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v-*myb* and v-*ets* Cooperate for the Mitogenic Stimulation of Primary Fibroblasts by Avian E26 Retrovirus

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By using a series of deletion mutants, we have shown that the stimulation of fibroblast growth by E26 requires the cooperation of the two oncogenes, v-*myb* and v-*ets*, fused in the nuclear viral product. Of the two DNA-binding domains, only one must be present to promote anchorage-independent growth, whereas that of v-*myb* is required to allow growth in low serum medium. Furthermore, the v-*ets* oncogene comprises multifunctional domains.

E26 is a defective avian acute leukemia virus inducing a mixed erythroid-myelomonocytic leukemia in chickens (20, 24). It does not cause solid tumor growth and is unable to transform chicken embryo fibroblasts (CEFs) in vitro (3, 19). Nevertheless, the E26-encoded oncogenic product does stimulate proliferation of either CEFs (9), neuroretina cells (1), or NIH 3T3 cells (28). The E26 retrovirus genome encodes, through a unique 5.7-kb genomic mRNA, a 135-kDa *gag-myb-ets* fusion protein (p135$^{gag\text{-}myb\text{-}ets}$) (15, 21) which localizes to the nucleus of infected avian myeloblasts (4, 13) and binds DNA in vitro (18).

The v-*myb* oncogene domain has derived from a cellular proto-oncogene, c-*myb* (8, 12). Both c-*myb* and v-*myb* are nuclear proteins with a DNA-binding domain composed of imperfectly conserved direct repeats. A transcriptional activation domain is located in the central part of both proteins (for a review, see reference 17). The c-*myb* oncogene appears to be involved in the control of cell proliferation or differentiation (7, 27; for a review, see reference 26). The second oncogene domain carried by E26, v-*ets*, displays similarities with several cellular genes coding for nuclear DNA-binding proteins. c-*ets* proteins are transactivating phosphoproteins sharing homology with several transcription factors (5, 23; for a review, see reference 11) which may play a role during cell growth (2, 25).

The unique feature of E26, therefore, is that it contains two fused genes, each of which encodes transcriptional activators involved in cell proliferation. Moreover, the E26-encoded fusion protein carries the respective v-*myb* and v-*ets* DNA-binding domains at opposite ends of the polypeptide, raising the possibility that p135$^{gag\text{-}myb\text{-}ets}$ can interact with target genes either through the v-*ets*, v-*myb*, or both DNA-binding domains.

To further analyze the role of v-*myb* and v-*ets* in the mitogenic activation of CEFs, we made E26 constructs with modifications in these two oncogenes. All mutants have been made from the XJE26 genome, a recombinant E26 containing in addition the neo^r gene (Fig. 1; 16). To test the role of v-*myb* in the growth stimulation of CEFs, we made the mutants XJE26-DA, deleted of 172 nucleotides within its transactivating domain (10), and XJE26-DD, deleted of the repeats within its DNA-binding domain. Two types of modifications were made within the v-*ets* oncogene: (i) deletions between three in-frame *Pvu*II restriction sites (mutants XJE26-2a, -3a, and -4a) and (ii) insertion of a stop codon at the respective positions 630 (XJE26-630), 1740 (XJE26-1740), and 1070 (XJE26-1070). Proteins encoded by these three truncated mutants do not contain the v-*ets* DNA-binding domain. All of these mutants are depicted in Fig. 1.

The plasmids carrying all of these mutants genomes were cotransfected into CEFs together with pRAV-1 and then selected in the presence of G418. As reported for E26 (9), expression of all XJE26-derived constructs was barely detectable in CEFs (data not shown), although they were able to transform hematopoietic cells in vitro (4a). Each mutant was tested for its ability to allow infected CEFs to both grow in low-serum medium and form minute colonies in semisolid medium. A positive control consisted of anchorage-independent avian erythroblastosis virus (AEV)-transformed CEFs able to give large colonies in agar and to grow in low-serum (0.5%) medium, whereas TXN-infected CEFs, unable to grow in such conditions, were the negative control (6).

Effects of v-*ets* modified mutants on growth stimulation of CEFs. The biological effects of all mutants could be classified into three types. (i) Mutants XJE26-3a and XJE26-1740 have the same effect as XJE26 virus: they can grow in low-serum medium (Fig. 2) and form minute colonies in agar (Fig. 3E; Table 1) which are comparable to those obtained with XJE26 (Fig. 3D). Thus, deletions of the v-*ets* DNA-binding domain (construct XJE26-1740) does not alter the mitogenic effect of the virus in the presence of v-*myb*. (ii) Mutants XJE26-630 and XJE26-4a, with the largest deletion within v-*ets*, have weak and no mitogenic activity, respectively (Fig. 2 and 3F; Table 1). This means that at least some regions of v-*ets*, together with an intact v-*myb*, are necessary to stimulate CEF growth in liquid and semisolid cultures. (iii) Unexpected results were obtained with both mutant XJE26-2a and mutant XJE26-1070. CEFs infected with these two constructs appeared to be highly independent of growth factors since they started to grow in 0.5% serum medium only after a 5-day lag period in culture and proliferated very rapidly thereafter (Fig. 2). Their growth ability was even higher than

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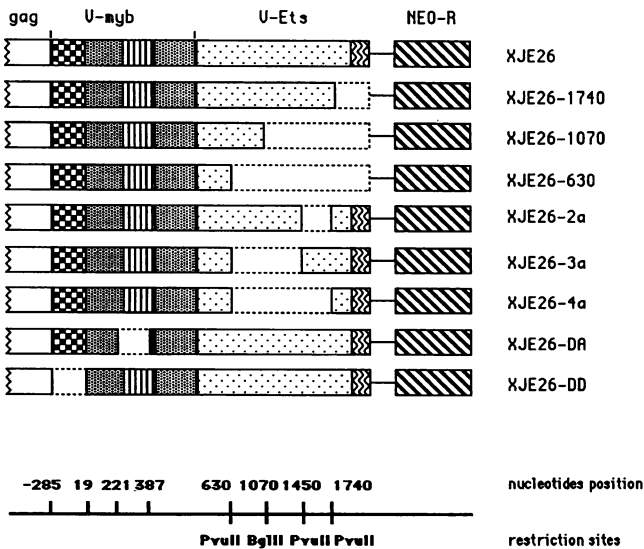

FIG. 1. Schematic representation of XJE26 mutants. Symbols: ▦, DNA-binding domain of the v-*myb* oncogene; ▥, transactivating domain of the v-*myb* oncogene; ▩, DNA-binding domain of the v-*ets* oncogene; ⬚, deleted part of the gene. Positions of nucleotides are according to the sequence published by Nunn et al. (21).

that of AEV-transformed fibroblasts. This is underscored by the high number and the large size of the colonies which were obtained in semisolid medium with CEFs infected with either of these two constructs (Fig. 3B and C; Table 1). The colonies were identical in size to those obtained with AEV-transformed CEFs (Fig. 3A). Therefore, the 290 bp of v-*ets* deleted in the XJE26-2a construct may define a functional

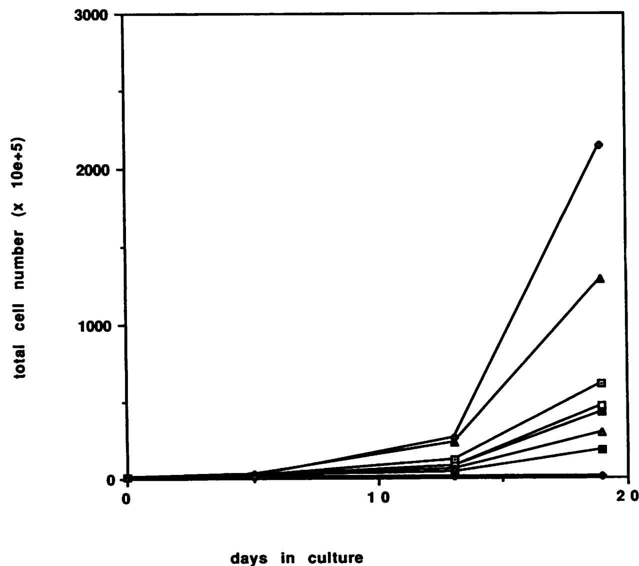

FIG. 2. Growth curves of XJE26 mutant-infected CEFs grown in low-serum medium. A total of 15 × 10^5 G418-selected CEFs were seeded at day 0 and grown in 0.5% fetal bovine serum containing medium. CEFs were infected with XJE26 or XJE26-1740 (□), XJE26-3a (■), XJE26-630 (-■-), XJE26-2a (◇), XJE26-DA or -DD (△), XJE26-1070 (▲), XJE26 -4a or -TXN (◆), and AEV (⊡).

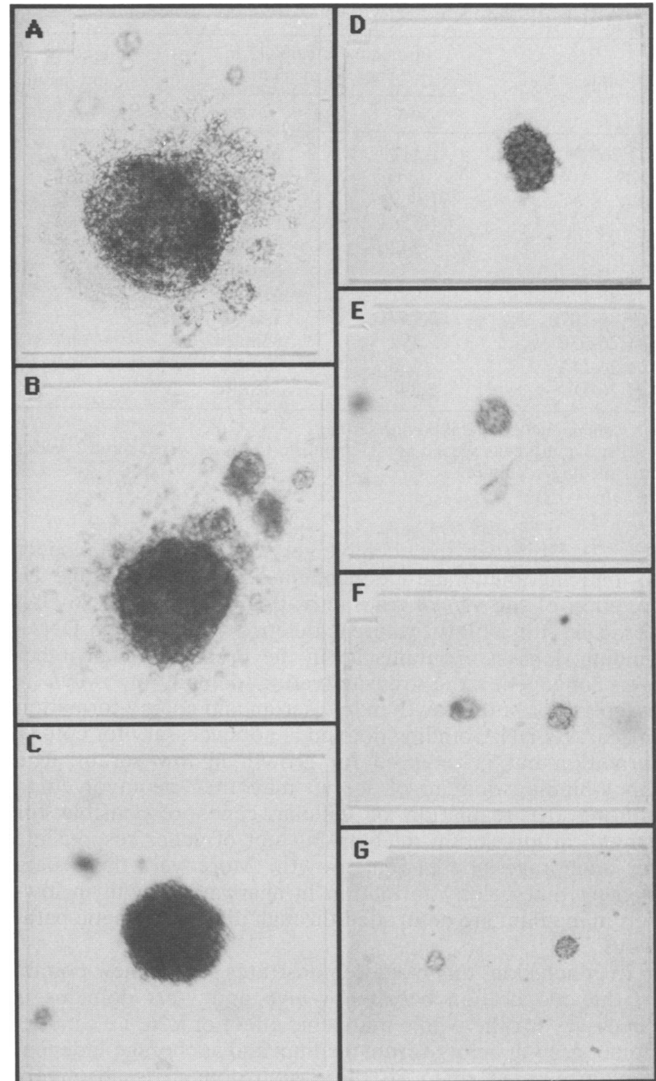

FIG. 3. Colony assay in semisolid medium. Infected CEFs (5 × 10^4 per 35-mm dish) were seeded in agar-containing medium. Colonies were numbered 2 weeks later. Colonies shown on the figure were obtained after infection of CEFs with AEV (A), XJE26-1070 (B), XJE26-2a (C), XJE26 (D), XJE26-3a (E), XJE26-4a (F), and TXN (G).

domain which has a regulatory effect inhibiting the activity of the p135$^{gag\text{-}myb\text{-}ets}$ protein in CEFs. This domain contains several serine residues which were shown to be targets for phosphorylation events in the c-*ets1* protein (14, 22).

From these results, we conclude that part of v-*ets*, excluding the DNA-binding domain, is required together with an intact v-*myb* for the mitogenic activation of CEFs by E26. In the presence of an intact v-*myb*, deletions within the v-*ets* oncogene either maintain, increase, or abolish the mitogenic effect of E26. This finding suggests that multifunctional domains exist within the v-*ets* oncogene.

Effects of v-*myb* deleted mutants on the growth stimulation of CEFs. Both XJE26-DA and XJE26-DD, the two mutants with deletions within the v-*myb* oncogene, have partly lost their ability to stimulate CEF growth in low-serum medium

TABLE 1. Mitogenic activity of XJE26 constructs on CEFs

Virus	Formation of colonies in semisolid medium		Growth in low-serum medium[a]
	No.[b]	Type	
XJE26	1,112	Minute	+
TXN	0		−
AEV	1,069	Large	+
XJE26-2a	1,556	Large	++
XJE26-3a	242	Minute	+
XJE26-4a	35	Small	−
XJE26-1740	992	Minute	+
XJE26-1070	2,757	Large	++
XJE26-630	192	Minute	+/−
XJE26-DA	138	Minute	+/−
XJE26-DD	1,248	Minute	+/−

[a] Containing 0.5% fetal bovine serum.
[b] Per 5×10^4 cells seeded per 35-mm dish. Colonies were scored 2 weeks after seeding in culture.

(Fig. 2). However, they behave very differently with respect to inducing anchorage-independent CEF growth (Table 1). Deletion of the v-*myb* transactivating domain (XJE26-DA) abrogates this ability, whereas deletion of the v-*myb* DNA-binding domain maintains it. In the presence of an intact v-*ets* oncogene, the transactivating domain of v-*myb* is essential for both growth in low serum and colony formation in agar. Its DNA-binding domain is not necessary for colony formation but is required for growth in low serum. The DNA-binding domain of v-*myb* may then be involved in altering the regulation of cellular genes responsible for growth in low-serum medium but not of genes responsible for anchorage-independent growth. Moreover, these data suggest that colony formation in agar and growth in low-serum medium are controlled through different genetic pathways.

In conclusion, this work demonstrates several new points: (i) the cooperation between v-*myb* and v-*ets* domains is necessary for the whole mitogenic effect of E26, i.e., induction of growth in low-serum medium and anchorage-independent growth in agar; (ii) the v-*myb* domain is absolutely necessary for the whole mitogenic effect of E26; (iii) the v-*myb* DNA-binding domain and transactivating domain contribute differently with respect to induction of growth in low serum and colony formation in agar; (iv) the v-*ets* DNA-binding domain is dispensable for both effects; and (v) the v-*ets* oncogene comprises a multifunctional domain capable of maintaining, increasing, or abolishing the mitogenic effect of E26. E26 and its mutants provide therefore helpful tools to investigate the various genetic processes which control cell proliferation and tumorigenic transformation.

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REFERENCES

1. **Amouyel, P., V. Laudet, P. Martin, R. Li, B. Quatannens, D. Stehelin, and S. Saule.** 1989. Two nuclear oncogenic proteins, $p135^{gag\text{-}myb\text{-}ets}$ and $p61/63^{myc}$, cooperate to induce transformation of chicken neuroretina cells. J. Virol. **63:**3382–3388.
2. **Bhat, N. K., C. B. Thompson, T. Lindsten, C. H. June, S. Fujiwara, S. Koizumi, R. J. Fisher, and T. S. Papas.** 1990. Reciprocal expression of human ETS1 and ETS2 genes during T-cell activation: regulatory role for the protooncogene ETS1. Proc. Natl. Acad. Sci. USA **87:**3723–3727.
3. **Bister, K., M. Nunn, C. Moscovici, B. Perbal, M. A. Baluda, and P. H. Duesberg.** 1982. Acute leukemia viruses E26 and avian myeloblastosis virus have related transformation specific RNA sequences but different genetic structures, gene products and oncogenic properties. Proc. Natl. Acad. Sci. USA **79:**3677–3681.
4. **Boyle, W. J., M. A. Lampert, A. C. Li, and M. A. Baluda.** 1985. Nuclear compartmentalization of the v-*myb* oncogene product. Mol. Cell. Biol. **5:**3017–3023.

4a. **Domenget, C., et al.** Unpublished data.

5. **Fujiwara, S., R. J. Fischer, N. K. Bhat, S. M. D. Espina, and T. Papas.** 1988. A short-lived nuclear phosphoprotein encoded by the human *ets-2* proto-oncogene is stabilized by activation of protein kinase C. Mol. Cell. Biol. **8:**4700–4706.
6. **Gandrillon, O., P. Jurdic, M. Benchaibi, J. M. Xiao, J. Ghysdael, and J. Samarut.** 1987. Expression of the v-erbA oncogene in chicken embryo fibroblasts stimulates their proliferation in vitro and enhances tumor growth in vivo. Cell **49:**687–697.
7. **Gewirtz, A. M., G. Anfossi, D. Venturelli, S. Valpreda, R. Sims, and B. Calabretta.** 1989. G_1/S transition in normal human T-lymphocytes requires the nuclear protein encoded by c-myb. Science **245:**180–183.
8. **Gonda, T. J., and J. M. Bishop.** 1983. Structure and transcription of the cellular homolog (c-*myb*) of the avian myeloblastosis virus transforming gene (v-*myb*). J. Virol. **46:**212–220.
9. **Jurdic, P., M. Benchaibi, O. Gandrillon, and J. Samarut.** 1987. Transforming and mitogenic effects of avian leukemia virus E26 on chicken hematopoietic cells and fibroblasts, respectively, correlate with level of expression of the provirus. J. Virol. **61:**3058–3065.
10. **Kalkbrenner, F., S. Guehmann, and K. Moelling.** 1990. Transcriptional activation by human c-myb and v-myb genes. Oncogene **5:**657–661.
11. **Karim, F. D., L. D. Urness, C. S. Thummel, M. J. Klemsz, S. R. McKercher, A. Celada, C. Van Beveren, R. A. Maki, C. V. Gunther, J. A. Nye, and B. J. Graves.** 1990. The ets-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. Genes Dev. **4:**1451–1453.
12. **Klempnauer, K. H., T. J. Gonda, and J. M. Bishop.** 1982. Nucleotide sequence of the retroviral leukemia gene v-myb and its cellular progenitor c-myb: the architecture of a transduced oncogene. Cell **31:**453–463.
13. **Klempnauer, K. H., G. Symonds, G. I. Evan, and J. M. Bishop.** 1984. Subcellular localization of proteins encoded by oncogenes of avian myeloblastosis virus and avian leukemia virus E26 and by the chicken c-myb gene. Cell **37:**537–547.
14. **Koizumi, S., R. J. Fisher, S. Fujiwara, C. Jorcyk, N. K. Bhat, A. Seth, and T. K. Papas.** 1990. Isoforms of the human ets-1 protein: generation by alternative splicing and differential phosphorylation. Oncogene **5:**675–681.
15. **Leprince, D., A. Gegonne, J. Coll, C. de Taisne, A. Schneeberger, C. Lagrou, and D. Stehelin.** 1983. A putative second cell derived oncogene of the avian leukemia retrovirus E26. Nature (London) **306:**395–397.
16. **Li, R., M. Duterque-Coquillaud, C. Lagrou, B. Debuire, T. Graf, D. Stehelin, and D. Leprince.** 1989. A single amino-acid substitution in the DNA-binding domain of the myb oncogene confers a thermolabile phenotype to E26-transformed myeloid cells. Oncogene Res. **5:**137–141.
17. **Lüscher, B., and R. N. Eisenman.** 1990. New light on myc and myb. Part II. Myb. Genes Dev. **4:**2235–2241.
18. **Moelling, K., E. Pfaff, H. Beug, P. Beimling, T. Bunte, M. E. Schaller, and T. Graf.** 1985. DNA-binding activity is associated with purified myb proteins from AMV and E26 viruses and is temperature-sensitive for E26ts mutants. Cell **40:**983–990.
19. **Moscovici, C.** 1975. Leukemic transformation with avian myelo-

blastosis virus: present status. Curr. Top. Microbiol. Immunol. **71**:79–101.

20. **Moscovici, C., J. Samarut, L. Gazzolo, and M. G. Moscovici.** 1981. Myeloid and erythroid neoplastic responses to avian defective leukemia viruses in chicken and in quail. Virology **113**:765–768.
21. **Nunn, M. F., P. H. Seeburg, C. Moscovici, and P. H. Duesberg.** 1983. Tripartite structure of the avian erythroblastosis virus E26 transforming gene. Nature (London) **306**:391–395.
22. **Pognonec, P., K. E. Boulukos, R. Bosselut, C. Boyer, A. M. Schmitt-Verhulst, and J. Ghysdael.** 1990. Identification of a Ets-1 variant protein unaffected in its chromatin and in vitro DNA binding capacities by T cell antigen receptor triggering and intracellular calcium rises. Oncogene **5**:603–610.
23. **Pognonec, P., K. E. Boulukos, J. C. Gesquière, D. Stehelin, and J. Ghysdael.** 1988. Mitogenic stimulation of thymocytes results in the calcium dependent phosphorylation of c-ets-1 proteins. EMBO J. **7**:977–983.
24. **Radke, K., H. Beug, S. Kornfeld, and T. Graf.** 1982. Transformation of both erythroid and myeloid cells by E26, an avian leukemia virus that contains the "myb" gene. Cell **31**:643–653.
25. **Seth, A., D. K. Watson, D. G. Blair, and T. Papas.** 1989. c-ets-2 protooncogene has mitogenic and oncogenic activity. Proc. Natl. Acad. Sci. USA **86**:7833–7837.
26. **Shen-Ong, G. L. C.** 1990. The myb oncogene. Biochim. Biophys. Acta **1032**:39–52.
27. **Thompson, C. B., P. B. Challoner, P. E. Neiman, and M. Groudine.** 1986. Expression of the c-myb proto-oncogene during cellular proliferation. Nature (London) **319**:374–380.
28. **Yuan, C. C., N. Kan, K. J. Dunn, T. S. Papas, and D. G. Blair.** 1989. Properties of a murine retroviral recombinant of avian acute leukemia virus E26: a murine fibroblast assay for v-*ets* function. J. Virol. **63**:205–215.