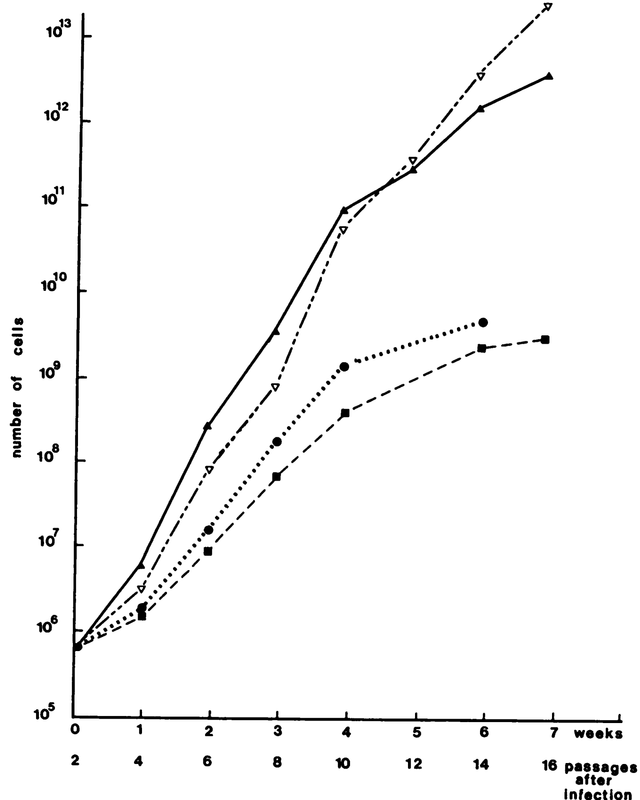


FIG. 2. Long-term growth kinetics of CEFs in regular medium containing 6% serum. The cells were passaged every 3 to 4 days and reseeded at $5 \times 10^5$ per plate. Numbers of cells are cumulative. Growth of uninfected CEFs (●) or CEFs infected with E26(▲), E26 AV (■), or AEV (▽).

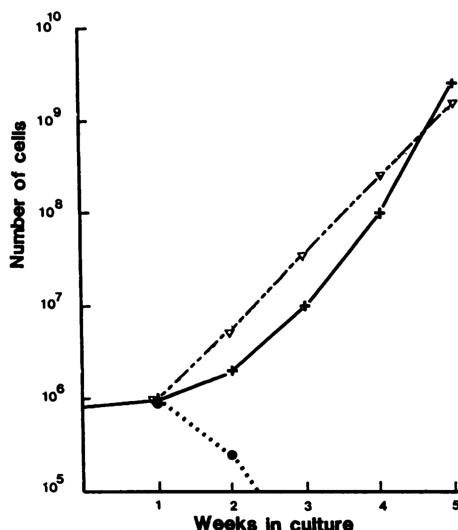


FIG. 3. Long-term growth kinetics of CEF in medium containing 0.5% serum. The cells were passaged once a week and reseeded at $7.5 \times 10^5$ per plate. Numbers of cells are cumulative. Growth of uninfected CEFs (●) or CEFs infected with E26 (+) or AEV (▽).

In low-serum medium, normal CEFs do not grow and enter quiescence (6a, 27). It is therefore likely that the inability of the cells to attach to the dishes was related to their quiescent state. However, we could not rule out an increased sensitivity of these cells to deleterious effects of trypsin. In contrast to normal CEFs, the CEFs infected with either AEV or E26 could be passaged and, after a 1-week lag, showed a sustained growth for at least 10 cell generations. These results show, therefore, that CEFs infected by E26 or AEV can overcome quiescence induced by serum deprivation.

**E26-infected CEFs can grow at low cell density in growth factor-depleted medium.** The E26-infected CEFs exhibited growth advantages over normal CEFs. We investigated further whether they would grow in stringent conditions which restrict the growth of normal cells. Infected or uninfected CEFs were seeded at low cell density ($4 \times 10^4$ cells per 35-mm dish) in low-serum medium. Twenty-four hours later, the cells were covered with a soft agar overlay containing low-serum medium. Crude agar has been shown to inhibit the growth of normal cells, presumably by trapping growth factors (27). We expected in these conditions that only residual traces of growth factors would be available to the cells. Two weeks after seeding, dense cell foci were observed in E26- and AEV-infected CEF cultures, whereas no focus ever developed in uninfected or E26AV-infected cultures (Fig. 4). The E26-infected CEFs that developed in these conditions displayed a fusiform shape and grew in parallel arrays (Fig. 4F), whereas the AEV-transformed CEFs showed a typical criss-cross growth pattern (Fig. 4E).

**Phenotype of the E26-infected CEFs.** The growth properties of E26-infected CEFs were very similar to those of CEFs transformed by AEV. However, previous reports have suggested that E26 virus does not transform CEFs (11; C. Moscovici, personal communication). We therefore analyzed our E26-infected CEFs for several features associated with the transformed state. The cells were assayed at the ninth passage after infection.

**(i) Morphology.** In liquid culture, the AEV-infected CEFs were refringent with a typical elongated shape and showed

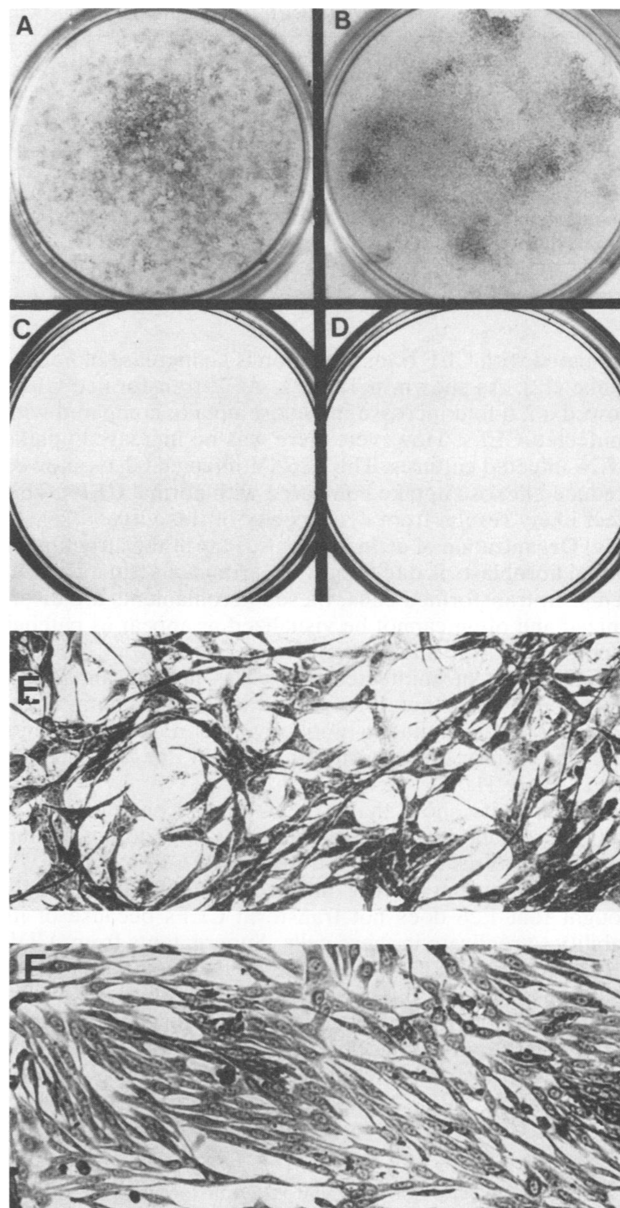

FIG. 4. Growth under low-serum agar overlay of CEFs infected with E26 (A), AEV (B), or E26AV (C), or uninfected CEFs (D). Photomicrographs of (E) the AEV- and (F) E26-infected cultures; magnification, ×485. Dishes were stained with hematoxylin and counterstained with Wright-Giemsa.

the characteristic criss-cross growth pattern of transformed fibroblasts. E26-infected CEFs were flat but slightly more fusiform than uninfected fibroblasts and grew in parallel arrays.

**(ii) Anchorage-independent growth.** Transformed fibroblasts gave rise to colonies when seeded in suspension in semisolid medium. We seeded the uninfected or infected CEFs in regular growth medium containing 0.36% agar. Colonies were counted 2 weeks later. Ten percent of the AEV-infected CEFs gave rise to typical transformed colonies, whereas no colony was ever detected with either uninfected or E26-infected CEFs (Table 1). This observation is in agreement with that previously published (11).

**(iii) Hexose uptake.** One of the changes most consistently

TABLE 1. Phenotype and growth properties of normal and infected CEFs

| CEFs | Growth (maximum no. of generations) | | Growth under low-serum semisolid medium | Cell morphology | Growth pattern in liquid culture | No. of colonies formed in soft agar (per $5 \times 10^4$ plated cells) | Relative hexose uptake | % of cells with organized actin microfilaments |
|---|---|---|---|---|---|---|---|---|
| | In presence of serum | Low-serum medium | | | | | | |
| E26AV infected | 20 | 0 | − | Flat | Parallel arrays | 0 | 0.3 | 80 |
| AEV transformed | 33 | >10 | + | Refringent, fusiform | Criss-cross | 5,700 | 2.6 | 5 |
| E26 infected | 30 | >10 | + | Flat, fusiform | Parallel arrays | 0 | 0.6 | 0 |
| Noninfected | 20 | 0 | − | Flat | Parallel arrays | 0 | 1 | 75 |

associated with CEF transformation is an increase of hexose uptake (12). As shown in Table 1, AEV-transformed CEFs showed a 2.6-fold increase in hexose uptake compared with uninfected CEFs. However, there was no increased uptake in E26-infected cultures. The E26AV-infected CEFs showed a reduced hexose uptake compared with normal CEFs. This effect likely results from cytotoxicity of this virus.

**(iv) Organization of actin filaments.** Part of the structure of normal fibroblasts is due to ordered arrays of actin microfilaments. In transformed cells, these microfilaments are disorganized and often cannot be visualized or appear as patches dispersed within the cells. The organization of actin microfilaments in uninfected as well as in E26- and AEV-infected cells was therefore studied by indirect immunofluorescence using antiactin antibody. Actin microfilaments were not seen in the majority of the AEV- or E26-infected CEFs (Table 1).

These results show that except for the organization of actin microfilaments, the E26-infected CEFs do not exhibit the other features specific to transformed CEFs.

**Virus production by the infected CEFs.** It is generally thought that E26 does not transform CEFs because of its inability to replicate in these cells. Supernatants from AEV- or E26-infected CEFs were collected between the first and eighth passage after infection and used to infect bone marrow cells from normal chickens. The transforming efficiency of the viruses on hematopoietic cells was assessed by the number of transformed colonies in infected bone marrow cultures (Table 2). Supernatants collected from AEV-infected CEFs grown for eight passages were still able to transform erythrocytic bone marrow target cells with the same efficiency. In contrast, bone marrow transformation with the supernatants of E26-infected CEFs decreased with continued passage of the infected CEFs. The titer of the E26 virus rescued from E26-infected CEFs after eight passages was about 10 times lower than that of the original virus stock suspension rescued from transformed myeloid cells. These results show that E26 virus replicates poorly in CEFs.

**Expression of the p135$^{gag\text{-}myb\text{-}ets}$ protein.** The effects of E26 on the growth of CEFs may be mediated through its gene product, p135$^{gag\text{-}myb\text{-}ets}$. We therefore investigated the presence of this protein in E26-infected cells.

E26-infected CEFs, eight passages after infection, and E26-transformed myeloblasts were labeled with [$^{35}$S]methionine and then lysed. The cell proteins were immunoprecipitated with either anti-*gag* or anti-*ets* antibodies or with anti-*ets* antibody previously incubated with an excess of the bacterially synthesized *ets* peptide. They were then analyzed by electrophoresis on a 10% polyacrylamide-SDS gel. The expected 135-kilodalton E26-specific protein was seen in E26-transformed myeloblasts after immunoprecipitation with anti-*ets* (Fig. 5, lane 1) but not with adsorbed antiserum (Fig. 5, lane 2). The p135 was never detected in lysates of E26-infected CEFs (Fig. 5, lane 4), whereas in the same cells the anti-*ets* antibody revealed the c-*ets* products at 54, 62, and 64 kilodaltons (7, 8). Protein p135 could not be detected in E26-infected CEFs even when the labeling period was increased from 1 to 3 or 6 h (not shown) or when the analysis

TABLE 2. Detection of AEV and E26 transforming particles in supernatants of infected CEF cultures[a]

| Origin of the viruses | No. of transformed colonies |
|---|---|
| Original E26 stock suspension[b] | 64[c] |
| Supernatant of E26-infected CEFs collected after[d]: | |
| 1 | 11[c] |
| 2 | 7[c] |
| 8 | 5[c] |
| Original AEV stock suspension[e] | 7[f] |
| Supernatant of AEV-transformed CEFs collected after eight passages[d] | 12[f] |

[a] All the virus samples were assayed in parallel in the same experiment. Numbers of transformed colonies are given for $2 \times 10^5$ chicken bone marrow cells infected with 0.1 ml of the tested suspension.
[b] Collected from E26-transformed myeloblasts.
[c] The hematopoietic lineage of the transformed colonies was not determined.
[d] Number of passages after infection.
[e] Collected from AEV-transformed erythroleukemic cells.
[f] Erythroid transformed colonies.

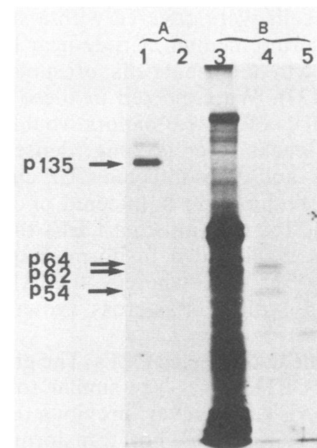


FIG. 5. Immunoprecipitation analysis of E26-infected cells. E26-transformed myeloblasts (lanes 1 and 2) and E26-infected CEFs (lanes 3, 4, and 5) were immunoprecitated with anti-*ets* antiserum (lanes 1 and 4), anti-*ets* preincubated with bacterial *ets* peptide (lanes 2 and 5), or an antibody raised against viral *gag* proteins (lane 3).

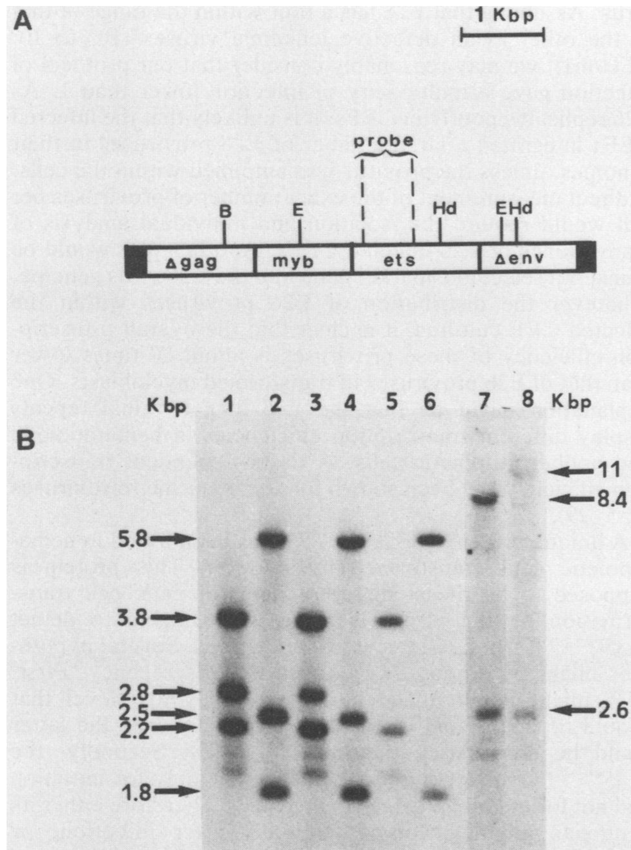


FIG. 6. (A) Schematic restriction endonuclease map of E26: E, *Eco*RI; B, *Bam*HI; Hd, *Hin*dIII. (B) Southern blot analysis of DNAs extracted from E26-transformed myeloblasts (lanes 1, 2, and 7), E26-infected CEFs (lanes 3, 4, and 8), and uninfected CEFs (lanes 5 and 6). DNAs digested with *Eco*RI (lanes 2, 4, and 6), *Hin*dIII (lanes 7 and 8), or a *Bam*HI-*Hin*dIII mixture (lanes 1, 3, and 5) were hybridized with a v-*ets*-specific probe. The weakness of the 11- and 8.4-kb bands in lane 8 is likely due to bad transfer.

was performed on CEFs at either 1, 4, or 11 passages after infection. These results show that the p135 is synthesized at an extremely low level, if at all, in E26-infected CEFs.

The numerous and intense bands obtained when E26-infected CEF lysate was immunoprecipitated with the antibody against viral *gag* proteins (Fig. 5, lane 3) clearly show that the helper virus proteins were actively produced in these cultures.

The absence of p135 in E26-infected CEFs could be explained by either of two possibilities. Either (i) only a few CEFs among the whole population have integrated and replicate the virus, or (ii) the majority of CEFs have integrated the provirus, but virus replication remains low because of transcriptional or posttranscriptional regulation. To determine which of these hypotheses is correct, we analyzed the integration of proviral E26 DNA and the production of viral E26 RNA in infected CEFs.

**Analysis of E26 proviruses in infected CEFs.** DNA was extracted from uninfected or E26-infected CEFs seven passages after infection and from cloned myeloblasts transformed by E26. After digestion with either *Eco*RI or a mixture of *Bam*HI and *Hin*dIII, the DNA was fractionated by electrophoresis on agarose gels and transferred to nitrocellulose filters. The endogenous c-*ets* and proviral v-*ets* sequences were revealed by hybridization with the v-*ets* probe shown in Fig. 6A.

According to the E26 restriction map (Fig. 6A), we expected a 2.5-kb viral band after digestion with *Eco*RI and a 2.8-kb viral band after digestion with the *Bam*HI-*Hin*dIII mixture. Figure 6B shows indeed that these two specific bands were present in the DNAs isolated from either E26-infected CEFs or E26-transformed myeloblasts, but absent in the DNA isolated from uninfected CEFs. Furthermore, all these DNAs shared common bands corresponding to part of the c-*ets* gene. We detected two c-*ets* bands at 5.8 and 1.8 kb after digestion with *Eco*RI and two major bands at 3.8 and 2.2 kb and a minor band at 1.9 kb after digestion with the *Bam*HI-*Hin*dIII mixture. These data match only partially with previous published results (14, 20, 26), probably due to the use of a different probe.

Figure 6B shows that in all cases the intensities of the proviral v-*ets* bands were at the level of those of the c-*ets* bands. There are, therefore, an average of two copies of E26 provirus per diploid genome in E26-infected CEFs as well as in E26-transformed myeloblasts. Analysis of the cell DNAs digested with *Hin*dIII should reveal provirus junction fragments. In these conditions, our v-*ets* probe should identify only one E26 junction fragment. This includes all the virus genome upstream from the *Hin*dIII restriction site within the *ets* domain, up to the first *Hin*dIII site within the adjacent cellular DNA; its predicted size should be over 4 kb (Fig. 6A).

It can be seen in Fig. 6B (lanes 7 and 8) that after digestion with *Hin*dIII, DNAs isolated from either E26-infected CEFs or E26-transformed myeloblasts share common bands of 8.4 and 2.6 kb, respectively. These bands probably represent c-*ets* sequences. In myeloblast DNA, the 11-kb band represents a junction fragment expected from the clonal origin of these cells. In E26-infected CEFs, no specific band greater than 4 kb could be detected, suggesting that these cells are not of clonal origin.

**Transcription of the E26 provirus in infected CEFs and transformed myeloblasts.** The expression of the E26 proviral genome was compared in E26-infected CEFs and E26-transformed myeloblasts. Total RNAs were extracted from E26-infected CEFs eight passages after infection or from uninfected secondary CEFs as controls. In parallel, RNAs were isolated from the same E26-transformed myeloblasts as those used for DNA analysis. The RNAs were fractionated by electrophoresis on agarose gel, transferred to nitrocellulose filters, and hybridized with either a v-*ets* or v-*myb* probe.

The v-*ets* probe identified a unique RNA transcript of 5.7 kb in RNAs isolated from E26-infected cells (Fig. 7). No signal was detected in noninfected CEFs. This transcript was identical to the genomic RNA described by Nunn et al. (20). The v-*myb* probe revealed exactly the same transcript in E26-infected CEFs, thus confirming the nature of this RNA as the E26 viral transcript. A large difference in the amount of viral E26 transcript was observed between myeloblasts and E26-infected CEFs. A similar signal intensity was observed when 1 μg of total RNA from transformed myeloblasts was compared with 20 μg of total RNA from E26-infected CEFs, suggesting that the average amount of E26 viral transcripts in infected CEFs was about 20-fold lower than in myeloblasts.

## DISCUSSION

The present study investigated the effect of E26 virus in CEFs. In agreement with other reports (11), we have shown

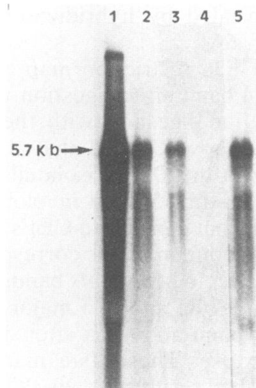


FIG. 7. Northern blot analysis of RNAs extracted from E26-transformed myeloblasts (5 μg, lane 1, and 1 μg, lane 2), E26-infected CEFs (20 μg, lanes 3 and 5), and uninfected CEFs (20 μg, lane 4). RNAs were hybridized with a v-*ets*-specific (lanes 1 through 4) or a v-*myb*-specific (lane 5) probe.

that this virus does not induce in these cells the expression of all the features usually associated with transformation (Table 1). In particular, the E26-infected CEFs did not show anchorage-independent growth, increased hexose uptake, or disorganized growth in liquid culture. Nonetheless, they exhibited an altered actin network and a fusiform morphology not seen in normal CEFs. Moreover, we have shown that E26 activates the growth of infected CEFs. Indeed, in contrast to normal CEFs, the E26-infected CEFs did not exhibit any growth latency upon replating and showed an increased life span and growth potential in vitro in either regular or low-serum medium. In addition, these cells could grow under an agar overlay at low cell density and in growth factor-depleted medium. All the growth properties of E26-infected CEFs are similar to those of CEFs transformed by AEV. These data suggest, therefore, that the E26-infected CEFs exhibit a phenotype intermediate between those of normal and transformed CEFs. Most of the growth properties of E26-infected CEFs are reminiscent of those of immortalized rodent fibroblasts (22, 28). However, we have no evidence that E26 induces immortalization of CEFs. No growth activation was ever observed after infection of CEFs with E26AV, which strongly suggests that E26 virus per se is responsible for the biological effect. Thus, either the virus induces alterations in growth control mechanisms in the infected cells, or it induces the selective growth of a subset of CEFs with a naturally enhanced growth potential. Primary CEFs have been shown to be heterogeneous in their growth potential, with a low percentage of cells able to grow more than 35 generations in vitro (1). We might therefore assume that E26 induces the selective growth of those CEFs with a higher life span. The growth lag of E26-infected CEFs in low-serum medium might argue in favor of such a selective process.

The analysis of the E26 provirus in the DNA of infected CEFs was performed seven passages after infection. At this time, the number of E26-infected CEFs produced exceeded that of normal CEFs by a factor of 50 (Fig. 2). Although we found an average of nearly two copies of E26 proviruses per cell genome, we have no direct evidence that all CEFs in the E26-infected cultures contained a provirus. It is possible that a few cells contain a great number of copies of provirus. We consider this hypothesis as unlikely for the following reason. Cultures ($10^6$ CEFs) were infected with 0.5 ml of E26 stock virus. Assuming that E26 has a titer within the range of that of the other avian defective leukemia viruses ($10^5$ to $10^6$ CFU/ml), we may reasonably consider that our protocol of infection gave a multiplicity of infection lower than 1. As E26 replicates poorly in CEFs, it is unlikely that the infected CEFs integrated a large number of E26 proviruses in their genomes, unless the provirus was amplified within the cells. A direct measurement of the exact number of proviruses per cell would require the isolation and individual analysis of many clones of E26-infected CEFs. Another way would be to insert a selectable marker gene into the E26 virus genome. Whatever the distribution of E26 proviruses within the infected CEF cultures, it is clear that the overall transcription efficiency of these proviruses is about 20 times lower than that of E26 proviruses in transformed myeloblasts. One explanation might be that the E26 long terminal repeats display different transcription efficiencies in hematopoietic and nonhematopoietic cells. A tissue-dependent transcription efficiency has been shown for some murine retroviruses (4, 5, 29).

A unique protein, $p135^{gag\text{-}myb\text{-}ets}$, has been found in hematopoietic cells transformed by E26 (3). This protein is supposed to be the mediator of hematopoietic cell transformation by the virus (13). We were unable to detect $p135^{gag\text{-}myb\text{-}ets}$ in E26-infected CEF cultures. Several hypotheses might be proposed to explain this discrepancy. First, p135 might be produced at an extremely low level that should be below that of the c-*ets* products since the latter could be detected in the infected CEFs. Secondly, the $p135^{gag\text{-}myb\text{-}ets}$ protein might be necessary only for initiation and not for maintenance of CEF growth. Therefore either its synthesis might occur immediately after infection, or $p135^{gag\text{-}myb\text{-}ets}$ packaged in the virions would be sufficient to activate cell growth. The third possibility is that the E26 provirus encodes an as yet unidentified viral protein. We cannot exclude the occurrence of minor E26 transcripts derived from the main genomic RNA through splicing out of minute introns. Such transcripts might not be discernible on Northern blots. It is unlikely that induction of cell growth by E26 provirus results from insertional activation of cellular genes, since no preferential provirus integration site within the cell genome was detected.

These results suggest, therefore, that E26 might exert different effects in hematopoietic and nonhematopoietic cells. In hematopoietic progenitor cells the provirus is highly transcribed and induces leukemic transformation. In CEFs, the E26 provirus is poorly transcribed and induces activation of cell growth and expression by the cells of features intermediate between those of normal and transformed CEFs. It might be speculated that, according to its level of expression, the *myb-ets* oncogene product would induce either cell growth activation or cell transformation. Such a dosage effect has been demonstrated for the adenovirus type 2 Ela gene (24). However, we have no evidence that overexpression of the $p135^{gag\text{-}myb\text{-}ets}$ protein in CEFs would induce transformation. This analysis would require modifying the E26 genome to enhance its expression in CEFs or selecting infected CEF clones showing high expression of the viral protein.

We have recently shown that the v-*erbA* oncogene carried by AEV activates the growth of CEFs similarly to E26 (6a). Interestingly, the v-*erbA* and *myb-ets* oncogenic sequences seem to induce similar effects on CEFs and are both involved in erythrocyte progenitor cell transformation.

The E26 genome contains several domains. We do not know yet whether the same sequences are responsible for

hematopoietic transformation and CEF growth stimulation. An answer to this question requires the production of E26 mutants with mutations specifically localized in defined regions of the virus genome.

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