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Biologically Active Proviral Clone of Myeloblastosis-Associated Virus Type 1: Implications for the Genesis of Avian Myeloblastosis Virus

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A biologically active myeloblastosis-associated virus (MAV) provirus was cloned from a bacteriophage recombinant library constructed from leukemic chicken myeloblast DNA. The restriction endonuclease map of this clone was consistent with that of a type 1 MAV (MAV-1). Interference assays of virus recovered from cultured chicken embryo fibroblasts after DNA transfection established that the provirus was infectious and confirmed that it belonged to avian retrovirus subgroup A (type 1). Antipeptide antibodies raised against the *env*-encoded carboxyl terminus of p48^{myb}, the transforming protein of avian myeloblastosis virus, specifically immunoprecipitated the gp37^{env} from quail cells transfected with MAV-1 proviral DNA but not from cells infected with MAV-2. This suggests that MAV-1 rather than MAV-2 is the progenitor helper virus from which avian myeloblastosis virus arose by the transduction of cellular proto-oncogene sequences.

The avian myeloblastosis-associated virus types 1 and 2 (MAV-1 and MAV-2) are replication-competent "helper" retroviruses isolated from the standard avian myeloblastosis virus (AMV) complex, which also includes the acutely transforming, replication-defective AMV proper (27, 28). Although these helper viruses are related to other members of the avian leukemia virus-avian sarcoma virus group, they are unique in two respects. (i) The U3 region of the MAV and AMV long terminal repeat (LTR) sequence, which has presumptive transcriptional enhancer and promoter functions (13, 14, 23, 24, 44), appears to be largely unrelated to those of any other known retroviruses (36). (ii) Like other helper viruses of the avian leukemia-sarcoma virus group, the MAV viruses cause nonacute neoplastic disease. However, in addition to the visceral lymphoid leukoses commonly caused by the Rous-associated viruses (RAVs), the MAVs also induce nephroblastomas and osteogenic osteoblastomas (9).

To understand the molecular basis of oncogenicity in these unique viruses, we have cloned a biologically active MAV-1 provirus. In addition, we have examined the structural relationships of this virus to AMV and to other members of the avian leukemia-sarcoma virus group.

MATERIALS AND METHODS

Purification of lambda-proviral recombinant DNA. The lambda Charon 4A library of leukemic chicken DNA partially digested with *Eco*RI (40) was screened by plaque hybridization (1), and the recombinants were purified as described previously (31). The lambda-proviral recombinant DNA was prepared from high-titer stock, obtained after infection of *Escherichia coli* DP50supF (5).

Restriction endonuclease digestion and gel electrophoresis. Restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, Md.) were used in the buffers recommended by the supplier. Digestions were performed with a 10-fold excess of the enzyme. The digested DNAs were electrophoresed in 0.8% agarose (type II; Sigma Chemical Co., St. Louis, Mo.) gels containing 0.5 μg of ethidium bromide per ml.

Blotting and hybridization. Southern blotting (38) of the DNA electropherograms has been described previously (31). The blots were hybridized in the presence of 50% formamide–10% dextran sulfate (43) to ^{32}P-labeled nick-translated probes (34).

Cloning. *E. coli* HB101 (7) and pBR322 (6) were the recipient strain and the plasmid vector, respectively, used in transformation experiments. Cloning of the purified DNA fragments was performed as described previously (31). Characterization of the recombinants was performed by colony hybridization (16) or by a miniscreen procedure (32).

Cells. Chicken embryo fibroblasts (CEF) were obtained from line 6_3 C/E embryos (Regional Poultry Research Laboratory, East Lansing, Mich.) and line 11 (C/E, chf$^-$/gs$^-$) embryos (SPAFAS, Inc., Norwich, Conn.). Cell line 16Q is a quail cell line transformed by and producing noninfectious Bryan high-titer Rous sarcoma virus [BH-RSV(−)], kindly provided by Helen Murphy (Imperial Cancer Research Fund Laboratories, London, England) (29). The chemically transformed QT6 quail cell line (26) was kindly provided by R. Guntaka (Columbia, Mo.) and J. T. Parsons (Charlottesville, Va.).

Transfection. Three different procedures were used for transfection. The first procedure followed the technique described by Graham and Van der Eb (15). Briefly, 1 μg of DNA to be transfected was dissolved with 15 μg of salmon sperm DNA as carrier in 0.75 ml of HEPES buffer (137 mM NaCl, 5mM KCl, 0.5 mM Na_2HPO_4, 5.5 mM dextrose, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2- ethanesulfonic acid], pH 7.1). The DNA was precipitated by the dropwise addition of 2 M $CaCl_2$ to a final concentration of 125 mM. After a 30-min incubation at room temperature, a very fine DNA precipitate resulted which was added to

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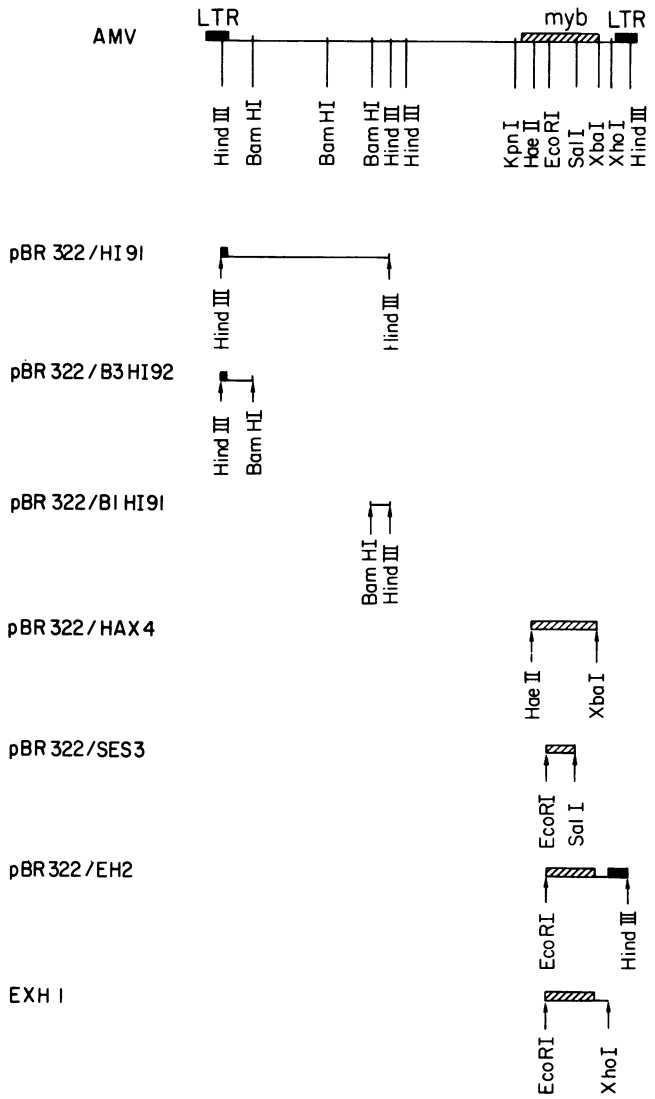

FIG. 1. Derivation of the hybridization probes representing specific viral subgenomic regions.

semiconfluent CEF cultures. After 30 min of exposure, culture medium was added and incubated for an additional 4 h before being replaced with fresh medium.

The second transfection procedure involved DEAE-dextran treatment. CEF were incubated for 15 min with medium containing 100 μg of DEAE-dextran per ml, molecular weight either 5×10^5 or 2×10^6. The medium was withdrawn and replaced with 1 ml of Tris-dextrose buffer (137 mM NaCl, 5mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM dextrose, 25 mM Tris hydrochloride, pH 7.2) containing 1 μg of the dissolved DNA to be transfected. After a 30-min incubation, fresh medium was added.

We used both techniques to transfect control dishes with salmon sperm DNA. All cultures were routinely passaged for up to 4 weeks. The culture supernatants were collected at different times and assayed for infectious virus by two methods, a standard interference assay (42) and the 16Q complementation assay (10). In the latter assay, line 11 CEF (SPAFAS) were infected with the experimental culture supernatants, passaged three times, and mixed with 2×10^5 16Q cells per 60-mm dish. The culture supernatants were harvested after 7 days, centrifuged, and assayed for the presence of focus-forming virus units on line 11 CEF (10, 12).

The third transfection procedure employed polybrene and dimethyl sulfoxide treatment as described previously (20), with 5 μg of carrier-free DNA per 100-mm tissue culture dish.

Cell labeling and immunoprecipitation. Cells were metabolically labeled with [^{35}S]methionine, lysed in detergent buffer, immunoprecipitated, and analyzed by electrophoresis in 10% polyacrylamide gels as previously described (8).

RESULTS

Isolation of a putative complete MAV proviral clone. A total of 1.5×10^6 plaques from a lambda Charon 4A library of leukemic chicken myeloblast DNA (40) were screened with an in situ hybridization technique (1), using the probes shown in Fig. 1. Triplicate nitrocellulose filter imprints were hybridized to either the HI91 probe (U5-*gag-pol* specific) (33), the SES3 probe (*myb* specific) (32), or the EXHI probe (*myb-c* region specific). Of 57 plaques that hybridized with the HI91 probe, 13 also hybridized with the SES3 probe and were discarded as presumptive AMV clones. Of the remaining 44 plaques, 8 did hybridize with the EXHI probe, suggesting that they were likely to contain a full-length MAV clone. These eight plaques were replated once and hybridized separately with the nonoverlapping B3HI92 (U5-*gag* specific), B1HI91 (*pol* specific), and EH2 (*myb-c* region-U3 specific) probes. One of the plaques that hybridized with these three probes was further purified and was again shown not to hybridize with the *myb*-specific SES3 and HAX4 probes (31). This putative λ-MAV proviral DNA recombinant (clone λ-311411) was replaqued twice more, and high-titer stocks were prepared as described previously (5).

Restriction endonuclease analysis of the λ-MAV provirus. A series of single and double digests of purified DNA from λ-311411 were electrophoresed in 0.8% agarose gels, blotted

TABLE 1. Interference assay[a]

CEF supernatant	Relative efficiency of focus formation[b]	
	RSV (RAV-1)	RSV (RAV-2)
Untreated	1.0	1.0
MAV-1 infected	<0.001	0.96
MAV-2 infected	0.59	0.02
Mock transfected[c]		
Calcium phosphate	0.91	0.80
DEAE-dextran	1.31	1.83
Transfected		
Calcium phosphate	<0.001	0.55
DEAE-dextran (M_r, 2×10^6)	<0.001	0.68
DEAE-dextran (M_r, 5×10^5)	<0.001	0.84

[a] Transfection was done by either the calcium phosphate precipitation or DEAE-dextran procedure as described in Materials and Methods. After transfection, all CEF cultures were transferred twice. Untreated CEF cultures were then infected with supernatants from the transfected cultures, passaged twice, and challenged with either RSV (RAV-1) or RSV (RAV-2). Foci were counted 7 days later.

[b] Average relative efficiency of focus formation from duplicate cultures. The efficiency of 1.0 in untreated cultures represents an average of 295 foci per plate at a dilution of 2,500:1 for RSV (RAV-1) and 212 foci per plate at a dilution of 250:1 for RSV (RAV-2).

[c] Transfected with salmon sperm DNA.

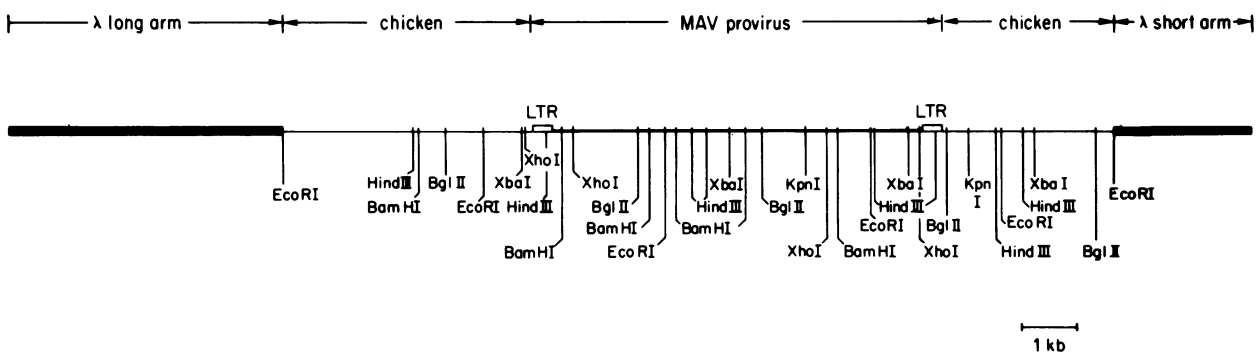

FIG. 2. Restriction endonuclease map of λ-311411, showing the lambda vector arms, cellular sequences, and MAV proviral sequences. The scale is shown in kilobases (kb). λ vector arms are not drawn to scale.

onto nitrocellulose as described by Southern (38), and hybridized to either the B3HI92 or HI91 ^{32}P-labeled probe (data not shown). These results allowed us to construct a restriction endonuclease map of this λ-MAV proviral clone (Fig. 2) that was more detailed than but consistent with that of the complete MAV-1 genome as established in previous studies of linear unintegrated viral DNA (2). Clone 311411 carried the *Hin*dIII site at 1.2 kilobases from the 3′ viral terminus which is present in MAV-1 linear DNA but absent in at least one isolate of MAV-2 linear DNA. In addition, the structure of the flanking cellular DNA regions established that the complete λ-311411 clone represented a different integrated provirus than that which had previously been cloned as a partial MAV provirus designated λ10A2-1 (39). Comparison of the λ-311411 restriction map with those reported for various strains of Rous sarcoma virus (RSV) revealed that, except for the LTR and *src* regions, the restriction site map of this MAV-1 clone was nearly identical to that reported for the Prague A and C strains (17, 19) and to that deduced from the nucleotide sequence of the Prague C strain (37). However, the MAV-1 restriction map appeared to be more divergent from that of the Schmidt-Ruppin A strain (11).

Infectivity and subgroup of the λ-MAV proviral DNA. Many proviral clones of retroviruses without gross deletions are nevertheless noninfective (30). To test the biological activity and establish the subgroup of the λ-311411 MAV proviral DNA, we used transfection experiments. In the first set of experiments, CEF were transfected by both the DEAE-dextran and calcium phosphate methods, and the culture supernatants were tested for virus production in an interference assay (Table 1). The virus produced by all the transfected cultures induced strong interference with RSV(RAV-1), a subgroup A virus, but not with RSV(RAV-2), a subgroup B virus.

As an additional test of biological activity, released virus was assayed by its ability to complement the envelope-defective BH-RSV(−) virions produced by 16Q cells. The supernatants from three CEF cultures independently transfected with λ-311411 DNA efficiently complemented BH-RSV(−) (not shown). More than 10^5 focus-forming units of BH-RSV(MAV-1) infectious transforming virus per ml were detected. Thus, transfection of λ311411 DNA resulted in the release of infectious virus. Furthermore, this virus was of subgroup A and was therefore MAV-1 rather than MAV-2.

Immunological cross-reactivity of MAV-1 gp37^{env} and AMV p48^{myb}. The acutely transforming virus AMV arose by recombination of an MAV-like helper virus with cellular proto-oncogene sequences (3, 41). The transforming protein of AMV, p48^{myb}, is predicted to share its 11 carboxyl-terminal amino acids with those of the envelope glycoprotein, gp37^{env}, of the progenitor helper virus from which AMV arose (21, 22, 35) (Fig. 3). For the identification of p48^{myb}, an antiserum was raised against the 19 amino acids predicted from DNA sequence analysis to comprise the carboxyl terminus of this protein (8). This antiserum successfully immunoprecipitated p48^{myb} from standard-AMV-transformed leukemic myeloblasts, but somewhat surprisingly did not immunoprecipitate any helper virus envelope glycoproteins in leukemic cells producing an infectious AMV pseudotype. Since the AMV(MAV) pseudotype released by leukemic myeloblasts transformed by standard AMV is predominantly subgroup B (25), we reasoned that the progenitor helper virus from which AMV arose and which is predicted to share its gp37^{env} carboxyl terminus with that of p48^{myb} might in fact be MAV-1, which is of subgroup A. To test the structural relationship of MAV-1 gp37^{env} and p48^{myb}, QT6 quail cells, which are permissive for subgroup A but not

AMV	pf hkdqtf TEYRKMQGGAV
PR-C	GLLLGLVVILLLVVCLPCLL QFVSSSIRKMIN SSINYHTEYRKMQGGAV
SR-A	----------I-CGN-----N--S-----K-L-KAYGQPESRIV
Y73	----------V----------N-FS-RE-CK-L-EACKQPERGI
RAV-0	----------I----------N--S-----K-L-KACRQPENGAV
RAV-2	----------C----------N-LG-RE--K-IT--L

FIG. 3. Comparison of the carboxyl-terminal amino acid sequences for p48^{myb} and various gp37^{env} proteins. The carboxyl-terminal sequence of AMV P48^{myb} was predicted from DNA sequence analyses (21, 35) and also represents the synthetic peptide used to generate the antiserum used in the experiment shown in Fig. 4. *myb*-specific amino acids are shown in lowercase letters. The carboxyl-terminal sequences of various gp37^{env} proteins were also predicted from DNA sequence analyses (see reference 4 for a review): Prague C RSV (Pr-C), Schmidt-Ruppin A RSV (SR-A), Y73 sarcoma virus (Y73), RAV-0, and RAV-2. Dashes indicate homology with the Prague C strain sequence.

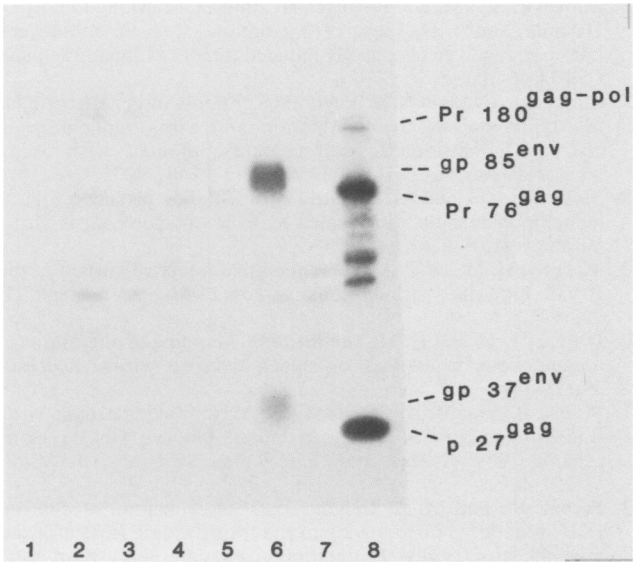

FIG. 4. Anti-p48^{myb} carboxyl-terminal peptide antiserum specifically immunoprecipitates the MAV-1 gp37^{env}. QT6 quail cells were transfected with or without 5 μg of λ-311411 DNA by the polybrene-dimethyl sulfoxide method (20) and passaged twice to allow viral spread. Cells were then metabolically labeled with [^{35}S]methionine, lysed and analyzed by immunoprecipitation and gel electrophoresis as previously described (8). Lanes 1 through 4, Mock infected cells; lanes 5 through 8, λ-MAV-1-transfected cells. Lanes: 1 and 5, normal rabbit serum; 2 and 6, anti-p48^{myb} carboxyl-terminal peptide antiserum; 3 and 7, anti-p48^{myb} carboxyl-terminal peptide antiserum in the presence of excess free peptide; 4 and 8, anti-p27^{gag} antiserum.

for subgroup B viruses (26), were transfected with λ-311411 MAV-1 proviral DNA and metabolically labeled with [^{35}S]methionine, and lysates of these cells were immunoprecipitated with anti-*myb* carboxyl-terminal peptide antiserum (Fig. 4). This antiserum specifically immunoprecipitated both gp37^{env} and the covalently linked gp85^{env} from the λ-MAV-1-transfected cells but not from the uninfected cells. In contrast, an antiserum specific for a non-carboxyl-terminal peptide of p48^{myb} did not immunoprecipitate gp37^{env} or gp85^{env} (data not shown). As expected, the *gag*- and *gag-pol*-encoded structural proteins were identified with anti-p27^{gag} antiserum only in the λ-MAV-1-transfected cells.

DISCUSSION

A complete, biologically active MAV-1 provirus has been cloned from a bacteriophage lambda recombinant library of leukemic chicken myeloblast DNA. Both restriction endonuclease mapping and interference assays of virus recovered from transfected CEF established this clone as a subgroup A (type 1) virus. This was somewhat surprising, since the leukemic myeloblasts from which this provirus was cloned were transformed by an isolate of AMV-B (40). This indicates that this isolate of AMV-B contains some MAV-1 virions even though it appeared to be predominantly of subgroup B.

The product of the AMV oncogene, p48^{myb}, is predicted from DNA sequence analysis to share its 11 carboxyl-terminal amino acids with those of the gp37^{env} protein of its progenitor helper virus. Antipeptide antibodies reactive with the p48^{myb} carboxyl terminus also specifically immunoprecipitated the gp37^{env} of the cloned MAV-1 provirus, but did not cross-react with the gp37^{env} of MAV-2. This indicates that MAV-1 rather than MAV-2 is the likely progenitor helper virus from which AMV arose by recombination with cellular proto-oncogene sequences.

Although MAV-1 (subgroup A) appears to be the progenitor helper virus for AMV, and although an isolate of an AMV pseudotype of subgroup A does cause leukemias (18), MAV-2 (subgroup B) appears to predominate in vivo in most AMV-induced leukemias. This suggests that AMV(MAV-2) may have a selective advantage over AMV(MAV-1) in leukemic myeloblasts from genetically nonrestrictive chickens. Interestingly, it has been reported that subgroup A helper viruses are unable to rescue AMV from transformed "nonproducer" C/O and C/E chicken yolk sac clones (25).

Comparison of restriction endonuclease maps and of the highly variable gp37^{env} carboxyl-terminal amino acid sequences and adjacent noncoding DNA sequences (Fig. 3; see reference 4 for a review) has revealed that MAV-1 and AMV are more closely related to the Prague strain RSVs than to RAV-0, RAV-2, or Schmidt-Ruppin strain RSVs. With the exception of *src*-specific sequences, MAV-1 appears to differ from the Prague C strain RSV only in the U3 sequences of its LTR. Thus, AMV appears to have arisen by two distinct recombinational events: (i) an en bloc replacement of the highly conserved U3 sequences of a Prague-related helper virus with putative promoter sequences of unknown origin to form MAV-1, and (ii) a subsequent replacement of most of the *env* sequences with *proto-myb* sequences to generate AMV itself. Since MAV-1 appears to differ from Prague-related viruses only in its unique U3 region, these sequences probably determine the unique oncogenic spectrum of the MAVs. The biologically active clone described in this paper will allow this hypothesis to be tested.

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