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Specific Amino Acid Substitutions Are Not Required for Transformation by v-*myb* of Avian Myeloblastosis Virus

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The protein product of the v-*myb* oncogene of avian myeloblastosis virus, p48$^{v\text{-}myb}$, differs structurally in several ways from its normal cellular homolog, p75$^{c\text{-}myb}$. We demonstrated that the 11 specific amino acid substitutions found in two independent molecular clones of this virus were not required for the transformation of myeloblasts by v-*myb*.

Avian myeloblastosis virus (AMV) causes acute myelomonocytic leukemia in chickens and transforms only myeloid cells in vitro. The v-*myb* oncogene of this virus arose by transduction of cellular sequences contained in the c-*myb* proto-oncogene (reviewed in reference 11). As a result of this transduction event, the protein product of v-*myb* differs structurally from its normal cellular counterpart in three ways (6, 7, 18). First, p48$^{v\text{-}myb}$ is truncated at both its amino and carboxyl termini relative to p75$^{c\text{-}myb}$. Second, p48$^{v\text{-}myb}$ has new amino and carboxyl termini which are encoded by portions of the viral *gag* and *env* genes, respectively. Third, p48$^{v\text{-}myb}$ contains 11 amino acid substitutions within the region of general homology with p75$^{c\text{-}myb}$. The presence of an identical set of such substitutions in two independent molecular clones of AMV suggests that they may have some biological significance (10, 19). The E26 leukemia virus has independently transduced a segment of c-*myb* which lacks these amino acid substitutions (15). However, the E26 virus also contains large coding segments of the *gag* and *ets* genes, which are not present in AMV and which might compensate for the lack of specific amino acid substitutions.

We have recently demonstrated that neither the *gag*- nor the *env*- encoded terminus of p48$^{v\text{-}myb}$ is required for myeloblast transformation (13; unpublished data). To further investigate the mechanism of activation of the c-*myb* gene by viral transduction, we have now constructed and tested a v-*myb* virus which lacks all 11 of the specific amino acid substitutions present in p48$^{v\text{-}myb}$ of AMV. The study of such a *myb* virus is of interest because other proto-oncogenes, most notably *ras*, can be activated by specific amino acid substitutions (2, 17, 21, 22).

Construction of a v-*myb* gene lacking the specific amino acid substitutions encoded by AMV. The 11 amino acid substitutions in p48$^{v\text{-}myb}$ of AMV are shown in Table 1. Although a few of the substitutions are conservative in nature (arginine to lysine, for example), many of them represent rather drastic changes in chemical or structural properties (valine to aspartate or leucine to proline, for example). The location of these 11 substitutions within p48$^{v\text{-}myb}$ is shown schematically in Fig. 1. Previous studies had shown that the extreme carboxyl terminus of p48$^{v\text{-}myb}$, which is encoded by DNA sequences between the *Bal*I restriction enzyme site and the p48$^{v\text{-}myb}$ termination codon, was dispensable for transformation (13). The two amino acid substitutions contained within this region are thus not required for transformation. We therefore began our constructions with the pUCmyb-LX4 plasmid, which contains a v-*myb* gene bearing this deletion.

To remove the first, most amino-terminal amino acid substitution, pUCmyb-LX4 was subjected to oligonucleotide-directed site-specific mutagenesis to alter nucleotide 223 (19) by the gapped duplex method (9, 16) using the following primer, with the mutated nucleotide indicated in bold type:

v-*myb*: CAGAACTTAACAAAGGTCCA
primer: CAGAACTTA**T**CAAAGGTCCA

Mutant colonies were detected by initial colony hybridization with ^{32}P-end-labeled primer as a probe with final washes in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 58°C. Plasmid DNAs from several positive colonies were then used to retransform bacteria, and representative colonies were rescreened by oligonucleotide hybridization. The presence of the desired mutation in several colonies was then verified by DNA sequencing (3).

The remaining eight amino acid substitutions were removed by replacing the *Nco*I-*Sal*I fragment of AMV with the corresponding fragment of pLEB. The latter is a partial plasmid clone of the E26 leukemia virus which itself does not encode any amino substitutions relative to c-*myb* in this region (15). This fragment exchange was verified by digestion with the *Sau*96I restriction enzyme, because the mutation in AMV which causes the sixth amino acid substitution simultaneously creates a novel recognition site for this enzyme which is not present in the E26-derived sequences.

The resulting plasmid, pUCmyb-LX4-NM, contains a v-*myb* gene with none of the specific amino acid substitutions present in wild-type AMV. To test the biological activity of this gene, plasmid pNEO-AMV-LX4-NM was constructed by using the *Kpn*I-*Xba*I fragment of pUCmyb-LX4-NM to replace the *Kpn*I-*Xba*I *env* fragment of the plasmid pNEO-MAV (14). A related proviral clone, pNEO-

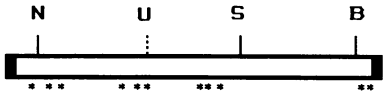

FIG. 1. Location of the specific amino acid substitutions in wild-type p48$^{v\text{-}myb}$. The primary amino acid sequence of p48$^{v\text{-}myb}$ is represented by the large box (*myb*-specific residues are unshaded; *gag* and *env*-encoded residues are shaded). The positions of the amino acid substitutions within the *myb* domain are indicated by asterisks below the box. The relative positions of restriction enzyme sites used in constructing the mutant v-*myb* gene are indicated above the box (N, *Nco*I; U, *Sau*96I; S, *Sal*I; B *Bal*I).

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TABLE 1. Amino acid substitutions in $p48^{v\text{-}myb}$

Protein	Predicted sequence[a] and substitutions
$p75^{c\text{-}myb}$	Ile-91 Leu-106 Val-117 Ile-181 Leu-199 Leu-207 Val-267 Val-270 Asn-285 Arg-438 Pro-441
$p48^{v\text{-}myb}$	Asn-26 His-41 Asp-52 Val-116 Pro-134 Pro-142 Ile-202 Ile-205 Thr-220 Lys-373 Thr-376

[a] Data for $p75^{c\text{-}myb}$ from reference 6. Data for $p48^{v\text{-}myb}$ (except six amino-terminal residues encoded by spliced *gag* leader) from references 10 and 19.

AMV, which coexpresses the dominantly selectable marker *neo* and the wild-type v-*myb* gene, has also been described (14).

Transformation of avian myeloid cells by the mutant v-*myb* retroviral vector. To introduce the mutant v-*myb* gene into avian hematopoietic cells, the NEO-AMV-LX4-NM proviral DNA was first cotransfected with proviral DNA of the MAV-1 helper virus into QT6 quail cells as previously described with DEAE-dextran at 200 μg/ml (4). These transfected QT6 cells were then selected for *neo* expression with the drug G418 at 200 μg/ml. Cells from the G418-resistant colonies were pooled and treated with mitomycin C at 10 μg/ml for 2 h to prevent cell division but not virus production. These cells were then cocultivated with myeloid cells freshly isolated from the yolk sacs of 12-day-old White Leghorn chicken embryos as described previously (13). After 24 h of cocultivation, the nonadherent yolk sac cells were replated in a fresh tissue culture dish, and the incubation was continued for several weeks.

Similar morphological transformation was evident in mutant NEO-AMV-LX4-NM (Fig. 2) and wild-type-NEO-AMV-infected cultures at 2 weeks of incubation but not in uninfected control cultures. The small, round, highly refractile NEO-AMV-LX4-NM-transformed cells had a morphology typical of v-*myb*-transformed myeloblasts when stained with Wright-Giemsa (Fig. 3) and were indistinguishable from NEO-AMV-transformed cells in this respect. Myeloblasts transformed by the NEO-AMV-LX4-NM virus were capable of forming colonies in soft agar (Fig. 4), as are cells transformed by the NEO-AMV virus (13). Thus, the NEO-AMV-LX4-NM virus lacking the specific amino acid substitutions present in the v-*myb* gene of AMV is competent for myeloblast transformation. However, cells transformed by the mutant virus grew more slowly than those transformed by wild-type virus and formed fewer colonies in soft agar. Both types of cells could be subcultured in the absence of the original yolk sac macrophage feeder layers, but the NEO-AMV-LX4-NM-transformed cultures consistently contained many more dead cells and debris than NEO-AMV-transformed cultures. This suggests that while the amino acid substitutions present in AMV are not required for transformation per se, they do confer a selective advantage on transformed cells, at least in the simple growth conditions used in our assay. These substitutions may therefore have been positively selected during the repeated passage of leukemogenic stocks of AMV in chickens.

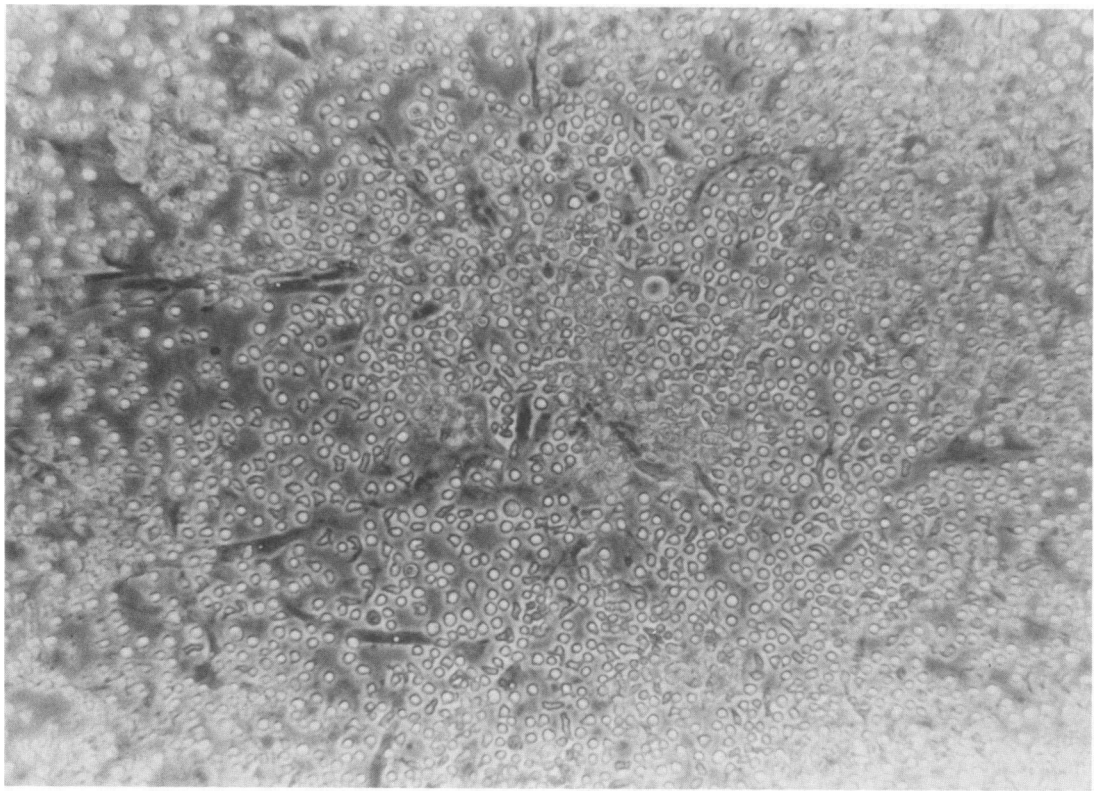

FIG. 2. Transformation of yolk sac myeloid cells by NEO-AMV-LX4-NM virus. Cells from 12-day-old chicken embryo yolk sac were cocultivated with adherent mitomycin-treated QT6 virus-producing cells for 24 h. Nonadherent cells were then transferred to fresh dishes and incubated for 3 weeks at 37°C. The small, round, highly refractile cells are transformed myeloblasts.

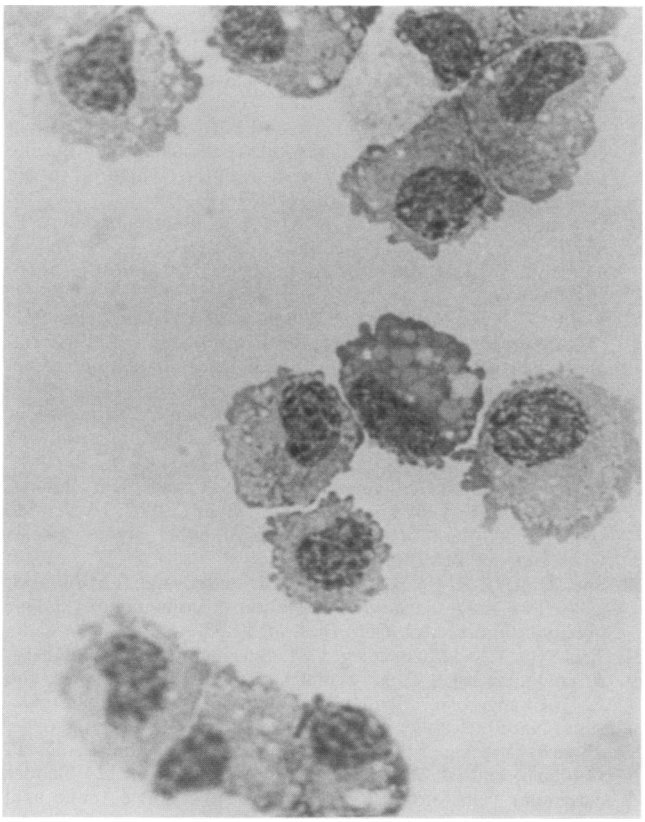

FIG. 3. Morphology of cells transformed by NEO-AMV-LX4-NM virus. Nonadherent cells from the culture shown in Fig. 2 were spread and air-dried with a cytocentrifuge and then stained with Wright-Giemsa.

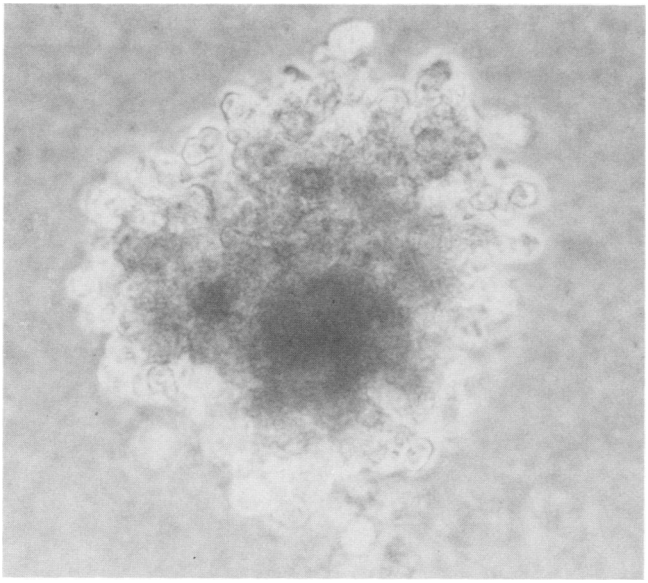

FIG. 4. Growth of NEO-AMV-LX4-NM-transformed myeloblasts in soft agar. Nonadherent cells from the cultures shown in Fig. 2 were seeded in medium containing 0.3% agar (Difco) and incubated at 37°C for 2 weeks.

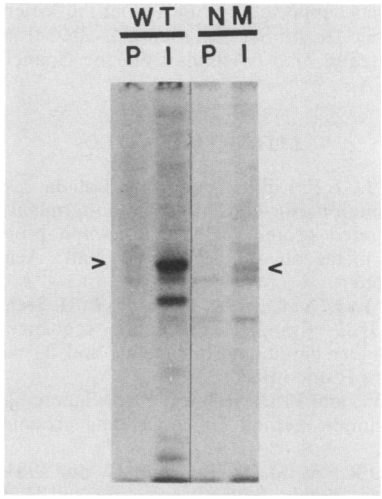

FIG. 5. Proteins encoded by v-*myb* and v-*myb*-LX4-NM. Myeloblasts transformed by either wild-type NEO-AMV (WT) or the mutant NEO-AMV-LX4-NM (NM) virus were metabolically labeled with [^{35}S]methionine and lysed in detergent buffer as described previously (13). Lysates were then immunoprecipitated with preimmune serum (P) or an *myb*-specific immune serum (I) as described previously (1). The arrows on the left and right indicate the migration of the wild-type and mutant v-*myb* gene products, respectively.

Expression of v-*myb* protein in myeloblasts transformed by NEO-AMV-LX4-NM. The protein product of the mutant v-*myb* gene was identified in transformed myeloblasts by metabolic radiolabeling and immunoprecipitation (Fig. 5). The product of the mutant v-*myb* gene migrated more rapidly than the wild type v-*myb* gene product, as would be predicted for a protein with a net deletion of 14 amino acids at its carboxyl terminus. The mutant gene product was, however, relatively less abundant than the wild-type gene product in transformed myeloblasts. This suggests that there may be a dosage effect in addition to a potential structural basis for the observed selective advantage of myeloblasts transformed by wild-type $p48^{v\text{-}myb}$ relative to the mutant.

Mechanism of activation of the c-*myb* proto-oncogene. Previous studies have demonstrated that the virally encoded amino and carboxyl termini of $p48^{v\text{-}myb}$ are not required for myeloblast transformation (13; unpublished data). The experiments in this report demonstrate that the specific amino acid substitutions present in $p48^{v\text{-}myb}$ are also not required for myeloblast transformation. These experiments rule out two possible modes of c-*myb* activation, leaving two additional models for the unmasking of the leukemogenic potential of c-*myb*. First, inappropriately high levels of expression of c-*myb* itself may be sufficient for transformation. This is plausible because c-*myb* expression is tightly regulated both during the myeloid differentiation pathway (5, 8, 26) and during the cell cycle (12, 23, 24). Second, the amino and/or carboxyl-terminal truncation of $p75^{c\text{-}myb}$ may be required for its activation. This latter model is supported by retroviral insertional mutagenesis of the c-*myb* gene in several murine myeloid malignancies (20, 25), although altered *myb* proteins have not yet been demonstrated in such tumors. The recent molecular cloning of c-*myb* cDNAs from hematopoietic cells should allow the construction of appropriate viruses to test these hypotheses.

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