Expression of Molecular Clones of *v-myb* in Avian and Mammalian Cells Independently of Transformation

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We demonstrated that molecular clones of the *v-myb* oncogene of avian myeloblastosis virus (AMV) can direct the synthesis of *p48^{v-myb}* both in avian and mammalian cells which are not targets for transformation by AMV. To accomplish this, we constructed dominantly selectable avian leukosis virus derivatives which efficiently coexpress the protein products of the Tn5 *neo* gene and the *v-myb* oncogene. The use of chemically transformed QT6 quail cells for proviral DNA transfection or retroviral infection, followed by G418 selection, allowed the generation of cell lines which continuously produce both undeleted infectious *neo-myb* viral stocks and *p48^{v-myb}*. The presence of a simian virus 40 origin of replication in the proviral plasmids also permitted high-level transient expression of *p48^{v-myb}* in simian COS cells without intervening cycles of potentially mutagenic retroviral replication. These experiments establish that the previously reported DNA sequence of *v-myb* does in fact encode *p48^{v-myb}*, the transforming protein of AMV.

Avian myeloblastosis virus (AMV) is a replication-defective retrovirus which causes acute myelomonocytic leukemia in chickens and transforms avian myeloid cells in vitro (1, 2). The virus contains sequences transduced from the chicken *c-myb* proto-oncogene which have replaced viral 3' *pol* sequences and almost all of the *env* gene (11, 57). The *v-myb* oncogene of AMV is expressed as a spliced, subgenomic viral mRNA by using a transduced cellular splice acceptor site (28). Antisera raised against *myb*-specific synthetic peptides or bacterial expression vector products have been used to identify a 48,000 *M_r* nuclear protein in AMV-transformed myeloblasts and AMV-infected fibroblasts (5, 6, 29, 30, 32). This *v-myb* gene product, *p48^{v-myb}*, is hypothesized to be a hybrid protein containing an amino terminus encoded by *gag* sequences upstream of the normal viral splice donor site (6 amino acids), internal *myb*-specific residues downstream of the transduced splice acceptor site (371 amino acids), and a carboxyl terminus encoded by the remaining *env* sequences (11 amino acids) (Fig. 1). In this report we demonstrate directly that the entire open reading frame of the previously reported DNA sequence of *v-myb* encodes *p48^{v-myb}*.

The mechanism of transformation by *p48^{v-myb}* is unknown at present. Directed mutagenesis of the *v-myb* oncogene and replacement of *v-myb* sequences with homologous regions of *c-myb* would be very useful in understanding this mechanism. However, several characteristics of AMV have made genetic analysis of its oncogene product very difficult. Unlike other oncogene-bearing, acutely transforming retroviruses, AMV does not transform nonhematopoietic tissues in vitro or in vivo, and levels of production of pseudotyped AMV by nontransformed cells are very low. In addition, unlike fibroblasts transformed by conditional mutants of sarcoma viruses, temperature-sensitive AMV-transformed

myeloblasts terminally differentiate and survive only briefly in tissue culture after shift to the nonpermissive temperature (40). These features have hampered detailed biochemical analysis of the products of conditional or nontransforming mutants of AMV.

To produce *v-myb* viruses and their gene products consistently regardless of transforming capacity, we therefore constructed molecularly cloned variants of AMV which coexpress the dominantly selectable marker *neo* (8, 24, 56) in addition to a *v-myb* gene of known DNA sequence (47). The strategy used to introduce a selectable marker while preserving the *gag* leader and viral splice donor site should also be applicable to the study of other avian retroviral genes which are expressed via a subgenomic message, such as *v-myc* of the OK10 and MH-2 viruses, *v-erbB* of the avian

FIG. 1. Structure of the *v-myb* oncogene of AMV. The cross-hatched box represents *myb*-specific coding sequences, the large black boxes represent *gag* and *env* coding sequences also encoding *p48^{v-myb}*, the large open boxes represent *gag* and *pol* sequences not encoding *p48^{v-myb}*, and the stippled boxes represent LTR sequences. The small black box represents the transduced *pol* termination codon, and the small open box represents the first internal *myb*-specific AUG. BP indicates the region encoding the bacterially expressed *myb* immunogen. K = *Kpn*I; E = *Eco*RI; X = *Xba*I; SD = splice donor site; SA = splice acceptor site.

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FIG. 2. Construction of pMAV-1 and pAMV. The cross-hatched boxes represent the *myb* gene, the solid boxes represent the retroviral LTRs, the striped boxes represent the simian virus origin of replication, and the open boxes represent bacteriophage lambda arms (not to scale). Only relevant restriction enzyme sites are shown: B = *Bam*HI; G = *Bgl*II; K = *Kpn*I; O = *Xho*I; S = *Sal*I; X = *Xba*I; * = sites destroyed by cross-ligation (*Bam*HI-*Bgl*II or *Sal*I-*Xho*I). Details of constructions are provided in Materials and Methods. To conserve space, the various bacteriophage and plasmids were not drawn to a common scale.

erythroblastosis viruses, *v-src* of Rous sarcoma virus, and the *env* genes of the avian leukosis viruses. In addition, transient expression of $p48^{v-myb}$ in simian COS cells should provide a rapid means of screening protein production by mutants of *v-myb*.

MATERIALS AND METHODS

Plasmid constructions. The plasmid pSV0d (35), a PBR322 derivative which contains simian virus 40 origin of replication and lacks sequences inhibiting plasmid replication in eukaryotic cells (33), was kindly provided by J. Lubinski (University of California at Los Angeles). The plasmid pUC18 (43) was kindly provided by J. Messing (Rutgers University). The plasmid pUCneo, which contains the *Hind*III-*Sma*I neo fragment of Tn5 cloned into pUC8 after addition of an *Hind*III linker at the *Sma*I site (13), was kindly provided by I. Chen (University of California at Los Angeles). Other plasmids were constructed as described below by standard procedures (34). These constructions were greatly facilitated by use of the MATILDA computer program kindly provided by D. Shalloway (Pennsylvania State University) (49).

pMAV-1. A recombinant Charon 4A bacteriophage containing a biologically active MAV-1 provirus (45) was partially digested with *Bgl*II, and the 9.8-kilobase (kb) DNA fragment containing the intact provirus and flanking chicken cellular DNA was isolated and ligated to pSV0d DNA which had been previously digested with *Bam*HI and dephosphorylated with calf intestinal alkaline phosphatase to yield pMAV-1 (Fig. 2).

pUCmyb. A recombinant Charon 4A bacteriophage containing a full-length AMV provirus (57) was completely digested with *Kpn*I and *Xho*I. The 1.4-kb *Kpn*I-*Xho*I *myb* DNA fragment was isolated and ligated to pUC18 DNA which had previously been digested with *Kpn*I and *Sal*I to yield pUCmyb (Fig. 2).

pAMV. The two *Xba*I restriction sites of pMAV-1 within the viral *pol* gene and the 5' flanking cellular DNA were removed by two successive rounds of partial digestion with *Xba*I, isolation of full-length linear DNA, treatment with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of all four deoxynucleotide triphosphates, and blunt-end ligation under conditions favoring recircularization. The resulting plasmid was then digested at its unique *Kpn*I and *Xba*I restriction sites flanking the *env* gene. The large pMAV-1 DNA fragment was isolated and ligated to the 1.3-kb *Kpn*I-*Xba*I *myb* DNA fragment of pUCmyb to yield pAMV (Fig. 2).

pNEO-MAV and pNEO-AMV. DNA of pMAV-1 was completely digested with *Bgl*II and dephosphorylated with calf intestinal alkaline phosphatase, and the large pMAV-1 DNA fragment was isolated. This fragment was ligated to a *Bgl*II-*Bam*HI fragment of pUCneo, which contained the entire neomycin phosphotransferase type II (*npt*II) coding region (3). This *Bam*HI site was derived from the pUC8 polylinker 3' to the *Hind*III site downstream from the Tn5 insert. A clone in which the *neo* fragment was inserted in the same orientation as MAV-1 was designated pNEO-MAV. The *Xba*I site within the 5' flanking cellular DNA was removed as described above for pAMV, and DNA from a clone which lacked this site was then digested at its unique *Kpn*I and *Xba*I sites. The large NEO-MAV fragment was isolated and ligated to the 1.3-kb *Kpn*I-*Xba*I *myb* fragment of pUCmyb to yield pNEO-AMV (Fig. 3).

pNEO-AMV-dBG. pNEO-AMV DNA was completely digested at its unique *Bam*HI and *Bgl*II sites and religated under conditions favoring intramolecular ligation to yield pNEO-AMV-dBG (Fig. 3).

pNEO-POL-AMV and pPOL-NEO-AMV. pAMV DNA was partially digested with *Bgl*II and dephosphorylated with calf intestinal alkaline phosphatase, and linear DNA forms were isolated. These DNAs were ligated to the *Bgl*II-*Bam*HI

neo fragment of pUCneo (see above), and the resulting colonies were screened by various restriction enzyme digestions of plasmid DNA. Plasmids were identified which contained the proviruses NEO-POL-AMV and POL-NEO-AMV with the *neo* fragments inserted in the same orientation as MAV-1 (Fig. 3).

Cells culture, DNA transfections, and viral infections. Simian COS cells producing simian virus 40 large-T antigen (16) were kindly provided by J. Gasson (University of California at Los Angeles) and maintained in BMII medium (5) supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. COS cell transfections were performed with DEAE-dextran and chloroquine, as previously described (18), with 10 µg of plasmid DNA per 10-cm dish of cells plated 24 h earlier at a concentration of 10^6 cells per dish. Cells were analyzed for protein production 72 h later as described below.

The chemically transformed QT6 quail cell line (41) was kindly provided by R. Guntaka (University of Missouri) and maintained in BMII medium supplemented with 10% tryptose phosphate (Difco Laboratories), 5% fetal calf serum, and antibiotics as described above. Defective proviral plasmid (10 µg), either alone or in the presence of 1 µg of pMAV-1, was transfected by the polybrene-dimethyl sulfoxide method (26) in the absence of carrier DNA onto a

FIG. 3. Structure of the proviruses used for DNA transfection. Each of these proviruses was contained in a plasmid derived from pMAV-1 or pAMV (Fig. 2) and constructed as described in Materials and Methods. The solid boxes represent the retroviral LTRs, the stippled boxes represent the *neo* gene, and the cross-hatched boxes represent the *myb* gene. Only those restriction enzyme sites relevant to the constructions are shown: B = *Bam*HI; G = *Bgl*II; K = *Kpn*I; X = *Xba*I. SD = splice donor site; SA = splice acceptor site.

FIG. 4. Transient expression of p48^{v-myb} in COS cells. COS cells were transfected with 10 µg of plasmid DNA, and 72 h later the cells were metabolically labeled with [³⁵S]methionine, lysed in detergent buffer, and analyzed by immunoprecipitation with 120 ng of affinity-purified anti-*myb* antibodies in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of excess unlabeled, purified immunogen. Lanes: 1 and 2, mock-transfected cells; 3 and 4, pAMV-transfected cells; 5 and 6, pMAV-1-transfected cells. ¹⁴C-methylated proteins were electrophoresed in parallel as molecular mass standards (arrowheads at left correspond to 200, 93, 69, 46, 30, and 14 kilodaltons [from top to bottom]).

10-cm dish of QT6 cells which had been plated as described above. For *neo* selection, fresh medium containing 200 µg of G418 (GIBCO Laboratories) was added 48 h later and changed as needed every 1 to 3 days. After 10 to 14 days of continuous selection, G418^r colonies were clearly visible. For virus production, G418-free tissue culture medium from pooled G418^r colonies was harvested 24 to 48 h after a medium change. Virus stocks were passed through 0.45-µm (pore size) nitrocellulose filters and stored at -70°C. For viral titration, 10-cm dishes of QT6 cells plated as described above were incubated with filtered viral stocks diluted as indicated into 3 ml of tissue culture medium for 1 h at 37°C with occasional rocking and then flooded with an additional 7 ml of tissue culture medium. G418 selection was begun 48 h later as described above.

The AMV-transformed BM-2 chicken myeloblast cell line (42) kindly provided by M. G. and C. Moscovici (University of Florida) was maintained in BMII medium supplemented with 10% tryptose phosphate, 5% heat-inactivated chicken serum, 5% calf serum, and antibiotics as described above.

Eucaryotic DNA isolation and analysis. Genomic DNA was isolated as previously described (4) and analyzed after complete restriction enzyme digestion by electrophoresis in 0.7% agarose gels and blotting onto nitrocellulose filters (55). ³²P-labeled, nick-translated DNA probes (~10⁸ cpm/µg) used for hybridization were the 0.92-kb *Pst*I *neo*-specific fragment of pNEO (56) and the 0.35-kb *Eco*RI *myb*-specific fragment of pUC165x (Boyle, Lipsick, and Baluda, Proc. Natl. Acad. Sci. USA, in press), which contains *v-myb* sequences from the splice acceptor site to the internal *Eco*RI site (Fig. 1).

Cell labeling and immunoprecipitation. Dishes (10-cm di-

TABLE 1. Titration of selectable AMVs

Plasmid or virus	Kanamycin resistance in <i>E. coli</i> ^a	Virus rescued from G418 ^r transfectants (U/ml)	Virus rescued from G418 ^r infected cells (U/ml)
NEO-AMV	+++	5 × 10 ³	3 × 10 ⁴
NEO-AMV-dBG	+++	2 × 10 ⁴	4 × 10 ⁴
NEO-POL-AMV	+++	No colonies	Not done
POL-NEO-AMV	+++	8 × 10 ²	8 × 10 ⁴

^a + + +, Growth in 50 µg of kanamycin per ml.

ameter) of cells were incubated in 3 ml of methionine-free BMII medium supplemented with 5% dialyzed calf serum for 60 min, 0.5 mCi of [³⁵S]methionine (>800 mCi/mmol; New England Nuclear Corp.) was added per 10-cm dish, and incubation was continued for an additional 90 min, at which time cells were lysed with 4 ml of ice-cold detergent buffer as previously described (5). Total incorporation of [³⁵S]methionine into cellular proteins was determined by trichloroacetic acid precipitation and scintillation counting, and for each experiment, individual lysates were normalized to contain equivalent concentrations of radiolabeled proteins by dilution with additional detergent buffer. Immunoprecipitation, sample analysis by electrophoresis in denaturing polyacrylamide gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and autoradiography were performed as previously described (5) except that unabsorbed protein A-Sepharose (Pharmacia) was used in place of preabsorbed, fixed *Staphylococcus aureus*. Anti-AMV p27^{rag} antiserum was kindly provided by D. Bolognesi (Duke University); anti-nptII antiserum was kindly provided by B. Reiss, M. Kaling, and H. Bohnert (University of Arizona); preparation and affinity purification of anti-BP52^{trpE-myb} antiserum will be described elsewhere (Boyle, et al., in press).

RESULTS

Transient expression of p48^{v-myb} in simian COS cells. A recombinant DNA clone of an AMV provirus has previously been isolated in our laboratory (57), and its *v-myb* DNA sequence has been completely determined (47). Injection of chickens with fibroblasts which had been transfected with this proviral clone resulted in disease typical of AMV, but only after a prolonged latency period (52). To prove that the *v-myb* sequences of this clone are in fact capable of directing the synthesis of p48^{v-myb} in the absence of mutation or recombination with cellular or retroviral sequences after viral replication and selection for transformation, we sought to express them transiently in the well-studied COS cell system (35).

To preserve the retroviral *gag* leader and splice donor site, which are essential for *v-myb* expression, and also to permit amplification and high-level transient expression in simian COS cells, the plasmid pAMV was constructed (Fig. 2). This plasmid contains DNA from three different sources: (i) a pSVOD backbone allowing replication and selection in bacteria and replication in COS cells (35); (ii) a biologically active proviral clone of type 1 myeloblastosis-associated virus (MAV-1), the helper virus from which AMV appears to have arisen (45); and (iii) the *KpnI-XbaI* restriction fragment of AMV which contains the entire transduced region of *v-myb* (47) and which was inserted in place of the MAV-1 *env* gene to reconstruct an AMV provirus.

Transfection of pAMV DNA into COS cells resulted in the expression of high levels of a 48,000 *M_r* protein identified by metabolic labeling with [³⁵S]methionine and subsequent im-

FIG. 5. Structure of integrated selectable AMV proviruses. High-molecular-weight genomic DNA was isolated from uninfected or pooled colonies of G418^r viral vector-infected QT6 cells. (A) A *neo*-specific probe was used to detect *HindIII*-restricted fragments after Southern blotting of (lanes): 1, uninfected QT6 cells; 2, NEO-AMV(MAV-1)-infected G418^r cells; and 3, POL-NEO-AMV(MAV-1)-infected G418^r QT6 cells; 4, NEO-AMV-dBG(MAV-1)-infected G418^r QT6 cells. The positions of coelectrophoresed *HindIII*-digested bacteriophage lambda DNA molecular size standards are indicated on the left in kilobases. (B) An *myb*-specific probe was used to detect *HindIII*-restricted (lanes 1 to 4) or *XhoI*-restricted (lanes 5 to 8) fragments after Southern blotting of (lanes): 1 and 5, uninfected QT6 cells; 2 and 6, NEO-AMV(MAV-1)-infected G418^r QT6 cells; 3 and 7, POL-NEO-AMV(MAV-1)-infected G418^r QT6 cells; 4 and 8, NEO-AMV-dBG(MAV-1)-infected G418^r QT6 cells. The positions of coelectrophoresed, *HindIII*-digested bacteriophage lambda DNA molecular size standards are indicated on the left in kilobases. (C) Abbreviated restriction endonuclease maps of the initially transfected DNAs indicating the fragment sizes expected for unrearranged proviral DNAs. Solid boxes represent retroviral LTRs, stippled boxes represent the *neo* gene, and cross-hatched boxes represent the *myb* gene. H = *HindIII*; O = *XhoI*.

munoprecipitation with anti-myb-specific antisera (Fig. 4). Mock transfection without DNA or transfection of pMAV-1 DNA, which differs from pAMV only in that the *env* gene replaces *v-myb*, did not result in the production of this protein. The antibodies used to detect p48^{v-myb} in COS cells were raised against a bacterial fusion protein containing the first 115 myb-specific amino acids, which are encoded entirely upstream of the first internal myb-specific AUG of AMV and which are preceded by an in-frame termination codon (47) (Fig. 1). In addition, antibodies directed against a carboxyl-terminal v-myb peptide also immunoprecipitated p48^{v-myb} in COS cells (data not shown). This implies that the

FIG. 6. Expression of *neo*-encoded proteins by selectable AMV proviruses. (A) The cells described in Fig. 5 were metabolically labeled with [³⁵S]methionine. Detergent lysates of these cells were analyzed by immunoprecipitation with 1:250 dilutions of either normal rabbit serum (lanes 1, 3, 5, and 7) or rabbit anti-nptII serum (lanes 2, 4, 6, and 8) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes: 1 and 2, uninfected QT6 cells; 3 and 4, NEO-AMV(MAV-1)-infected G418^r QT6 cells; 5 and 6, POL-NEO-AMV(MAV-1)-infected G418^r QT6 cells; 7 and 8, NEO-AMV-dBG(MAV-1)-infected G418^r QT6 cells. Molecular mass markers are as indicated in Fig. 4. (B) The predicted structure of the *gag-neo* junction in NEO-AMV as deduced from the sequence of Pr-C Rous sarcoma virus, which is very closely related to MAV-1 (45, 48), and from that of Tn5 (3). The preserved *Bgl*III site is boxed, and the coding potentials of the predicted *gag* and *neo* reading frames are indicated.

FIG. 7. Stable expression of p48^{v-myb} by selectable AMV proviruses. Samples of the QT6 cell lysates described in Fig. 6 and of lysates of similarly radiolabeled AMV-transformed BM-2 cells were analyzed by immunoprecipitation with a 1:2,500 dilution of normal rabbit serum (lanes 1, 5, 9, 13, and 17), a 1:2,500 dilution of rabbit anti-p27^{gag} serum (lanes 2, 6, 10, 14, and 18), 120 ng of affinity-purified anti-myb antibody (lanes 3, 7, 11, 15, and 19), or 120 ng of affinity-purified anti-myb antibody blocked with excess bacterial myb antigen (lanes 4, 8, 12, 16, and 20). Lanes 1 to 4, uninfected QT6 cells; 5 to 8, NEO-AMV(MAV-1)-infected G418^r QT6 cells; 9 to 12, POL-NEO-AMV(MAV-1)-infected G418^r QT6 cells; 13 to 16, NEO-AMV-dBG(MAV-1)-infected G418^r QT6 cells; 17 to 20, BM-2 cells.

COS cell p48^{v-myb} utilizes the entire predicted *v-myb* open reading frame and is very likely to employ the same *gag* AUG initiation codon as authentic p48^{v-myb} of AMV. Thus, the previously reported sequence of *v-myb* is indeed capable of directly expressing p48^{v-myb}, the transforming protein of AMV.

Design and construction of selectable AMVs. To allow production of infectious AMVs and their oncogene products regardless of transforming capacity, we wished to introduce an independent selectable marker gene into the virus. Transmissible retroviral vectors with selectable, nononcogenic marker genes have recently been constructed from the murine leukemia and sarcoma and structurally related avian reticuloendotheliosis viruses (51, 58, 60). Retroviral vectors that successfully utilize internal promoters to drive two independent transcription units have been reported (36); however, such vectors often display a high frequency of deletion and transcriptional suppression (12, 13). In contrast, all known replication-competent retroviruses normally use a single 5' promoter to coexpress at least two distinct gene complexes, *gag-pol* and *env*, via regulated partial mRNA splicing. Viruses of the avian leukosis group differ from the murine leukemia and sarcoma and avian reticuloendotheliosis viruses by using a single translation initiation site for these two gene complexes. This is achieved by the position of the splice donor site downstream of the *gag* initiation codon (19, 48), which results in a common *gag* leader peptide on the primary translation products of *gag*, *gag-pol*, *env*, and numerous oncogenes, including *v-myb* of AMV.

The *v-myb* gene contains its own splice acceptor site of cellular origin and requires precise splicing for efficient translation, since the amino terminus of its gene product, p48^{v-myb}, is encoded by the viral *gag* sequences upstream of the splice donor site (5, 28, 29, 32). To preserve this viral splice donor site and preceding *gag* sequences, we placed the selectable marker Tn5 *neo* within the partially spliced retroviral intron in place of or in addition to various *gag* and *pol* sequences. This dominant gene confers resistance to neomycin and kanamycin upon procaryotes and resistance to the otherwise lethal aminoglycoside G418 upon eucaryotic

cells (8, 24, 56). We also retained the *cis*-acting sequences which are essential for retroviral transcription initiation and termination, packaging, reverse transcription, and integration (59).

The structure of the various selectable AMVs and the parental proviruses from which they were derived are shown in Fig. 3. Because deletion of the avian leukosis virus *Bgl*II fragment, which contains a portion of the *gag* and *pol* genes, did not disturb RNA splicing and *env* expression after microinjection of proviral DNA (10), we replaced this fragment with *neo* (pNEO-AMV). We also replaced a larger *Bam*HI-*Bgl*II fragment (pNEO-AMV-dBG), which contains a putative viral packaging site (46). Finally, since intronic sequences themselves have been shown to regulate retroviral gene expression (7, 23), we also constructed two vectors in which the entire viral intron was retained with the addition of the *neo* gene at different distances from the 5' viral long terminal repeat (LTR) pNEO-POL-AMV and pPOL-NEO-AMV).

Transfection of QT6 cells and selection of proviral DNAs. Although chicken embryonic fibroblasts cannot generally be stably transfected with DNA (9), the chemically transformed QT6 quail cell line has been shown to integrate a nontransmissible expression vector (Rous sarcoma virus LTR-*neo*-Rous sarcoma virus LTR) stably (37). This cell line had previously been shown to be clonable and to be apparently devoid of endogenous retroviruses which might recombine with the transfected viral genomes (41). Transfection of QT6 cells with pNEO-MAV, pNEO-AMV, pPOL-NEO-AMV, or pNEO-AMV-dBG DNAs (Fig. 3) alone, followed by selection with G418, yielded approximately 2 to 20 G418^r colonies per microgram of DNA per 10⁶ transfected cells (data not shown). These transfected cells stably maintained their G418^r phenotype in the prolonged absence of selection during 2 months of passaging. Transfection with pMAV-1 DNA or mock transfection did not result in any G418^r colonies under similar selection.

Surprisingly, transfection with pNEO-POL-AMV did not result in any G418-resistant colonies, although the sequences 5' to the *neo* gene were identical to those in pNEO-AMV (Fig. 3). The *neo* gene of pNEO-POL-AMV was shown to be functionally intact by its ability to confer kanamycin resistance upon *E. coli* despite the absence of its normal promoter (Table 1). This bacterial expression of *neo* was presumably driven by the MAV-1 LTR, which has a U5 region nearly identical to that of Rous sarcoma virus (47). The latter has previously been shown to contain a functional procaryotic promoter (38).

Production of infectious selectable viral stocks. To generate infectious viral stocks, QT6 cells were cotransfected with individual vector DNAs and cloned helper virus DNA (pMAV-1) in a 10:1 ratio to allow the spread of pseudotyped defective virus. QT6 cells are permissive for the replication of subgroup A retroviruses including MAV-1, which has a particularly low incidence of cytopathicity for avian cells (39, 41, 45). Tissue culture supernatants were first harvested at various times in the absence of selection and tested on fresh QT6 cells for transmissible G418^r virus production. Barely detectable titers (<10 G418^r U/ml) of selectable virus were found during the first week after transfection. In contrast, if similar cultures were first selected for G418^r before virus production was assayed, titers of greater than 10⁴ G418^r U/ml could be obtained (Table 1). Surprisingly, high-titer virus was rescued from cells transfected with pNEO-AMV-dBG DNA, which lacks a putative viral packaging site (46). The independence of calculated viral titer

with respect to viral dilution indicated single-hit kinetics for G418^r, implying that only one defective viral genome was required for resistance. The presence of helper virus in the various infected G418^r colonies was verified by immunoprecipitation of viral *gag* and *env* proteins which were not present in uninfected cultures (for an example, see Fig. 7).

Structure of selectable AMV proviruses in infected cells. Genomic DNA from pooled colonies of QT6 cells which had been infected with viral stocks and selected with G418 for 2 weeks was analyzed for the presence of integrated proviral DNA by Southern blotting after restriction endonuclease digestion. Uninfected cells contained no *neo*-specific DNA sequences (Fig. 5A, lane 1). Both uninfected and infected cells contained an *myb*-specific *Hind*III-restricted DNA fragment of 5.2 kb, which corresponds to the single-copy quail *c-myb* gene as previously described (4) (Fig. 5B, lanes 1 to 4). The *myb*-specific, 20-kb, *Xho*I-restricted DNA fragment present in both uninfected and infected cells also presumably represents the quail *c-myb* gene (Fig. 5B, lanes 5 to 8).

Novel DNA fragments containing additional single-copy sequences homologous to *neo* and *myb* were identified in vector-infected cells relative to uninfected cells. In the case of NEO-AMV(MAV-1)- and NEO-AMV-dBG(MAV-1)-infected cells, the sizes of the major *Hind*III-restricted DNA fragments (Fig. 5A, lanes 2 and 4, and B, lanes 2 and 4) were as expected from the structure of the originally transfected proviral plasmid DNAs (Fig. 5C). This indicated the presence of predominantly unrearranged, single-copy proviruses in these cells and furthermore demonstrated that the NEO-AMV-dBG provirus had not regained its deleted putative packaging site by recombination with helper virus.

Digestion with *Xho*I confirmed these results in that a single 4.2-kb DNA fragment was identified with an *myb*-specific probe in NEO-AMV(MAV-1)-infected cells (Fig. 5B, lane 6), as predicted (Fig. 5C). No proviral, *myb*-specific, *Xho*I-restricted DNA fragments were expected or seen in NEO-AMV-dBG(MAV-1)-infected cells (Fig. 5B, lane 8) since the initially transfected pNEO-AMV-dBG proviral DNA lacked an *Xho*I site adjacent to the deleted putative packaging site, leaving a single internal *Xho*I site (Fig. 5C). Proviral-cellular DNA junction fragments cannot be identified in polyclonally infected cells owing to random viral integration.

In contrast, G418^r cells infected with POL-NEO-AMV(MAV-1) contained two novel single-copy 2.0- and 2.7-kb *neo*-specific *Hind*III-restricted DNA fragments (Fig. 5A, lane 3) rather than the single expected 2.3-kb fragment (Fig. 5C). A single novel 2.1-kb *myb*-specific *Hind*III fragment was identified (Fig. 5B, lane 3) rather than the expected 2.5-kb fragment (Fig. 5C). In addition, an unexpectedly small *myb*-specific fragment was detected after digestion with *Xho*I (Fig. 5B, lane 7). These results suggest that only significantly rearranged proviruses were present in the G418^r POL-NEO-AMV(MAV-1)-infected cell population. Such gross rearrangements have been observed in various other retroviral vectors after transfection and selection.

Expression of *neo* gene products in virally infected cells. Since no background G418^r colonies were obtained with QT6 cells which were uninfected or infected only with the MAV-1 helper virus, the presence of the *neo* gene appeared to be absolutely required for the G418^r phenotype. To prove that *neo* was in fact expressed by the proviruses, we used an antiserum raised against nptII to immunoprecipitate metabolically radiolabeled proteins from virally infected G418^r QT6 cells. The *Bgl*II-*Sma*I *neo* DNA fragment used in the vector constructions is promoterless but does contain the

bacterial ribosome binding site, initial AUG, and complete coding sequence for nptII, a protein of 29,000 molecular weight (3). An in-frame termination codon is present within this fragment upstream of the normal *neo* AUG (Fig. 6B), as are three downstream in-frame termination codons (3).

All virally infected G418^r cells examined expressed *neo*-specific proteins (Fig. 6A). NEO-AMV(MAV-1)-infected cells contained three *neo*-encoded proteins: the predicted 29,000 *M_r* species and two additional larger proteins of 44,000 and 72,000 *M_r*. NEO-AMV-dBG(MAV-1)-infected cells produced only the expected 29,000 *M_r* nptII protein. In contrast, the rearranged proviruses in POL-NEO-AMV(MAV-1)-infected cells produced two large *neo*-encoded proteins but not the expected 29,000 *M_r* species. These results imply that either the expected 29,000 *M_r* nptII protein or the larger *neo*-fusion proteins are sufficient to confer G418 resistance upon QT6 cells.

The origin of the unexpectedly large *neo*-encoded proteins is unclear. They exceed the coding capacity of the predicted *neo* open reading frame described above. The larger open reading frame(s) required for their translation could result from RNA splicing or frameshift suppression as proposed for the normal *gag* and *pol* reading frames, which produce both a 76,000 *M_r* *gag* precursor and an additional 180,000 *M_r* *gag-pol* precursor protein (48, 61). Alternatively, in the case of NEO-AMV(MAV-1)-infected cells, which produce both the expected nptII and the larger *neo*-encoded proteins, distinct viruses with subtle mutations not detectable by Southern blotting could be present within the population.

Expression of p48^{v-myb} in G418^r virally infected cells. Viral expression of the unselected *v-myb* gene product, p48^{v-myb}, was documented by immunoprecipitation of lysates of metabolically radiolabeled cells with an anti-*myb*-specific antiserum. G418^r cells infected with NEO-AMV(MAV-1) and NEO-AMV-dBG(MAV-1) coexpressed significant amounts of p48^{v-myb}, although less than was present in an AMV-transformed myeloblast cell line (Fig. 7). p48^{v-myb} was not detected in uninfected cells or G418^r cells infected with POL-NEO-AMV(MAV-1), which harbor rearranged proviruses (see above). The specificity of immunoprecipitation with anti-*myb* antibodies was demonstrated by complete blocking with purified, unlabeled immunogen. The QT6 cells which expressed p48^{v-myb} showed no changes in growth rates or morphology, arguing against any selective advantage or toxicity due to *myb* expression. As expected, approximately equal amounts of the helper virus-encoded p27^{gag}, Pr76^{gag}, and Pr180^{gag-pol} were immunoprecipitated from all three infected cell types but not from uninfected cells (Fig. 7).

The antibodies used to detect p48^{v-myb} in infected QT6 cells were raised against bacterially expressed *myb* determinants which are encoded entirely upstream of the first internal *myb*-specific AUG (Fig. 1). This fact, along with the comigration of vector-produced p48^{v-myb} and AMV-produced p48^{v-myb} in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 7), strongly argues that the NEO-AMV and NEO-AMV-dBG viruses produce this protein in the same fashion as AMV, by splicing and translation of a *gag* leader peptide which provides the p48^{v-myb} initiation codon.

DISCUSSION

This is the first report of a molecular clone capable of synthesizing the *v-myb* gene product in eucaryotic cells. As such it provides direct physical evidence that the previously reported DNA sequence of *v-myb* actually encodes p48^{v-myb},

the protein observed in AMV-transformed leukemic myeloblasts. Transient expression of p48^{v-myb} achieved in mammalian cells should allow testing of the *v-myb* gene product for various activities such as transcriptional *trans*-activation of cellular genes as has been reported for the nuclear products of the *myc* (27) and adenovirus E1A oncogenes (15, 17).

In addition, we demonstrated that the *v-myb* oncogene can be stably expressed in a transformed quail fibroblast cell line, although *v-myb* itself does not transform fibroblasts. This suggests that the poor viral production of AMV by fibroblasts is not due to toxicity of *v-myb*. Rather, it is likely due solely to the rapid spread of helper virus and resultant inhibition of secondary viral infection.

The novel *neo*-containing proviruses described here provide two significant experimental advantages for studying AMV: (i) continuous production of infectious viruses and their oncogene products from cloned cell lines, regardless of viral transforming capacity, which should prove useful in directed mutagenesis of *v-myb*; and (ii) stable, selectable expression of *v-myb* gene products in cells which are not targets for transformation, which should be of help in understanding the unique tissue specificity of AMV. In addition, the design of these viruses allows relatively easy and accurate titration of the defective component of an infectious viral stock regardless of its transforming capacity.

Cocultivation of embryonic chicken yolk sac cells with NEO-AMV(MAV-1)-infected QT6 cells, but not with uninfected or NEO-AMV(MAV-1)-infected QT6 cells, results in transformation similar to that seen with wild-type AMV (unpublished data). This indicates that the p48^{v-myb} produced by NEO-AMV is biologically active. In addition to producing p48^{v-myb}, these transformed hematopoietic cells are also G418 resistant and therefore continue to express *neo* in the presence of selection for *v-myb* rather than for *neo* itself.

The strategy described here for *v-myb* should also be useful for studying other avian leukemia and sarcoma virus genes which are expressed from subgenomic mRNAs and are predicted to initiate translation in the *gag* gene upstream of the viral splice donor site. These genes include the *erbB* oncogene of the avian erythroblastosis viruses (21, 62), the *myc* oncogene of the OK10 and MH2 viruses (20, 25), the *src* oncogene of Rous sarcoma virus (22), and the retroviral *env* gene itself (14).

The dominantly selectable retroviruses described here provide a useful complement to the previously described nonselectable, Rous sarcoma virus-derived retroviral vectors for the introduction of genes into avian cells (53). They are also of value in understanding structure-function relationships within the avian leukosis viruses themselves. For example, three putative packaging sites have previously been identified in the viral genome: (i) a sequence 5' to the *gag* AUG and nearby splice donor site (31, 50); (ii) a sequence 3' to the splice donor site within *gag* coding sequences and the viral dimer linkage site (46); and (iii) a sequence 3' to the *env* termination codon, which is present in at least one copy in all known avian leukemia and sarcoma viruses and as a repeated element flanking the *src* gene in nondefective strains of Rous sarcoma virus (54). While this work was in progress, transfection of proviral DNA lacking the second putative packaging site was reported to yield infectious virus (44). However, the resulting viruses were not analyzed for recovery of these sequences by recombination with helper virus, which occurs frequently among retroviruses. One of the infectious viruses reported here,

NEO-AMV-dBG, has been shown to lack this putative packaging site yet still gives high G418^r titers of stable nonrecombinant virus. This implies that this second putative site is in fact not essential for viral packaging.

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