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Myeloblasts transformed by the avian acute leukemia virus E26 are hormone-dependent for growth and for the expression of a putative *myb*-containing protein, p135 E26

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Avian leukemia virus E26 contains the *myb* oncogene and transforms erythroid and myeloid hematopoietic cells *in vivo* and *in vitro*. E26-transformed nonproducer myeloblasts but not avian erythroleukemia virus (AEV)-transformed erythroblasts nor MC29-transformed macrophages were shown to be dependent for growth on factor(s) present in supernatants from Concanavalin A-stimulated chicken spleen cells. The same factor enhanced the synthesis of p135 E26, the candidate transforming protein of E26, but did not induce the synthesis of the transforming proteins of AEV and MC29 viruses nor that of helper virus-derived structural proteins. P135 E26 was shown to contain sequences related to the viral *gag* gene as well as sequences which may be related to the *myb* gene product. P135 E26 might constitute the first example of a viral *onc* protein whose synthesis is regulated directly or indirectly by an exogenous hematopoietic growth factor.

Key words: viral oncogenes/growth factors/differentiation/hematopoietic cells

Introduction

The avian leukemia virus strain E26 causes both erythroid and myeloid leukemia *in vivo* (Sotirov, 1981; Moscovici *et al.*, 1981; Radke *et al.*, in preparation). *In vitro*, it was originally described as inducing the outgrowth of transformed immature myeloid cells from infected cultures of chick bone marrow (Graf *et al.*, 1979; Beug *et al.*, 1979). Recently, however, the same virus was found to transform erythroid cells *in vitro* (Radke *et al.*, 1982) when infected bone marrow cells were cultivated under conditions allowing the survival and differentiation of normal erythroid progenitor cells, CFU-E. The genome of E26 contains the cell-derived *myb* oncogene together with part of the *gag* and *env* virus structural genes, and in addition contains an RNA sequence of unknown nature (Roussel *et al.*, 1979; Stéhelin *et al.*, 1980; Bister *et al.*, 1982).

Attempts to identify E26-coded protein(s) in transformed nonproducer myeloblasts by immunoprecipitation suggested the existence of a *gag*-related protein with a mol. wt. of 135 000 (Graf *et al.*, 1980; Hayman, 1981; Bister *et al.*, 1982). Two experimental problems, however, impeded further study of this E26-specific protein. First, E26 nonproducer myeloblast clones isolated from bone marrow Methocel cultures were difficult to grow in mass cultures, requiring the presence of a macrophage or fibroblast feeder layer (H. Beug, unpublished observations). Also, the few clones that could be propagated synthesized only minute amounts of the 135-K protein, thus precluding its analysis by peptide mapping.

Here we report that E26-transformed myeloblasts can be grown efficiently in medium containing growth factor(s) obtained from Concanavalin A (Con-A) stimulated chick spleen cells and that E26 myeloblasts synthesize enhanced amounts of the 135-K protein. This effect seems to be specific for immature hematopoietic cells of the myeloid lineage. A preliminary characterization of the 135-K protein by immunoprecipitation and tryptic peptide analysis is also presented.

Results

An activity in Con-A spleen supernatants stimulates the growth of E26-transformed myeloblasts but not AEV-transformed erythroblasts or MC29-transformed macrophages

During the course of experiments designed to identify factors that stimulate the growth of normal chicken hematopoietic cells, an activity was found in cell-free supernatants from Con-A-stimulated spleen cells ('growth factor') which not only stimulated the growth of normal myeloid cells (T. Graf, unpublished observations) but also promoted the growth of myeloblasts transformed by E26 and avian myeloblastosis (AMV) viruses. Using a semi-quantitative screening assay (described in Materials and methods) it was possible to obtain spleen cell supernatants that exhibited a significant growth stimulatory activity on E26 myeloblasts at concentrations of $<0.1\%$. The availability of these preparations allowed us to study the effect of the presumptive growth factor on E26-transformed myeloblasts more closely.

Figure 1 shows the growth curves of E26-transformed myeloblasts as a function of growth factor concentration. The doubling time of E26 myeloblasts maintained in the absence of growth factor was ~39 h and decreased to 14.5 h in the presence of 2% growth factor. Concentrations of 0.4 and 0.08% growth factor resulted in doubling times of 16 and 24 h, respectively, indicating that the cells respond to the factor in a concentration-dependent fashion. These results were confirmed by measuring DNA synthesis in the same cells pulse-labeled with [^{3}H]thymidine (data not shown). The stimulatory activity of the factor appears to be rather stable since a single addition of the factor to the cells enhanced their growth rate for at least 4 days. The E26 myeloblasts cultured in the absence of growth factor tended to aggregate and were smaller, more irregularly shaped, and less refractile than when cultured in the presence of growth factor.

To determine whether or not the growth factor also stimulates the multiplication of other types of virus-transformed hematopoietic cells, we tested its effects on AEV-transformed erythroblasts and MC29-transformed macrophages. As shown in Table I, it did not stimulate the growth of these other types of cells. On the contrary, especially with MC29-transformed macrophages, it was even slightly inhibitory. In the presence of growth factor, these cells became more adherent and resembled mature normal macrophages. No morphological changes were observed with AEV-transformed erythroblasts.

The growth factor preparations described greatly facilita-

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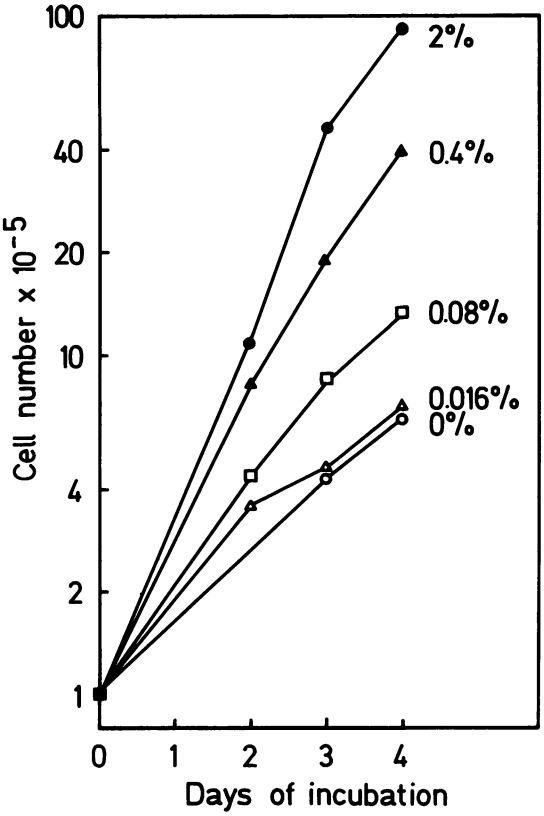

Fig. 1. Growth stimulation of E26-transformed myeloblasts by a growth factor obtained from Con-A-treated spleens. E26-transformed myeloblasts (producer clone 3/21) were seeded at 1 x 10^5 cells in 2 ml growth medium supplemented with the concentrations (v/v) of growth factor indicated. Where necessary, the cells were subcultured and the values obtained from counting corrected for the corresponding dilution factor. Cell numbers were determined using a Coulter Counter and represent the average of two determinations.

Table I. Effect of growth factor on the multiplication rate of hematopoietic cells transformed with E26, AEV, and MC29 viruses

Cell type	Clone No.	Increase in cell number with		Ratio $\frac{+GF}{-GF}$
		2% growth factor added	No growth factor added	
E26-myeloblasts	1	12.5[a]	2.4	5.2
	2	7.2	1.0	7.2
AEV-erythroblasts	1	7.2	8.1	0.9
	2	5.4	9.0	0.6
MC29-macrophages	1	4.2	10.2	0.4
	2	15.5	28.0	0.6

[a]Factor of cell multiplication respective to initial cell number seeded (5 x 10^4/ml), 3 days after seeding. Cells were washed once prior to their plating in standard growth medium. GF, growth factor. Values shown represent the mean of two independent determinations.

ted the isolation of E26 myeloblast clones exhibiting a long life span. To isolate nonproducer clones, which are useful for the characterization of E26 encoded proteins, bone marrow cultures were infected with E26 at a low multiplicity of infection and seeded in Methocel under standard conditions. Of 22 transformed colonies isolated 10 days after infection and seeded in growth medium plus 2% growth factor, 18 colonies could be propagated within 7 days to >2 x 10^7 cells each. This is in contrast to earlier trials, where >90% of the clonal cultures stopped growing 4–7 days after transfer to ordinary growth medium. Of the 18 colonies grown, 12 proved to be nonproducer colonies as determined by reverse transcriptase and cell transformation assays.

The growth factor enhances the synthesis of p135 in E26-transformed myeloblasts but not that of erb and myc proteins in AEV-transformed erythroblasts and MC29-transformed macrophages

To study whether or not the growth factor(s) contained in Con-A spleen supernatants influences the synthesis of the E26 specific 135-K protein (p135 E26), 2 x 10^6 E26 nonproducer cells were seeded in 2 ml of medium containing 5%, 2% or no growth factor and cultured for 2 days. A 3-fold stimulation of protein synthesis (relative to cell number) as measured by total incorporation of [^{35}S]methionine was observed in the cultures containing growth factor. After preparation of detergent lysates, aliquots containing the same amounts of total radioactivity were immunoprecipitated with an anti-*gag* serum. Figure 2 shows that only negligible amounts of p135 E26 were immunoprecipitated from cells cultured in the absence of growth factor, whereas in cells cultured with 2% and 5% growth factor, the synthesis of p135 E26 was stimulated 8- to 10-fold, respectively, as measured by densitometer tracings of fluorographs obtained from the gel. This effect was not restricted to the particular clone tested, since seven of nine other E26 nonproducer clones tested also showed a high expression of p135 E26 when cultured in the presence of growth factor (data not shown).

These results raised the possibility that the growth factor stimulates the synthesis of transforming proteins encoded by other acute leukemia viruses. We therefore tested the effect of growth factor on the synthesis of the *erb* gene products p75 AEV (*gag-erb*A), p65 AEV (*erb* B) and the *myc* gene product p110 MC29 (*gag-myc*). This was done by growing AEV-transformed nonproducer erythroblasts and MC29-transformed nonproducer macrophages with and without growth factor and analyzing them by immunoprecipitation. As can be seen from Figure 2, neither the synthesis of the *erb* proteins nor that of the *myc* protein was stimulated by culturing the corresponding cells in the presence of growth factor. On the contrary, there was even a slight decrease in the synthesis of p110 MC29.

The growth factor does not significantly induce the synthesis of gag-related virus structural proteins in E26-transformed producer myeloblasts

The results described raised the possibility that the growth factor leads to a general induction of the synthesis of virus-coded proteins in E26-transformed myeloblasts. We thus tested two E26-transformed clones which were superinfected by the helper virus RAV-2 and which therefore synthesized virus structural proteins in addition to p135 E26. As shown in Figure 3 and Table II, there was an 8-fold increase in the synthesis of p135, but only a 1.5- to 2-fold increase in the synthesis of p27, pr66, and pr180 when compared with pr60, a *gag* precursor protein chosen arbitrarily as an internal standard. Thus, the growth factor stimulates the synthesis of the E26 candidate transforming protein 4- to 8-fold more than it does the helper virus-derived *gag* proteins.

P135 E26 is probably a gag-myb fusion protein

To determine the relationship of p135 to other virus structural proteins, detergent lysates prepared from [^{35}S]methio-

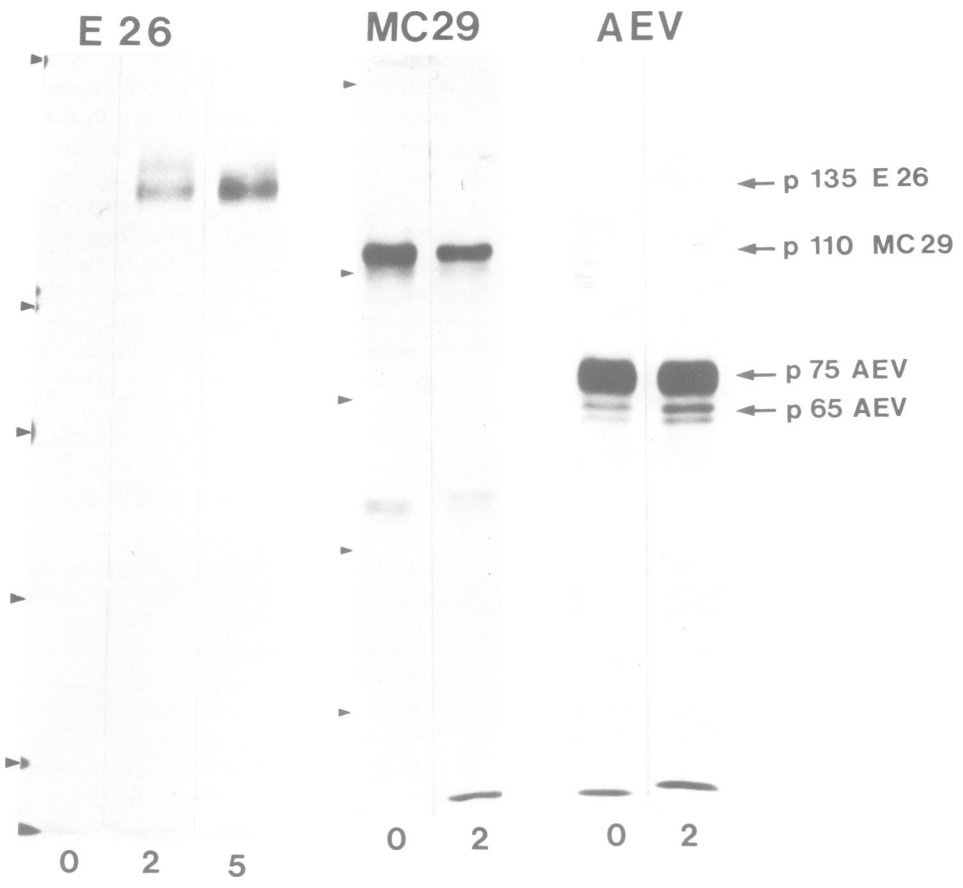

Fig. 2. Effect of growth factor on the expression of p135 E26. Extracts from cells labeled for 2 h with [^{35}S]methionine were immunoprecipitated with an anti-*gag* serum (Hayman, 1978) for E26 and MC29 cells and for AEV cells with a rat serum (ATia 3/1) directed against *erbA* and *erbB* proteins (Hayman *et al.*, unpublished data). The numbers shown on the bottom of each track indicate the percentage of growth factor which had been used during culturing of the cells for 3 days at 37°C. The mol. wt. markers indicated by arrows correspond to 200 K, 92 K, 69 K, 45 K and 30 K (from top to bottom). For the E26-transformed cells nonproducer clone 10 was used; the MC29- and AEV-transformed nonproducer cells used were clone 9 and LSCC HD3 cells, respectively. A doublet in the region of p135 E26 in the track corresponding to E26 cells grown in the presence of 2% growth factor was occasionally also seen with other E26-transformed cells immunoprecipitated with anti-*gag* serum. It is not dependent on the clone used.

nine-labeled E26 nonproducer myeloblasts grown in the presence of 2% growth factor were immunoprecipitated with various antisera. As shown in Figure 4A, p135 was precipitated by anti-*gag* sera (lanes 2 and 3) but not by anti-glycoprotein or by anti-reverse transcriptase serum (lanes 4 and 5). These results suggest that p135 E26 contains sequences related to the *gag* gene product but no sequences related to the *pol* and *env* gene product of avian retroviruses.

Immunoprecipitated p135 was also subjected to two-dimensional tryptic peptide fingerprinting (Hayman *et al.*, 1979a). Figure 4B shows that it contains peptides which are related to the *gag*-protein p19 when compared with tryptic peptide maps of pr76 (Hayman *et al.*, 1979a,1979b). In addition, at least five peptides were detected that are unrelated to any of the other *gag* proteins. These peptides also differed in mobility from the tryptic peptides of the *env*- and *pol*-derived viral structural proteins, from the *erb*A peptides obtained from p75 AEV and from *myc* peptides of p110 MC29 and p90 CMII that we have characterized previously (Kitchener and Hayman, 1980; Hayman *et al.*, 1979a,1979b). These novel peptides might therefore correspond to the *myb*-related portion of the p135 E26 protein.

A characteristic property of avian retrovirus-coded *gag-onc* fusion proteins is that they can be cleaved into defined fragments by the virus-coded protease p15 (Vogt *et al.*, 1979). When p135 was subjected to p15 digestion as described earlier (Beug *et al.*, 1981), three major fragments with mol. wts. of 32, 78, and 100 K were obtained. No traces of the *gag* proteins p17, p19/15, or p12 could be detected, suggesting that p135 contains only a small part of the *gag* gene coded sequences (data not shown).

Discussion

Our results demonstrate a correlation between the induction of cell proliferation and the induction of p135 in E26-transformed myeloblasts by a growth factor(s) from Con-A-treated spleen cultures. The factor seems to affect selectively the growth of early myeloid cells, since AEV-transformed erythroblasts and MC29-transformed macrophages are not stimulated. In addition, the factor induces the growth of AMV-transformed myeloblasts and the formation of large granulocyte-macrophage colonies from normal bone marrow but does not affect the formation of CFU-E colonies (T. Graf, unpublished results). Structural proteins synthesized by helper virus in E26-transformed myeloblasts are not significantly enhanced. The factor also does not stimulate the synthesis of *erb* and *myc* proteins in AEV-transformed erythroblasts and MC29-transformed macrophages. This could either mean that it is specific for p135 or that the failure of erythroblasts and macrophages to respond to the factor is due to the absence of hormone receptors on their surfaces.

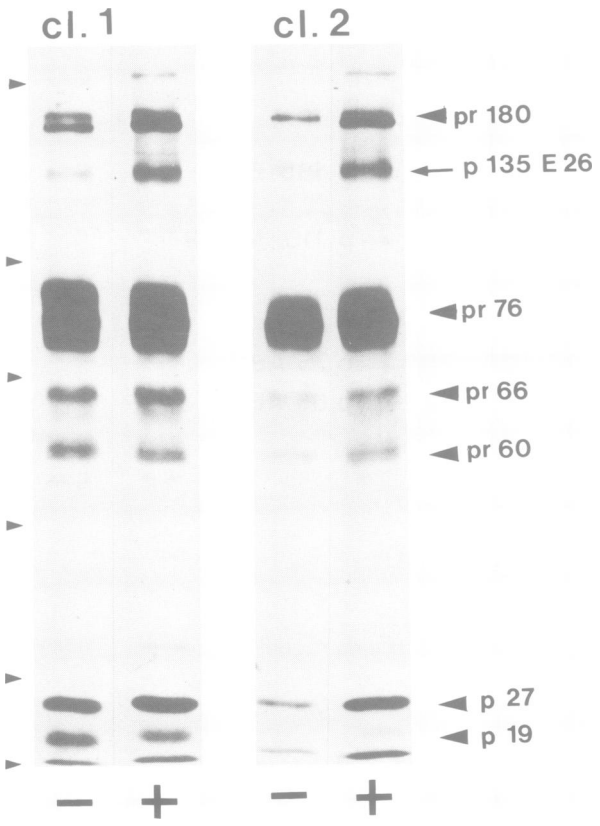

Fig. 3. Effect of growth factor on the expression of p135 E26 in virus-producing myeloblasts. Cells from clone 1 and clone 2 E26-transformed producer myeloblasts were grown in the absence (−) or in the presence (+) of 2% growth factor for 3 days. They were then treated as described in the legend of Figure 2.

The finding that MC29-transformed macrophages were slightly inhibited in their growth (as were macrophages transformed by other *myc*-containing viruses; T. Graf., unpublished observations), and in their synthesis of p110 *gag-myc* suggests that these cells are capable of responding to the factor and therefore argues against the latter interpretation.

The following observations suggest that p135 synthesized in E26-transformed myeloblasts represents a *myb*-containing *gag*-related protein: (1) the protein can be immunoprecipitated with anti-*gag* sera but not with either anti-reverse transcriptase or anti-glycoprotein sera; (2) p135 contains *gag*-specific tryptic peptides as well as peptides unrelated to other structural virus proteins and which also differ from *erb*- and *myc*-related peptides; and (3) these peptides are likely to be derived from the *myb* gene of E26 since *myb* sequences have been located in the 5′ half of the genome in proximity to the *gag* sequences of the virus (Bister *et al.*, 1982). Assuming that

Table II. Relative synthesis rate of virus-related proteins in E29 producer cells

	Clone 1	Clone 2
pr60:p135	7.3	8.3
pr60:p180	2.2	1.8
pr60:pr66	1.6	1.7
pr60:p27	1.5	1.4

The relative levels of *gag*-related proteins synthesized by E26 producer cells clone 1 and clone 2 were determined by tracing the bands shown in Figure 3 with a microdensitometer (Joyce Loebl) and weighing the areas under each peak. The values shown represent the ratios of the synthesis rates of p135 E26, pr180, pr66, and p27 relative to pr60 in cells grown with and without growth factor. Pr76 was not evaluated since the corresponding bands were overexposed.

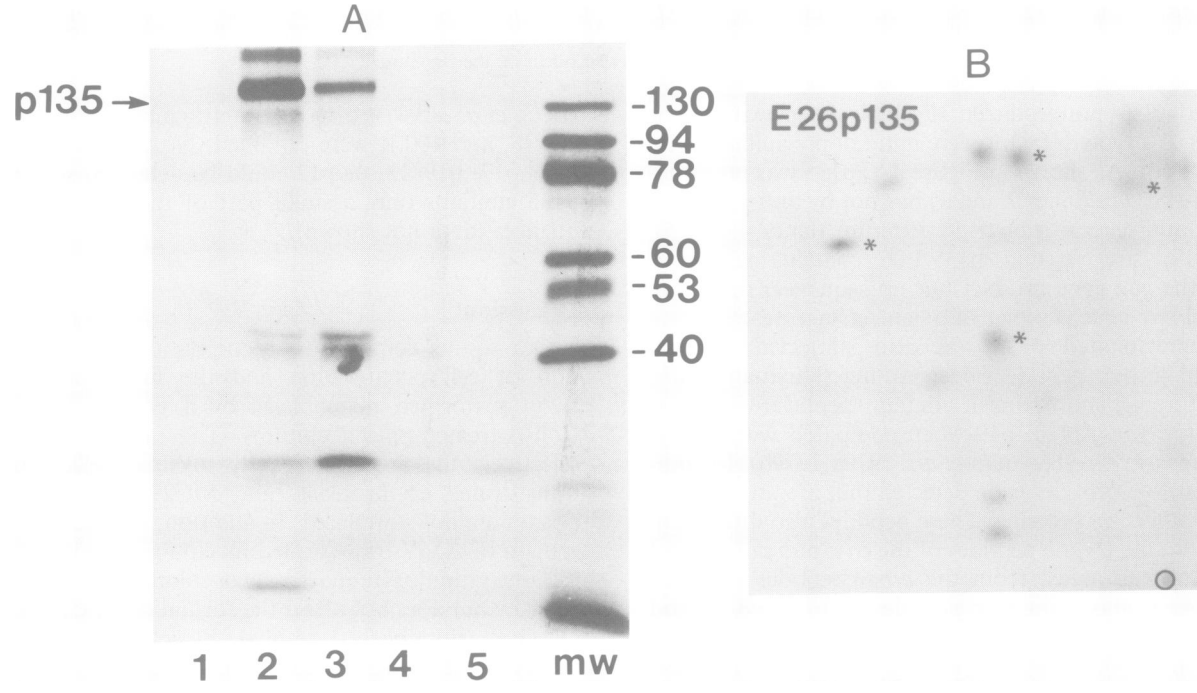

Fig. 4. Characterization of p135 E26 by immunoprecipitation with various sera and by tryptic peptide mapping. **(A)** E26 nonproducer clone 10 cells were grown in the presence of 2% growth factor and labeled and immunoprecipitated as described in the legend of Figure 2. **Track 1,** nonimmune serum; **track 2,** anti-whole virus serum; **track 3,** anti-*gag* serum; **track 4,** anti-envelope glycoprotein serum; **track 5,** anti-reverse transcriptase serum. Mol. wt. markers are shown in the track at the right. The additional high mol. wt. band precpitated with anti-whole virus serum represents a cellular protein which is also precipitated occasionally from uninfected cells using this serum. **(B)** In the tryptic peptide map all those spots corresponding to p19 (*gag*) peptides are indicated at their right with an asterisk.

p135 E26 contains about half of the information encoded for by the *gag* gene (~40 K) plus that of the *myb* gene (~35 K, Rushlow *et al.*, 1982), there is still at least 60 K of protein that is not accounted for by either *gag* or *myc* sequences. This portion of the molecule might represent the product of sequences with unknown genetic content known to also exist in the E26 genome (Stéhelin *et al.*, 1980). P135 E26 therefore probably represents in fact a *gag-myb-x* protein.

Definitive evidence that p135 E26 has a role in cell transformation will have to await the development of mutants defective for transformation.

It is interesting that the blast-like fractions of normal chick bone marrow express high levels of *myb* RNA (Chen, 1980; S. Saule, D. Stéhelin, H. Beug, and T. Graf, unpublished observations). In addition, the human myeloid leukemia cell line HL60 has been shown to express relatively high levels of *myb* RNA which decrease following the induction of differentiation (Westin *et al.*, 1982). These observations together with the findings that our growth factor stimulates the growth of normal myeloid cells as well as the synthesis of p135 suggest that the hypothetical protein encoded by the cellular *myb* gene may play a role in the growth regulation of immature myeloid cells.

Although our experiments suggest that the factor induces the synthesis of p135 E26 in transformed myeloblasts, it is not ruled out that it stabilizes the putative transforming protein. If the former possibility proves to be correct, it remains to be determined whether it acts at the level of transcription or translation. Since it is also not clear whether or not the factor obtained from ConA-stimulated spleen cells represents a mixture of different hormones it is still possible that its growth stimulatory activity and its enhancing effect on p135 E26 are separable. We are currently trying to purify the factor in order to clarify this question. Regardless of the outcome, the answers to some of these questions may provide new insights into the role of leukemia virus oncogenes during hematopoietic cell differentiation.

Materials and methods

Cells and viruses

The origin of E26 (RAV-2), the E26 strain used in these studies, as well as the isolation of nonproducer myeloblasts (transformed by E26 in the absence of helper virus) has been described previously (Graf *et al.*, 1979). The identification of E26-transformed cells as myeloblasts followed published procedures (Beug *et al.*, 1979). MC29- and AEV-transformed nonproducer cells were isolated following transformation of chick bone marrow cells with the corresponding viruses and seeding the cells in Methocel (Graf *et al.*, 1981).

Preparation and characterization of ConA-treated chick spleen supernatants

1 x 10^8 spleen cells from 3- to 4-week-old chicks were incubated for 3–6 days in 25 ml growth medium (Graf, 1973) supplemented with 10 μg/ml ConA (Pharmacia, Uppsala, Sweden). The cell-free supernatants of such cultures were then screened for their ability to stimulate myeloid cell growth by seeding 2 x 10^3 and 2 x 10^4 E26-transformed myeloblasts into 96 well microtiter plates (Falcon Plastics) containing 45 μl growth medium. 5 μl of samples to be tested was then added at various dilutions to the wells and the cells examined microscopically 2–3 days later. In addition, in some experiments the cell number in each well was determined in a Coulter Counter.

Immunoprecipitation and tryptic peptide mapping

Cell labeling and the preparation of detergent lysates were as described in Hayman *et al.* (1979a, 1979b). The antisera against the viral structural proteins have been characterized previously (Hayman, 1978). Immunoprecipitation was performed using fixed *Staphylococcus aureus* (Kessler, 1975) and the immunoprecipitated proteins analyzed on SDS-polyacrylamide gels followed by fluorography (Hayman *et al.*, 1979a, 1979b). Proteins to be peptide mapped were eluted from the gel, oxidized with performic acid, trypsinized and analyzed by two-dimensional separations as described earlier (Hayman *et al.*, 1979a, 1979b).

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