Definition of Functional Domains in P135\textsuperscript{gag-myb-ets} and p48\textsuperscript{v-myb} Proteins Required To Maintain the Response of Neuroretina Cells to Basic Fibroblast Growth Factor

CARMEN GARRIDO,1 DOMINIQUE LEPRINCE,2 JOSEPH S. LIPSICK,3 DOMINIQUE STEHELIN,2 DENIS GOSPODAROWICZ,1 AND SIMON SAULE1,2*

Cancer Research Institute, University of California Medical Center, San Francisco, California 94143-0128; INSERM U 186/CNRs UA 041160, Institut Pasteur de Lille, 59019 Lille cedex, France2*; and Department of Microbiology, State University of New York at Stony Brook, Stony Brook, New York 11794-86213

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The v-myb- and v-ets-containing E26 retrovirus induces the proliferation of chicken neuroretina (CNR) cells in minimal medium. Proliferation of E26 CNR cells is strongly stimulated by basic fibroblast growth factor (bFGF). The v-myb-containing avian myeloblastosis virus also induces the proliferation of infected CNR cells stimulated by bFGF. Both E26 CNR and avian myeloblastosis virus CNR cells are able to form colonies in soft agar in the presence of bFGF. This suggests that the v-myb product, a nuclear sequence-specific DNA-binding protein which activates gene expression in transient transfection assays, plays a role in the proliferative response of the infected CNR cells. To determine the structure-function relationships of P135\textsuperscript{gag-myb-ets} and p48\textsuperscript{v-myb}, we have used deletion mutants expressed in retroviral vectors and have analyzed their effect on CNR cell proliferation as well as their effect on the CNR cell response to bFGF. We show that v-ets is not required for bFGF stimulation but increases the proliferation of CNR cells in minimal medium. In the v-myb mutants, the gag sequences derived from the helper virus increase the potency of the myb gene. The carboxyl-terminal domain required for the growth and transformation of myeloid cells and needed for maximal trans-activation in transient DNA transfection assays in fibroblasts was not required for the growth and bFGF response of CNR cells. In contrast, the domain encompassing amino acids 240 to 301 (containing part of the transcriptional activation domain of v-myb) was absolutely required for the response of CNR cells to bFGF and could be functionally replaced by the carboxyl-terminal transcriptional activation domain of the VP16 protein of herpes simplex virus.

The v-myb oncogene was initially identified as the cell-derived sequence of the avian myeloblastosis virus (AMV) that transforms exclusively myeloid cells (28, 35, 37). The v-myb protein product, p48\textsuperscript{v-myb}, is a doubly truncated version of its normal cellular counterpart and contains viral amino acids at both its amino (6-amino-acid) and its carboxyl (11-amino-acid) termini (8, 15, 22). The v-myb product is present within the cell nucleus, has short half-life, and possesses an intrinsic, sequence-specific DNA-binding activity (4, 6, 23, 27). Several lines of evidence suggested that myb-related genes may control cellular differentiation through a direct regulation of gene expression. The level of c-myb decreases dramatically upon differentiation of immature myeloblasts, erythroleukemic cells, and neuroblastoma cells (11, 36, 41). In addition, constitutive expression of c-myb prevented the differentiation of erythroleukemic cells (9). In DNA transfection assays p48\textsuperscript{v-myb} can function as a transactivator of gene expression (19, 24, 30, 44). To date three functional domains have been identified in the c-myb product: an amino-terminal DNA-binding domain, a transcriptional activation domain, and a negative regulatory domain (39). These experiments also suggest that p48\textsuperscript{v-myb} possesses 40% of the transcriptional activation potential of the c-myb protein and 75% of its transcriptional repression activity.

The v-myb gene is also present in the avian erythroblastos virus E26. After infection with E26, chickens develop mixed leukemias involving both myeloid and erythroid lineages (29, 35). The E26 retrovirus contains the v-myb-ets sequence fused in the same reading frame with the residual gag sequence (25, 32). A unique 5.7-kb genomic RNA encodes the 135-kDa gag-myb-ets protein which is located in the nucleus (6, 23): 272 codons encode the retroviral gag portion, 283 codons encode the myb part, and 491 codons encode the ets domain (25, 32, 33).

Mutants of E26 temperature sensitive for myeloblast transformation have been isolated (3). These mutants have been molecularly cloned, and a nonconservative mutation, located in the conserved v-myb DNA-binding sequence, was found to be responsible for the thermosensitive phenotype exhibited by the viruses (13, 26). On the other hand, a deletion in the carboxy terminus of v-ets removing 26 amino acids from the ets protein abolished only the erythroid potential of the mutant virus (31). These data suggest that the myb sequence is responsible for the E26-induced myeloid transformation and that the ets sequence may be additionally required for the erythroid potential of the virus. However, a single point mutation in the v-ets sequence has been shown to affect both erythroid and myelomonocytic cell differentiation (16), suggesting a cooperation between these two sequences in the transforming potential of E26. The function of the ets domain is unknown, but c-ets-l encodes transcription factors recognizing the PEA3 DNA motif and is able to cooperate with the c-fos and c-jun products for transcriptional activation in transient DNA transfection assays (42).

Biological systems unrelated to the hematopoietic system (20) could help to define the respective functions of myb and
ets. Since E26 is able to induce the proliferation of chicken neuroretina cells (1), we have studied the effect of E26, AMV, and mutants of both viruses on chicken embryo neuroretina (CNR) cells. We have found that these cells, following infection with E26 or AMV, are induced to proliferate in defined medium and are strongly stimulated by pituitary-derived basic fibroblast growth factor (bFGF). The ets domain is dispensable for the E26 effect on CNR cells, and the v-myb domain, including amino acid 240 to 301, is absolutely required for response of CNR cells to bFGF.

MATERIALS AND METHODS

Materials. Conalbumin and insulin were obtained from Sigma (St. Louis, Mo.). Dulbecco's modified Eagle's medium H16 (DMEM), F12 medium, and vitamin X100 were obtained from Gibco (Grand Island, N.Y.). Fetal calf serum (FCS) was obtained from Hyclone (Sterile Systems Inc., Logan, Utah). Tissue culture dishes were from Falcon Plastics (Oxnard, Calif.), Geneticin was from Schering Co. (Kenilworth, N.J.), and fungizone was from E.R. Squibb & Sons (Princeton, N.J.). Gelatinized dishes were coated with 0.2% gelatin in phosphate-buffered saline (PBS) overnight at 4°C. Bovine pituitary bFGF was purified as previously described (17).

Viruses and molecular clones. AMV and its mutants (18, 19) as well as E26 mutants are shown in Fig. 1. The various internal deletion mutants in v-ets were derived from the XJE26 as follows. The 3.5-kbp BamHI-SalI (partial digest) gag-myb-ets fragment of the XJE26 proviral DNA was subcloned into plasmid pKH47. This DNA was then partially digested by the restriction enzyme PvuII since the v-ets gene contains three in-frame PvuII sites, at positions 630, 1450, and 1740 (32). Plasmid clones containing the expected deletions (630 to 1450 in mutant 3A and deletion 630 to 1740 in mutant 4A) were obtained by purification of the appropriate fragments by agarose gel electrophoresis followed by religation and transfection into HB101. We then used a unique XhoI site located in the gag gene and a unique HindIII site located in v-ets at position 1770, upstream of the more distal PvuII site. The XhoI-HindIII fragment of each clone was prepared and inserted into the XhoI-HindIII proviral DNA of the XJE26 to yield the XJE26 3A and 4A clones. LinR8 resulted from a recombination between Rous-associated virus type 1 (RAV-1) and AMV and was isolated from AMV-infected pigmented retinas. This virus encodes a P68gag-myb fusion protein. The corresponding provirus was molecularly cloned, and the junction between gag and myb was sequenced; in addition, the restriction enzyme map of the LinR8 myb was found identical to that of AMV. The viruses used were obtained by cotransfection of the molecular clones DNA with pRAV-1 DNA onto CNR cells as previously described (1).

Cell culture, growth assay, and soft agar cloning. Dissociated CNR cells dissected from 7-day-old chicken embryos were plated in F12-DMEM (1:1 [vol/vol]) containing 10x FCS, 1x vitamin X100, and 10x of conalbumin per ml (complete medium). Dishes (60 mm) containing 5x10⁶ dissociated cells were infected with various viruses in the complete medium at 37°C in 5% CO₂. The infected cells were passaged four times on gelatin-coated dishes to allow virus to spread. Transfected cells selected in medium containing genetin (400 µg/ml) were passaged twice on gelatin-coated dishes before testing.

To study the growth kinetics of CNRs, 4x10⁶ cells were seeded in 22-mm gelatin-coated cell wells in F12-DMEM containing 5 µg of insulin per ml, 1% vitamin, and 10 µg of conalbumin per ml (defined medium). Every day cells from two wells were treated with trypsin-EDTA and counted in a Coulter particle counter. To study the sensitivity of the cells to growth factors, 4x10⁴ cells were seeded in 22-mm wells in defined medium. Growth factors were added every other day beginning on the day of plating. After 4 days, the cultures were trypsinized and cell density was determined with a Coulter counter. Similar results were obtained in three distinct experiments.

To assess the anchorage-independent growth of CNR, 4x10⁶ cells were suspended in 2 ml of soft agar containing medium (complete medium plus 0.4% Noble agar [cloning medium]). This medium was layered on a hardened base layer of medium containing 1.2% agarose, in a 35-mm tissue culture dish. bFGF (20 ng/ml) was added to duplicate cultures in the cloning medium. bFGF was added every other day on the top of the soft agar until the end of the experiment. Colonies were counted 14 days later by using a phase-contrast microscope (Biophot, Nikon).

Cell labelling and immunoprecipitation. Infected CNR cells starved in methionine-free medium for 30 min were incubated for 45 min in the presence of 30 µCi of L-[35S]methio-
nine (specific activity, 1,000 Ci mmol\(^{-1}\)) ml\(^{-1}\), lysed in radioimmunoprecipitation assay buffer, and immunoprecipitated as described previously (1). Serums used were rabbit anti-\textit{ets} serum, rabbit anti-myb (7), and rabbit anti-gag. Immunoprecipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then by fluorography.

**RESULTS**

Effects of wild-type and mutant E26 viruses on the proliferation and bFGF sensitivity of CNR cells. Infection of CNR cells in complete medium (see Materials and Methods) with E26 retrovirus resulted in the appearance of morphologically modified, actively dividing cells after two passages. Cells infected with viruses bearing a truncated \textit{ets} sequence, XJ E26 3A or XJ E26 4A (E26 \textit{ets} mutants [Fig. 1]), had a morphology similar to that of E26 wild-type CNR cell cultures. All cultures were morphologically homogenous after four passages and had an untransformed fibroblastic appearance. By contrast, infection with helper virus RAV-1 did not result in the appearance of actively dividing cells. In addition, these cultures were heterogenous, being mostly composed of fibroblast and epithelial-like cells. The growth of low-density cultures of transfected CNR cells was analyzed in serum-free medium. Uninfected cells as well as helper-infected cells did not survive. In contrast, CNR infected with wild-type E26 proliferated well and cultures expressing the mutants mentioned above (XJ E26 3A and 4A) did survive (Fig. 2). Thus, proliferation in this defined medium discriminates between normal and oncogene-expressing cells.

We next analyzed the effect of bFGF on these cells in serum-free medium (Fig. 3). Primary cells responded positively to bFGF, increasing by threefold their final cell density (data not shown). Propagated normal cells or cells expressing helper virus did not proliferate in response to bFGF. CNR E26 cells were strongly responsive, and the bFGF effect was dose dependent (50% effective dose, 0.7 ng/ml). Similar results were obtained with the E26 mutants (Fig. 3).

The myb gene product alone is responsible for the bFGF sensitivity of infected CNR cells. E26 and E26 \textit{ets} induced similar responses in infected CNR cells. This suggested that the myb domain was essential in the response of the CNR cells to bFGF. Therefore, we tested the biological properties of the myb-containing AMV and mutants of this virus (Fig. 1) on CNR. Transfection of CNR with molecular clones as well as infection of these cells with the viral progeny resulted in the appearance of morphologically modified cells for the AMV wild type and the mutants LX4, LX3, D4, D3, and VP16. All these cells were much less fibroblastic and differed morphologically from E26 CNR cells. LX2 and LX1 CNR cells had a morphology similar to that of the helper virus-infected cells. In contrast to E26 CNR cells, AMV CNR cells did not proliferate when maintained in minimum medium (Fig. 2). AMV CNR cells were also responsive to bFGF, but to a lesser extent than the E26 or E26 \textit{ets} mutant CNR cells (Fig. 3). The difference in the CNR response between AMV and E26 mutants could be due either to the \textit{gag} sequence or to the residual \textit{ets} sequence fused in frame with the \textit{myb} product. Therefore, we tested the biological activity of a AMV mutant virus (LiNR8) which expresses a P6\textit{gag}-\textit{myb} fusion protein. This mutant, isolated from pigmented retinal epithelial cells, has been molecularly cloned and the junction between \textit{gag} and \textit{myb} has been sequenced (Fig. 1). LiNR8
TABLE 1. Effects of bFGF on CNR transformation

<table>
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<tr>
<th>Virus</th>
<th>No. of colonies on agar</th>
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<td>XJ E26 3A</td>
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<td>LiNR8</td>
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FIG. 4. Comparative study of the effect of bFGF on the growth of AMV wild-type and mutant-infected cells in different conditions. (A) AMV and AMV mutants deleted in the carboxyl terminus; (B) AMV and other AMV mutants. Cells were tested as described in the legend to Fig. 2 in defined medium (solid black columns), in medium supplemented with 1% FCS (hatched gray columns), or in both media but with 4 ng of bFGF per ml added every other day (defined medium, solid white columns; medium supplemented with 1% FCS, hatched columns). After 4 days in culture, duplicate wells were trypsinized and cells were counted. Cells used are listed below each column. Similar results were obtained with three distinct cultures.

resulted from a recombination event between RAV-1 and AMV, removing the neo gene from the NEO AMV (18).

CNR cells infected with LiNR8 did survive, as well as AMV-infected cells, when maintained in minimal medium. However, while AMV cells hardly responded to bFGF, cells infected with LiNR8 responded to the growth factor as well as cells infected with E26. This suggests that, in that assay, the gag gene is able to increase the activity of the myb product.

We next analyzed the effect of bFGF on the CNR cells infected with AMV mutants in serum-free medium versus medium supplemented with 1% serum. This last condition was chosen because of the low proliferative potential, in serum-free medium, of v-myb-expressing cells. As shown in Fig. 4A, wild-type AMV, LX4, and LX3 responded to bFGF, while LX1 and LX2 did not respond to it even in the presence of 1% serum. This suggests that for bFGF response, a critical region of the myb product lies between amino acids 240 and 302 (18) (Fig. 1). A second series of mutants confirmed these results. CNR cells transfected or infected with mutant D3, lacking amino acids 240 to 301, were totally unresponsive to bFGF, while CNR cells transfected or infected with mutant D4, which contained this domain, did respond to bFGF (Fig. 4B). Mutant VP16 in which the transcriptional activation domain of VP16 protein of herpes simplex virus is linked to the amino-terminal domain of inactive D3 mutant also induces the CNR cells to respond to bFGF (Fig. 4B).

Effects of bFGF on the anchorage-independent growth of infected cells. The ability of cells to grow under anchorage-independent conditions has been shown to correlate with transformation (21). We therefore tested the ability of CNR cells to respond to bFGF when maintained in soft agar in the presence of serum-supplemented medium. As shown in Table 1, none of the infected cells formed colonies in the absence of FGF, and helper-infected cells exposed to bFGF were unable to grow. In contrast, cells expressing myb-ets or various myb products did grow in soft agar when bFGF was present. As in monolayer culture, LX1, LX2, and D3 were unresponsive to bFGF. In addition, it was observed that colonies developing in the presence of bFGF were larger for E26-transfected CNR cells than for cells transfected with AMV.

Protein products of mutant v-myb-ets and v-myb genes. To confirm that the expected proteins were found in CNR cells expressing mutant viruses, cells were labeled with [35S]methionine and lysates were immunoprecipitated with ets and myb antisera. As a control we also used gag antiserum. Results are shown in Fig. 5. Comparable amounts of P135gag-myb-ets and mutant proteins were found in E26, XJ E26 3A-, and XJ E26 4A-infected cells. Since the epitope recognized by the anti-ets serum lies downstream from the deletion site (14), mutants proteins were immunoprecipitated with anti-myb and anti-ets antibodies.

The relative mobilities of the mutant v-myb proteins were consistent with their predicted molecular weights as previously published (18). All of the v-myb mutant viruses tested produced nearly wild-type levels of stable protein products. In addition, anti-gag antibodies revealed similar amount of Pr76 RAV-1 product in each of the AMV mutant-infected CNR (Fig. 5B, lanes 1). This suggests that the efficiency of the helper infection was equivalent in all experiments, consistent with the similar number of G418-resistant colonies found in each culture after selection.
FIG. 5. Analysis of viral proteins in infected CNR. (A) E26 wild-type and mutant-infected CNR. Serums used were as follows: lanes 1, rabbit anti-myb serum; lanes 2, rabbit anti-ets serum. The mobility of P135gag-myb is indicated on the left. (B) AMV- and AMV mutant-infected CNR. Serums used were rabbit anti-gag serum (lane 1) and rabbit anti-myb serum (lane 2). Cell types used are listed across the top. The relative mobility of wild-type (wt) p48\textsuperscript{wt} and Pr76\textsuperscript{wt} are indicated on the left. (C) AMV- and AMV mutant-infected CNR. Serum used was the rabbit anti-myb serum. Cell types are listed across the top. The mobility of p48\textsuperscript{wt} is indicated.

DISCUSSION

The E26 and AMV leukemia viruses have been difficult to study in chickens, because unlike other oncogene-bearing viruses, they transform only hemopoietic cells. In this work we have extended our previous study which showed that E26 was able to induce a sustained proliferation of resting CNR cells. We observed that E26-infected CNR cells were able to proliferate in defined medium and exhibit a transformed phenotype in the presence of bFGF. Using this new biological assay, we have studied E26 mutant viruses deleted in the ets sequence, as well as AMV and AMV myb mutant viruses to define the respective roles of myb and ets in the response of CNR cells to bFGF.

The major difference between E26 (myb-ets) and AMV (myb) was seen in their anchorage-dependent growth in minimal medium. E26 CNR cells proliferate more actively than AMV CNR cells. The cause of this higher activity was the presence of the ets oncogene as demonstrated by the study of CNR cells infected with E26 mutants lacking most of the ets sequence. In minimal medium these cells exhibit a reduced proliferative potential, comparable to that of AMV CNR. Although all these infected cells were able to respond to bFGF, the response of E26 ets CNR was more pronounced than AMV CNR. The study of LiNR\(_8\), a spontaneously AMV mutant encoding a gag-myb fusion protein, suggested that the gag sequence is able to activate the myb gene rendering CNR cells more responsive to bFGF. Since previous reports have already shown that the gag gene was able to activate kinase-encoding oncogenes (10, 12, 34), it is conceivable that the gag sequences modify the conformation of the myb gene product, resulting in increased protein activity.

We have shown above that the presence of ets sequences in E26 increases the growth of infected CNR cells in minimal medium. ets sequences (c-ets-1 and c-ets-2) have been shown to encode transcriptional activators (5, 42) recognizing the PEA3 DNA sequence (42). The PEA3 sequence is a nuclear target for transcription activation by nonnuclear oncogenes (43). Therefore, the ets part of the P135 oncogenic protein of E26 may substitute for the signal transduction pathway which acts through the PEA3 motif and thereby activate genes required for cell growth in minimum medium. Alternatively, the ets sequence could modify the transactivation properties of the myb sequences and/or the nature of the myb-regulated genes.

The above results indicate that the v-myb product is able to confer bFGF responsiveness to the CNR cells. myb encodes a transcriptional activator containing an amino-terminal DNA-binding domain, a transcriptional activation domain, and a transcriptional negative regulation domain (39). CNR cells expressing v-myb are not transformed but are able to grow in soft agar in the presence of bFGF, suggesting a cooperative effect between the myb functions and the bFGF signal transduction pathway. Previous studies of v-myb deletion and insertion mutants demonstrated that transformation of hemopoietic cells correlates with transactivation of gene expression (19, 24). We have studied representative mutants in the CNR system (Fig. 6). Some mutants, D4 and LX3, although greatly diminished of

FIG. 6. Structure and function of the protein products of wild-type and mutant v-myb genes on CNR and hemopoietic cells. The results with the hemopoietic cells come from reference 18.
transactivating activity on fibroblasts, are still able to allow the CNR cells to grow in soft agar in the presence of bFGF. Particularly interesting are mutants D3 and VP16. In D3 a deletion was made in the domain defined to be necessary and sufficient for transcriptional activation (24, 39, 44). D3 was unable to induce the formation of CNR cell colonies in soft agar in the presence of bFGF. When this sequence was replaced with the carboxy-terminal transactivation domain of the VP16 protein of herpes simplex virus (19), a virus biologically active on CNR cells was obtained. This demonstrates that the transactivation domain of v-myb is critical for the response of infected CNR cells. The fact that D4 and LX3 mutants are not fully active in the hemopoietic cell system or in transactivation of gene expression in fibroblasts but retained a full biological activity on CNR cells could be due in CNR to the presence of a set of accessory proteins required to enhance the activity of the myb protein. Presumably, D3 is negative in all of these assays because of the deletion in the domain required to transactivate gene expression.

The most remarkable difference between the biological responses induced by myb expression in hemopoietic cells versus CNR cells is in the cell dependence on growth factors. In myeloid cells AMV, but not E26, induces a transformed phenotype independent of growth factors (2). Mutant LX3, which lacks the carboxyl-terminal domain of v-myb, is still able to maintain a transformed phenotype if macrophase-derived growth factors are present (18). This deletion is also found in the myb gene of E26. Thus, in the hemopoietic cells, the deleted form of v-myb reveals a cooperation between myb and growth factors. In CNR cells, both AMV- and E26-infected cells are dependent on bFGF to exhibit a transformed phenotype. This cooperation takes place with the wild-type and mutant proteins. However, in the CNR model, there are differences in the degree of cooperation. Mutants D4 and LX3 are reproducibly more effective in eliciting the bFGF response than the wild-type AMV or LX4 mutant. This suggests that deletions could enhance the cooperativity of myb with the growth factor. Many transcriptional activators act after binding to other proteins and, as discussed previously, the ets sequence associated with myb increases the growth response of infected CNR cells in minimal medium. Therefore, bFGF could induce the expression or posttranslational modification of a subset of proteins which, in CNR, would allow the myb product to form a more active transcriptional complex. Comparison of myb-associated proteins in hemopoietic and CNR cells could help to challenge this hypothesis.

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