

# Cells Transformed by a v-Myb-Estrogen Receptor Fusion Differentiate into Multinucleated Giant Cells

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**In order to make conditional alleles of the v-myb oncogene, we constructed and tested avian retroviruses which produce a number of different fusion proteins between v-Myb and the human estrogen receptor (ER). We found that the portion of the ER used in making these fusions profoundly affected their transcriptional activation. However, all the fusions tested were only weakly transforming in embryonic yolk sac assays and there was no direct correlation between the level of transcriptional activation and strength of oncogenic transformation. Nevertheless, transformation by a v-Myb-ER fusion was estrogen dependent, and upon withdrawal of the hormone, monocytic-lineage cells differentiated into multinucleated giant cells. Surprisingly, the withdrawal of estrogen caused a dramatic increase in the stability of the fusion protein, although it remained unable to promote cell growth or block differentiation.**

Conditional alleles of retroviral oncogenes have provided powerful tools with which to dissect the mechanism of oncogenic transformation and the biology of transformed cells (17, 29). Classically, such conditional alleles were derived by mutagenesis followed by selection for a temperature-sensitive phenotype. More recently, studies of the nuclear receptor proteins have revealed that they contain a portable, hormone-inducible negative regulatory domain (18). Picard and colleagues initially showed that the constitutively active adenovirus E1A protein could be converted to a hormone-inducible transcriptional activator by fusion with the hormone-binding domain of the glucocorticoid receptor (34). Subsequently, a number of retroviral viral oncogenes, including *myc*, *abl*, *fos*, *raf*, and *rel*, were fused to the hormone-binding domain of either the glucocorticoid receptor or estrogen receptor (ER) in order to produce hormone-inducible variants (6, 13, 23, 36, 41). In most cases, these fusion proteins have been used to study phenotypic changes in established cell lines, although in some cases the hormone-inducible transformation of primary cells has also been achieved.

The v-myb oncogene of the avian myeloblastosis virus (AMV) causes a rapidly fatal monoblastic leukemia in chickens and transforms cells of the monocytic lineage in culture (2). The v-Myb protein is a doubly truncated form of the normal c-Myb protein, which itself is required for normal hematopoiesis. Both v-Myb and c-Myb are located in the nucleus, bind to specific DNA sequences, and can regulate the transcription of various reporter genes (22). Although transcriptional regulation by v-Myb appears to be essential for its oncogenic capacity, mutants which activate transcription but do not transform cells in culture have recently been isolated (9). Despite considerable effort, the key target genes which are regulated by v-Myb to cause transformation remain unknown. A conditional allele of v-myb would be very useful in this regard.

A temperature-sensitive mutant of AMV has been previously reported (30). However, this mutant is somewhat difficult to work with and the basis for its temperature sensitivity remains unclear (37). Temperature-sensitive mutants of the E26 leukemia virus, which contains both the v-myb and v-ets onco-

genes, have been isolated and analyzed in considerable detail (3, 4). A differential cDNA screen has identified a target gene, *mim-1*, which is directly activated by the Gag-Myb-Ets protein of the E26 virus (31). However, this gene is not expressed in monoblasts transformed by AMV and therefore appears unlikely to be important for oncogenic transformation (11). Although a single substitution in the DNA-binding domain of the E26 fusion protein is sufficient for temperature sensitivity, introduction of the same mutation into v-Myb of AMV does not result in temperature sensitivity (16, 26).

The production of a fusion between v-myb and the human ER offers another means of producing a conditional allele of this oncogene. A fusion of a hybrid AMV/E26 v-myb with the hormone-binding domain of the human ER has previously been reported (7). This protein was capable of inducing a morphologic change in the v-myc-transformed HD11 macrophage cell line, and it was also shown to activate the *mim-1* gene in these cells. However, no evidence of oncogenic transformation of primary cells by this v-Myb-ER fusion was reported. Therefore, we constructed a series of v-myb (AMV)-ER fusions and tested their ability to regulate transcription and to cause the leukemic transformation of primary hematopoietic cells in culture.

## MATERIALS AND METHODS

**Plasmid constructions.** DNA restriction and modifying enzymes were purchased from New England Biolabs (Beverly, Mass.). Recombinant DNA manipulations were carried out by standard techniques (35). NEO-dGE and N-dGE are previously described retroviral constructs which express both the Tn5 *neo* gene and a variant of v-myb that is deleted of both the *gag* and *env* sequences of wild-type AMV (11, 20). Plasmid pHEO, which contains a human ER cDNA, was kindly provided by Pierre Chambon (19). To construct Myb-E, the E domain of the ER was isolated by digestion with *EagI* and *SacI* and inserted into the *MscI* site near the 3' end of the v-myb open reading frame. To construct Myb-DE, the D and E domains of the ER were isolated together by digestion with *Cfr10I* and *SacI* and inserted into the *MscI* site near the 3' end of the v-myb open reading frame. To construct the E-Myb and DE-Myb fusions, the same domains of the human ER were amplified by PCR so that they could be inserted in frame into the *KpnI* site at the 5' end of the v-myb-dGE open reading frame. The 282-Myb fusion was constructed in a similar fashion to encode a C-terminal fragment of the human ER previously defined by a linker insertion mutant at residue 282 (44).

**Reporter genes.** The KHK-TK-CAT and KHK-E1B-CAT genes have previously been described (12, 21). Each reporter has the same fragments of chicken genomic DNA containing nine Myb-binding sites (5).

**Cells and media.** Quail QT6 fibroblasts were grown in Dulbecco's modified essential medium supplemented with 5% fetal calf serum, 4.5 g of glucose per

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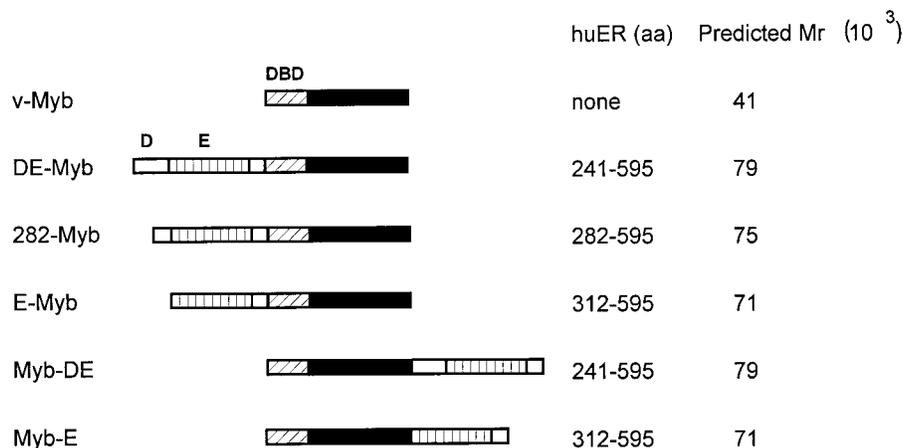


FIG. 1. Schematics of v-Myb-ER fusion proteins. Linear representations of the v-Myb and v-Myb-ER fusion proteins are shown. Diagonal cross-hatching indicates the v-Myb DNA-binding domain (DBD). Black bars indicate the remainder of the v-Myb protein. Vertical cross-hatching indicates the hormone-binding domain of the human ER (huER). Domains D and E of the ER are indicated for the DE-Myb protein. The amino acid (aa) residues from the human ER present in each fusion and the predicted relative molecular weights of proteins are also shown.

liter, 1× nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µg of streptomycin per ml, and 100 U of penicillin per ml in a humidified 10% CO<sub>2</sub>-90% air incubator at 37°C. Yolk sac cells were grown in Iscove's medium supplemented with 10% fetal calf serum, 5% chicken serum that had been heat inactivated for 1 h at 56°C, 4.5 g of glucose per liter, 1× nonessential amino acids, 1× MEM vitamins, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µg of streptomycin per ml, and 100 U of penicillin per ml in a humidified 5% CO<sub>2</sub>-95% air incubator at 37°C. Where indicated, estradiol was added at a final concentration of 1 µM.

**DNA transfections and immunoblotting.** Transient transfections into quail QT6 fibroblasts were performed by a modification of the calcium phosphate procedure (8, 21). Activator plasmids (3 µg) were cotransfected with reporter DNA (1 µg), tRNA (5.5 µg), and 0.5 µg of a plasmid expressing β-galactosidase from the cytomegalovirus promoter as an internal control for transfection efficiency. Half of the cells from each transfection plate were scraped in phosphate-buffered saline and then solubilized by boiling for 4 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading dyes. Normalized volumes of each were subjected to SDS-PAGE through 10% gels, and the proteins were transferred to nitrocellulose (BA-S 83; Schleicher & Schuell, Keene, N.H.). Transiently expressed Myb proteins were detected with monoclonal anti-Myb-2.2 and -2.7 antibodies (15). Blots were developed by incubation with anti-mouse or -rabbit immunoglobulin G-conjugated alkaline phosphatase (Promega, Madison, Wis.) and BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium)-Nitro Blue Tetrazolium.

**Transformation assays.** Each Myb-ER fusion protein was assayed for transformation by cocultivation of virus-infected QT6 cells with hematopoietic cells from chicken embryonic yolk sacs. QT6 cells were first cotransfected with the replication-defective *neo-myb* virus and DNA of a cloned MAV-1 helper virus. Then G418-resistant colonies were pooled and used as a source of virus to infect fresh QT6 cells (28). Hematopoietic cells from 12- to 13-day-old embryonic yolk sacs were infected with the different viruses by cocultivation for 24 h with adherent, mitomycin-treated, G418-resistant, virally infected QT6 cells (20, 27).

**DNA transfections for CAT activity.** Transient transfections into quail QT6 fibroblasts were performed as described above. The phase extraction method was employed to determine the chloramphenicol acetyltransferase (CAT) activity in normalized volumes of extract as previously described (38). All experiments were performed at least twice. Appropriate dilutions of cell extracts were assayed in those cases where the level of CAT activity indicated that the substrate was limiting. A background value of generally 100 to 300 cpm was obtained by extracting CAT assay mix to which no cell extract had been added.

**Cell cycle analysis.** Cells were harvested by low-speed centrifugation, washed in phosphate-buffered saline, and then fixed in 70% ethanol. The DNA content was determined with a fluorescence-activated cell sorter (FACS) after RNase treatment and staining with propidium iodide (24). The preparation of nuclei and DNA staining with propidium iodide were performed as previously described (43).

## RESULTS

### Construction and expression of v-Myb-ER fusion proteins.

In order to make estrogen-inducible forms of v-Myb, we constructed several avian retroviruses in which different portions

of the human ER were fused at either the amino or carboxyl termini of v-Myb (Fig. 1). A comparison of the vertebrate and avian ERs as well as functional studies have resulted in the definition of five domains, A through E, from the amino terminus to the carboxyl terminus (25). Domain C contains the highly conserved DNA-binding domain, whereas domain E contains the hormone-binding domain, which functions in dimerization and hormone inducibility, and a latent transcriptional activation domain. Lying between the C and E domains is the D domain, which was initially thought to function simply as a hinge connecting the C and E domains. However, evidence that the D domain can also function as a strong negative regulator of transcriptional activation has previously been presented (1). We therefore fused v-Myb with either the E domain or the D and E domains together. In addition, we constructed a fusion of v-Myb with a portion of the ER demarcated by a linker insertion mutation (residue 282) because this segment of the protein had previously been used in other hormone-inducible fusion proteins.

Production of the predicted protein was assayed by transient transfection of these proviral DNAs into the quail QT6 fibroblast cell line followed by immunoblotting with anti-Myb monoclonal antibodies (Fig. 2). Immunoreactive proteins of the predicted sizes were observed in all cases. Proteins which contained fusions of the ER at their carboxyl termini, not their amino termini, were also consistently accompanied by specific, faster-migrating forms (Myb-E and Myb-DE). This is a property shared by the unfused v-Myb protein itself.

### Transcriptional activation by v-Myb-ER fusion proteins.

Fusion proteins were assayed for the ability to activate transcription from a reporter containing an array of Myb-binding sites upstream of a simple TATA box from the adenovirus E1B gene and the CAT gene (Fig. 3). In the presence of estrogen, both fusions of the E domain to v-Myb were nearly as strong as v-Myb itself in activating the reporter gene. In contrast, fusions which contained both the D and E domains of the ER were very weak activators. The fusion containing all of the E domain and only part of the D domain (282-Myb) was intermediate in transcriptional activation. None of the v-Myb-ER fusion proteins were able to activate this reporter in the absence of estrogen, whereas v-Myb itself was estrogen independent.

The ability of different v-Myb-ER fusion proteins to activate transcription was dependent upon the promoter tested. For

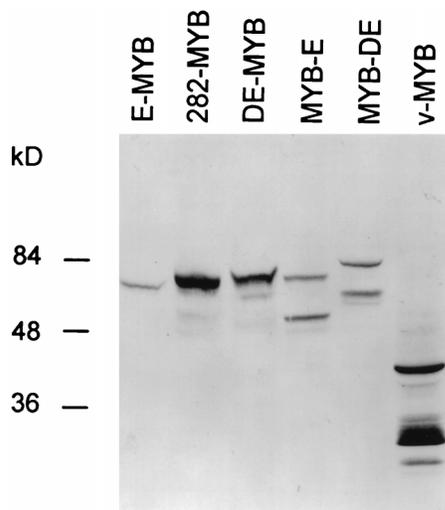


FIG. 2. Protein production by v-Myb-ER proviruses. The indicated proviral DNAs were transfected into quail QT6 fibroblasts, and 2 days later, cell lysates were analyzed by SDS-PAGE and immunoblotting with anti-Myb monoclonal antibodies. The mobilities of coelectrophoresed molecular mass (in kilodaltons [kD]) markers are indicated on the left.

example, although the fusions containing both the D and E domains of the ER were very weak activators of a reporter gene containing an array of Myb-binding sites upstream of the E1B TATA box, the same fusions were quite strong estrogen-dependent activators of a reporter gene containing the same array of Myb-binding sites upstream of the herpes simplex virus thymidine kinase (TK) promoter (Fig. 4). These results suggest that various estrogen fusion proteins might differentially activate endogenous cellular genes as well, presumably due to differential interactions with other proteins bound to the promoters of these genes.

**Leukemic transformation by v-Myb-ER fusion proteins.** In order to produce infectious retroviruses, each of the proviral DNAs encoding both a neomycin resistance protein and a

v-Myb-ER fusion was cotransfected into quail QT6 cells along with DNA of the MAV-1 helper virus. After selection with G418, cells were treated with mitomycin to prevent DNA replication but not the production of infectious viral particles. These cells were then cocultivated with freshly isolated hematopoietic cells from 12- to 13-day-old chicken embryos, which are a rich source of myelomonocytic precursor cells. In the absence of *v-myb*, these cells differentiate into mature granulocytes and macrophage within 7 to 10 days in culture. However, infection with retroviruses containing *v-myb* causes the outgrowth of small round monoblasts which fail to differentiate into macrophage (20, 27).

All of the viruses encoding v-Myb-ER fusion proteins were capable of weakly transforming embryonic yolk sac cells. Cells transformed by v-Myb-ER were apparent only at 3 to 4 weeks of culture rather than at 1 to 2 weeks of culture with wild-type v-Myb. In addition, v-Myb-ER-transformed cells could not be expanded beyond  $10^7$  cells, whereas v-Myb-transformed cells could be routinely expanded to  $10^9$  cells from a single 10-cm-diameter dish. Furthermore, estrogen was required for transformation by v-Myb-ER but not by wild-type v-Myb. Cytoentrifugation and staining as well as transmission electron microscopy revealed that v-Myb-ER-transformed cells were morphologically very similar to monoblasts transformed by v-Myb (Fig. 5 and 6). However, upon the withdrawal of estrogen, v-Myb-ER-transformed cells became adherent and displayed a more abundant and highly vacuolated cytoplasm typical of differentiated macrophage. In addition, the formation of large, multinucleated giant cells was observed within 48 to 72 h after estrogen withdrawal. Electron microscopy revealed that multiple nuclei were indeed present within single, highly vacuolated cells bounded by a single plasma membrane (Fig. 6). When estrogen was added back within 48 h after withdrawal, most of the cells in the culture rounded up, detached from the dish, and reverted to a transformed phenotype (data not shown). However, the readdition of estrogen at 96 h after withdrawal was unable to cause dedifferentiation.

The DNA content of v-Myb-ER-transformed cells in the presence or absence of estrogen was determined by propidium

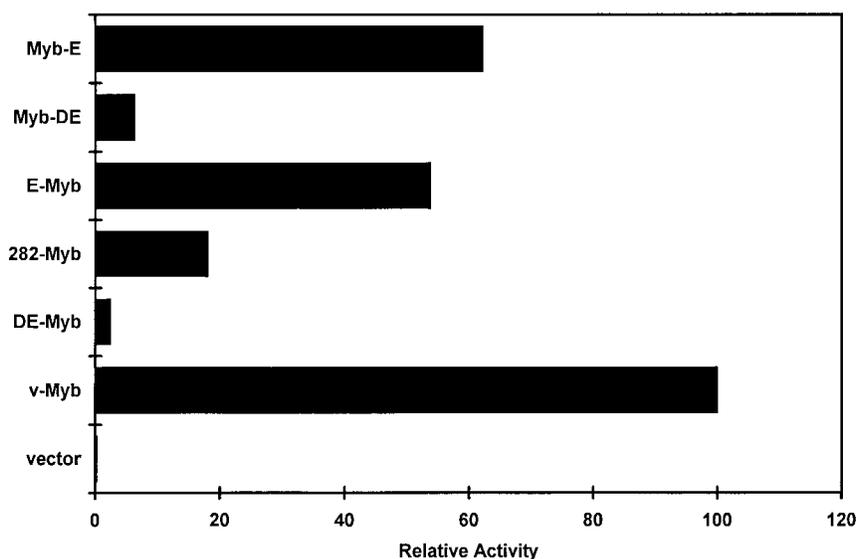


FIG. 3. Relative transcriptional activation by v-Myb-ER fusion proteins. The indicated proviral DNAs were transfected into quail QT6 fibroblasts along with the Myb-responsive reporter plasmid KHK-E1B-CAT. One day later, estrogen was added to each plate. Two days later, cell lysates were prepared and assayed for CAT activity. Transcriptional activation is reported as relative CAT activity, with the value for v-Myb set at 100.

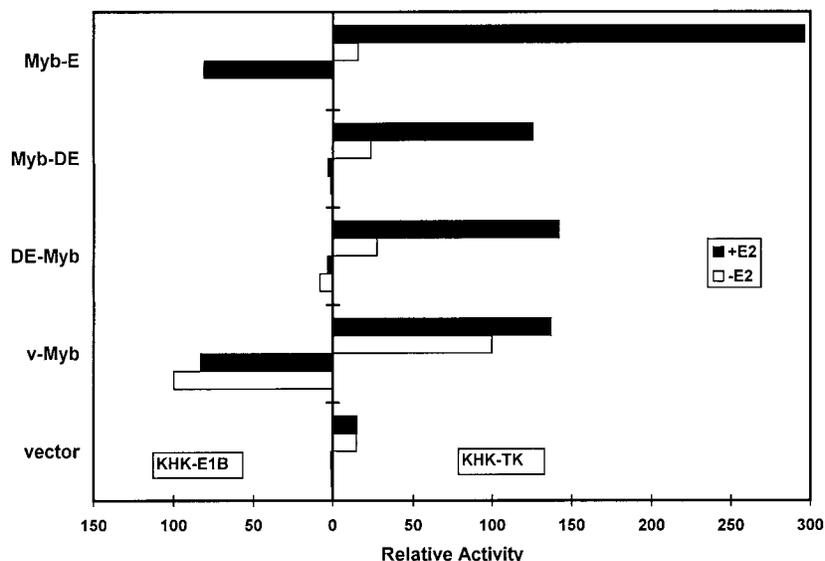


FIG. 4. Differential promoter activation by v-Myb-ER fusion proteins. Transfections were performed as described in the legend to Fig. 3, except that two reporter genes were used (indicated within each panel) and each assay was performed in the presence (+) or absence (-) of estrogen (E2). Transcriptional activation is reported as relative CAT activity, with the value for v-Myb in the absence of estrogen for the indicated reporter set at 100. The KHK-TK-CAT reporter gave a threefold-higher absolute value than did the KHK-E1B-CAT reporter when activated by v-Myb in the absence of estrogen.

iodide staining and FACS analysis (Fig. 7). When whole cells were analyzed, increased numbers of cells with 4-, 6-, 8-, and 10-N DNA contents were seen in the absence of estrogen. However, when nuclei were isolated from the same cells and similarly analyzed, it was clear that there was no change in DNA content per nucleus in the presence or absence of estrogen. Thus, the giant cells which differentiate upon the removal of estrogen contain multiple independent nuclei with a largely diploid DNA content.

**Estrogen withdrawal stabilizes a v-Myb-ER fusion protein.**

The ER hormone-binding domain is generally thought to effect negative regulation by binding to the hsp90 protein in the absence of ligand (33). Upon the binding of ligand, hsp90 is released, allowing functional protein folding to occur. However, it is also possible that ER fusion proteins are regulated by differential stability in the presence or absence of ligand. To address this question, we examined the levels of v-Myb-ER in transformed yolk sac cells before and after estrogen withdrawal (Fig. 8). Surprisingly, the fusion protein was barely detectable in transformed cells in the presence of estrogen,

whereas a remarkable stabilization occurred after the withdrawal of estrogen as differentiation progressed. The v-Myb-ER levels obtained after estrogen withdrawal were comparable to the wild-type v-Myb levels in transformed cells. These results suggest that the relatively weak transforming abilities of v-Myb-ER fusion proteins are due to their relative instability in the presence of estrogen in cells of the monocytic lineage.

**DISCUSSION**

We constructed and tested avian retroviruses encoding five different v-Myb-ER fusion proteins. These viruses were capable of weakly transforming primary myelomonocytic cells in culture in an estrogen-dependent fashion. Although v-Myb-ER-transformed cells were blocked in their differentiation in a fashion similar to that of cells transformed by v-Myb itself, the former cells failed to proliferate as rapidly in culture. In an attempt to promote the growth and/or survival of v-Myb-ER-transformed cells, additional viruses which successfully coexpressed either an activated Raf kinase known to cooperate

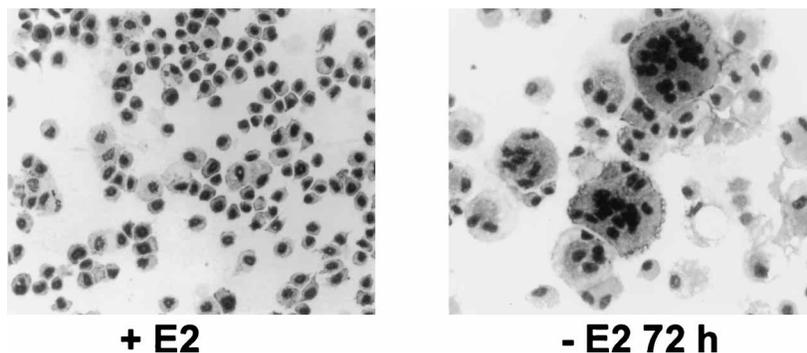


FIG. 5. v-Myb-E-transformed cells differentiate into multinucleated giant cells. Primary chicken yolk sac cells transformed by the v-Myb-E virus in the continuous presence of estrogen were incubated for 72 h in the presence (+) or absence (-) of estrogen (E2). Then cells were examined by cytocentrifugation, histochemical staining, and photomicroscopy.

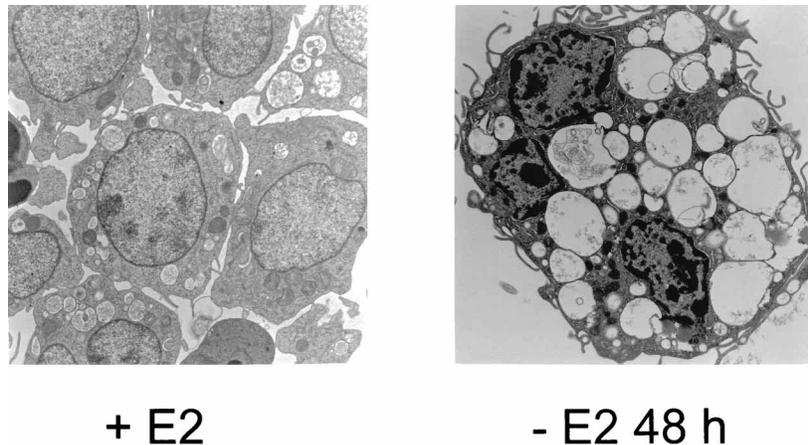


FIG. 6. Ultrastructure of v-Myb-E-transformed cells. Primary chicken yolk sac cells transformed by the v-Myb-E virus in the continuous presence of estrogen were incubated for 48 h in the presence (+) or absence (-) of estrogen (E2). Then cells were examined by transmission electron microscopy.

with the v-Myc oncoprotein in the MH2 virus or the Bcl2 antiapoptosis protein via an internal ribosomal entry site were constructed (data not shown). However, neither of these additional proteins caused a significant increase in the proliferation of v-Myb-ER-transformed cells.

Somewhat surprisingly, the stability of the v-Myb-ER fusion protein in transformed monoblasts was greatly increased after estrogen withdrawal. These results suggest that the failure of the Myb-ER proteins to promote the rapid outgrowth of transformed cells is due to the low steady-state levels of the v-Myb-ER protein in transformed cells.

Three different overlapping segments of the human ER were fused to the v-Myb protein. The abilities of the resulting fusion proteins to activate transcription from a promoter consisting of Myb-binding sites and a simple TATA box differed greatly. In general, our results demonstrated that the E domain of the receptor alone permits the strongest transcriptional activation, whereas increasing amounts of the adjacent D domain result in

decreasing activation by fusion proteins. This is consistent with a previous report that rather than acting as a neutral hinge, the D domain can function in transcriptional repression in a manner independent of DNA binding (1). Placing the same domain(s) at either the amino or carboxyl terminus of v-Myb gave similar results, arguing that protein function rather than simple steric hindrance is responsible for the differences in transcriptional activation observed between fusions of the E domain and those of the D and E domains. When the same proteins were tested for transcriptional activation of a more complex promoter containing the same array of Myb-binding sites upstream of the herpes simplex virus TK promoter, different results were obtained. In this case, the D-E fusions, which were inactive on the simple TATA box, were as active as the wild type v-Myb protein. Presumably this is due to a specific interaction between the ER domain(s) and the complex of endogenous cellular factors, including Sp1 and C/EBP, which assemble on the TK promoter. These results suggest that the failure

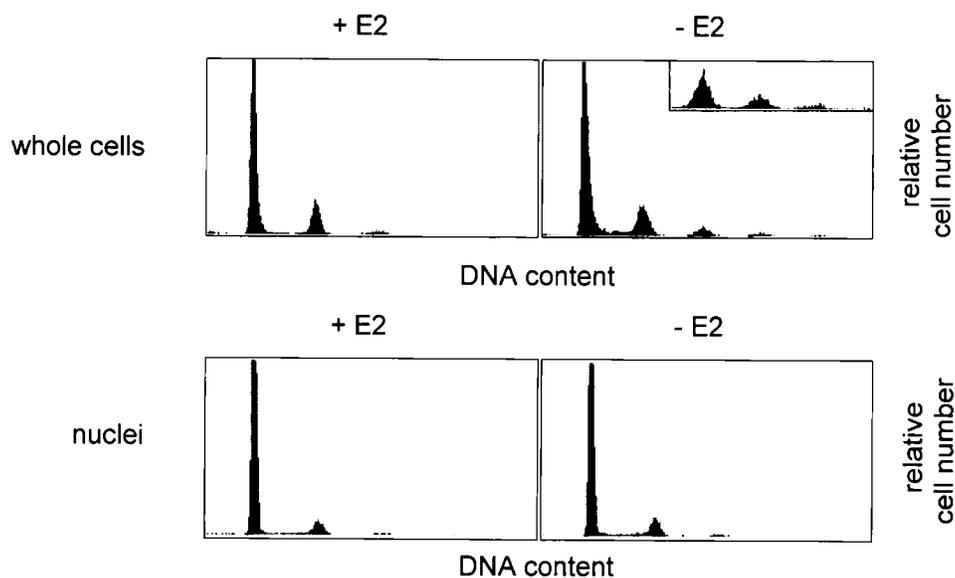


FIG. 7. DNA contents of v-Myb-E-transformed cells before and after differentiation. Primary chicken yolk sac cells transformed by the v-Myb-E virus in the continuous presence of estrogen were incubated for 72 h in the presence (+) or absence (-) of estrogen (E2). Then the DNA contents of either whole cells or nuclei were examined by staining with propidium iodide and FACS analysis. The inset represents a magnification of the peaks directly below it.

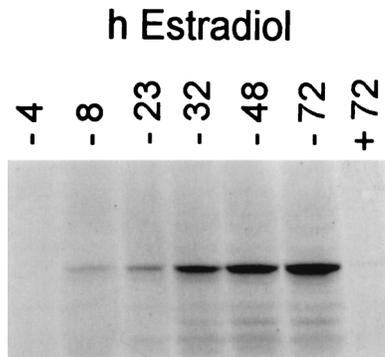


FIG. 8. Estrogen withdrawal increases protein stability. Primary chicken yolk sac cells transformed by the v-Myb-E virus in the continuous presence of estrogen were incubated for 72 h in the presence (+) or absence (-) of estrogen. Aliquots were removed at the indicated times and assayed for v-Myb-E protein by SDS-PAGE and immunoblotting with anti-Myb monoclonal antibodies.

of the Myb-ER proteins to promote the rapid outgrowth of transformed cells is instead due to differential interactions with the cellular target genes that are crucial for transformation by wild-type v-Myb. Further analysis of this point will require the identification and isolation of such target genes, which remains a major hurdle in the analysis of all the oncoproteins which function as transcription factors.

The differentiation of v-Myb-ER-transformed cells into multinucleated giant cells was unexpected. These cells resemble both the bone-marrow-derived osteoclasts which function in bone resorption and the giant cells which accumulate in response to persistent foreign bodies which cannot be easily phagocytized (10). Phorbol esters can induce v-Myb-transformed cells to differentiate into macrophage, but multinucleated giant cells are not generally observed during this process (32, 39, 42). The retinoic acid receptor- $\alpha$  can also act in a dominant fashion to suppress transformation by v-Myb (40). In this case, retinoic acid induces the differentiation of multinucleated cells which resemble the giant cells observed here. FACS analysis of DNA content has shown that tetradecanoyl phorbol acetate causes v-Myb-transformed cells to arrest and differentiate in both the G<sub>1</sub> and G<sub>2</sub> phases of the cell cycle (14). In contrast, although v-Myb-ER-transformed cells form multinucleated cells, the individual nuclei appear to arrest in G<sub>1</sub>, not in G<sub>2</sub>. Similarly, retinoic acid-treated v-Myb-RAR cells differentiate primarily in the G<sub>1</sub> phase of the cell cycle (39a). These results demonstrate that at least two pathways of differentiation are open to v-Myb-transformed monoblasts. These pathways differ not only in their morphological outcomes but also in their modes of cell cycle arrest.

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