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Vertebrate DNAs Contain Nucleotide Sequences Related to the Transforming Gene of Avian Myeloblastosis Virus

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Avian myeloblastosis virus contains a continuous sequence of approximately 1,000 nucleotides which may represent a gene (*amv*) responsible for acute myeloblastic leukemia in chickens. This sequence appears to have been acquired from chicken DNA and to be substituted for the envelope gene in the viral genome. We used hybridization probes enriched for the *amv* sequences and conditions that facilitate annealing of partially homologous nucleotide sequences to show that cellular sequences related to *amv* are present in the genomes of all vertebrates ranging from amphibians to humans but were not detected in fish, sea urchins, or *Escherichia coli*. In contrast to the preceding findings, nontransforming endogenous proviral nucleotide sequences closely related to the remainder of the avian myeloblastosis virus genome and to the entire myeloblastosis-associated helper virus are present only in chicken DNA. The *amv*-related cellular sequences appear to be highly conserved during evolution and to be contained at only one or a few locations in the genome of vertebrates. Within closely related species, they appear to share common evolutionary genetic loci. These findings and similar ones obtained with other highly oncogenic retroviruses containing a transforming gene suggest a general mechanism for acquisition of viral oncogenic sequences and an essential role for these sequences in the normal cellular state.

The DNA of normal vertebrates contains sequences homologous to the oncogenes of murine and avian retroviruses (6, 8, 16, 17, 25, 26). These putative progenitors of viral oncogenes are present as distinct normal genetic loci in uninfected cells and are not affiliated with the multiple cellular loci encoding for endogenous proviral DNA (13, 24). For the avian sarcoma virus *src* gene, the normal cellular analog is not even located on the same chromosome as the chicken endogenous proviruses (15).

The genome of avian myeloblastosis virus (AMV) in DNA form is a 4.9×10^6-dalton double-stranded molecule that contains a continuous cellular sequence of approximately 1,000 base pairs apparently substituting for the entire viral envelope gene (21-23, 24). The cellular sequence lies between the *Kpn*I and the 3'-proximal *Xba*I sites and contains one *Eco*RI site (see Fig. 1). This insertion appears to be responsible for acute myeloblastic leukemia in chickens (21, 22; Baluda, unpublished data). This putative leukemogenic sequence is not present in the natural helper of AMV, myeloblastosis-associated virus types 1 and 2 (MAV-1 and MAV-2), or in Rous-associated virus type 0 an endogenous virus produced by certain strains of chickens (20-22). Sequences homologous to the putative AMV oncogene were detected not only in uninfected chicken DNA but also in duck DNA (20, 24), which does not contain sequences homologous to chicken endogenous proviruses (18).

In this study, we surveyed many vertebrate species for the presence of normal DNA sequences related to the AMV leukemogenic sequences (*amv* gene). The results show that normal DNA sequences related to *amv* were present in all of the vertebrates tested, starting with the amphibians. We also found that the normal cellular homologs are present at one or a few distinct loci within the genome of these vertebrates.

MATERIALS AND METHODS

DNA sources. *Escherichia coli* DNA was a gift from Dan Ray, UCLA. Sea urchin DNA prepared from whole ground animals was a gift from Mike Grunstein, UCLA. Pigeon (mixed stock) erythrocyte DNA was a gift from Bernice Wenzel, UCLA. Rabbit (white laboratory) spleen DNA, cat (City Pound, Los Angeles, Calif.) spleen DNA, and baboon thymus DNA were gifts from Marge Nicholson, Los Angeles Children's Hospital. Japanese macaque spleen DNA was a gift from Robert Gallo, National Cancer Institute, Bethesda, Md. Human normal leukocyte (buffy coat) DNA from a UCLA patient without malignancies was a gift from Howard Stang, UCLA. Another sample of normal human leukocytes (buffy coat) was from a

healthy donor (Kathy Bergmann). Acute myelogenous leukemia leukocytes (buffy coat) from two UCLA patients and chronic myelogenous leukemia leukocytes from a UCLA patient were gifts from Faramarz Naiem, UCLA. The leukemic buffy coats consisted of at least 80% leukemic cells as determined by standard cytological examination. The sources of other vertebrate cellular DNAs were: erythrocytes from a Japanese koi fish (Friedmann Fish Farm, Malibu, Calif.); erythrocytes from a bullfrog (College Biologicals, Escondido, Calif.); C/O chicken embryos negative for group-specific antigen and chicken helper factor (gs^-chf^-) H&N Farms, Redmond, Wash.); Japanese quail embryos and erythrocytes from a Bronze turkey (Baer Bird Farm, La Habra, Calif.); Peking duck embryos, Mallard duck and Emden goose erythrocytes (Mission Labs, Rosemead, Calif.); and NIH Swiss mouse embryos from pregnant mice (Curd's Caviary, La Puente, Calif.).

Phage λ-proviral hybrids and plasmid-proviral hybrids. The phage λ-proviral DNA recombinants have been previously described (22, 24). λ-Proviral hybrid 11A1-1 contains the entire AMV provirus flanked at both ends by chicken DNA sequences, and hybrid 10A2-1 contains 85% of the MAV helper provirus with chicken sequences only at the 5′ terminus (22). The AMV *Hin*dIII 2.6-megadalton (Md) DNA fragment was purified by recloning from the λ vector (11A1-1) into plasmid pBR322. The MAV *Eco*RI 2.6-Md fragment was recloned from the λ vector (10A2-1) into plasmid pBR322. The recombinant plasmids were purified from HB101 by the procedure of Curtiss et al. (9).

Isolation of DNA fragments for nick translation. DNA from the two λ-proviral hybrids or pBR322-proviral fragment hybrids was treated with the appropriate restriction endonuclease and fractionated electrophoretically in 0.7% Seaplaque gel (Seakem low-temperature-melting agarose; Marine Colloids, Rockland, Maine) (22). The DNA fragments were eluted from the gel as previously described (22, 24). The DNA was labeled with [α-^{32}P]dCTP by a modification of the nick translation procedure of Maniatis et al. (14) to a specific activity of approximately 10^8 cpm/μg.

Purification and restriction endonuclease cleavage of cellular DNAs. High-molecular-weight DNA (larger than 30×10^6) was extracted from decapitated 13-day-old chicken embryos, 19-day-old duck embryos, 20-day-old mouse embryos, erythrocytes, or leukocytes as previously described (3). Restriction endonuclease digestion of cellular DNA has also been described (2).

Agarose gel electrophoresis, transfer, hybridization, and autoradiography. Restriction endonuclease-treated cellular DNA was electrophoresed in 0.7% agarose gels (low electroendoosmosis; Sigma Chemical Co., St. Louis, Mo.) as previously described (21, 22). The DNAs in the gels were transferred to nitrocellulose paper by the Southern technique (19). [α-^{32}P]dCTP-labeled DNA was hybridized to DNA in Southern blots as described by Wahl et al. (28), washed (5), and autoradiographed (20).

Physical and biological containment. This work was carried out at the P2-EK2 containment levels as specified in the revised guidelines of the National Institutes of Health (1978).

RESULTS

Presence of sequences related to *amv* in normal DNA of widely separated avian species. High-molecular-weight (greater than 30×10^6) DNA from embryos or erythrocytes of chickens, quails, turkeys, ducks, geese, and pigeons was digested with either *Eco*RI or *Hin*dIII, electrophoresed in agarose gels, and transferred to nitrocellulose paper by the Southern procedure (14, 19, 20). The blots were hybridized under relaxed conditions (28) with the ^{32}P-labeled AMV *Hin*dIII 2.6×10^6-dalton (2.6-Md) fragment containing the entire cellular substitution and some sequences shared with MAV on either side (Fig. 1). All avian species tested contained sequences related to this probe (Fig. 2).

The control hybridization probe was the *Eco*RI 2.6-Md fragment of the MAV-1-like helper, which contains over 1,000 base pairs located on the 3′ side of the *Kpn*I site (Fig. 1). When Southern blots of the various avian DNAs were hybridized with this MAV *Eco*RI 2.6-Md probe, only chicken DNA showed homology (Fig. 3, lanes c and j): an *Eco*RI band of 2.6 Md and three *Hin*dIII bands of 3.6, 2.3, and 1.3 Md representing endogenous proviral DNA (1, 20). The 13.8- and 5.8-Md fragments in *Eco*RI-digested chicken DNA, which hybridized to the AMV *Hin*dIII 2.6-Md fragment (Fig. 2, lane a)

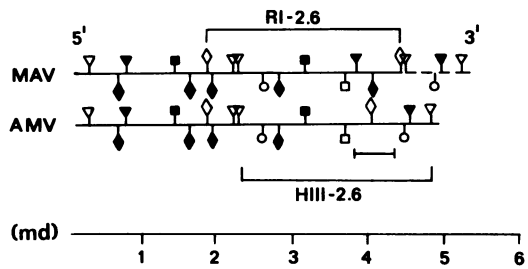

FIG. 1. *Restriction enzyme maps of AMV and MAV helper genomes. Restriction endonuclease sites were localized for the proviral DNA of AMV in the λ-proviral DNA hybrid λ11A1-1 (21, 23). The location of the enzyme sites in MAV-1 was determined with the partial λ-proviral DNA hybrid λ10A2-1 (21) (solid line) and with linear viral DNA (dashed line) (5). Enzyme sites: ▽, HindIII; ◇, EcoRI; ○, XbaI; □, KpnI; ◆, BamHI; ■, BglII; and ▼, XhoI. The bar under the AMV map represents the region of cellular substitution. The brackets above the MAV map and below the AMV map represent the MAV EcoRI 2.6-Md probe and the AMV HindIII 2.6-Md hybridization probe, respectively. The lower line represents the genomic scale in Md.*

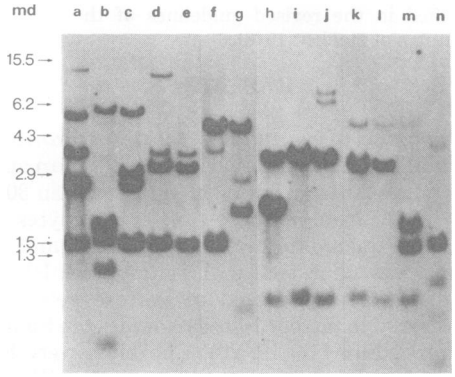

FIG. 2. *Southern blot analysis of avian DNAs hybridized to the AMV HindIII 2.6-Md probe containing the amv sequences. Purified high-molecular-weight DNA from avian erythrocytes or embryos was digested with either EcoRI or HindIII, electrophoresed, and Southern blotted. The digests contained 15 μg of DNA for all of the species except E. coli, in which 0.15 μg was used.The digests were hybridized with the ^{32}P-labeled AMV HindIII 2.6-Md probe, washed, and autoradiographed as described in the text. Lanes a through g are EcoRI digests and lanes h through n are HindIII digests of DNA from: a and h, H&N C/O chicken embryos; b and i, Japanese quail embryos; c and j, Bronze turkey erythrocytes; d and k, Peking duck embryos; e and l, Mallard duck erythrocytes; f and m, Emden goose erythrocytes; and g and n, pigeon erythrocytes. The molecular weight standards in Md (column 1) were HindIII fragments of phage λ DNA run in parallel.*

but not to the MAV *Eco*RI 2.6-Md fragment, contain endogenous proviral sequences (1, 20) related to the 3′ end of MAV and AMV. Two *Eco*RI bands (3.7 and 1.5 Md), as previously reported (20, 24), and one *Hin*dIII band (1.0 Md) in chicken DNA which hybridize to AMV do not appear to be related to endogenous proviral DNA. Also, these bands do not hybridize with RNA probes representing the entire genome of the MAV helper (Bergmann, unpublished data) or Rous-associated virus type 0 (1, 20), whereas they hybridize to AMV RNA (20). Consequently, they must represent cellular sequences, denoted as *proto-amv*, homologous to the *amv* sequences.

With the hybridization probes used above, we cannot exclude the possibility that other *Eco*RI or *Hin*dIII chicken DNA fragments may contain homology to *amv* but have the same size as some endogenous proviral fragment(s). This possibility was investigated with a probe which is more specific for *amv* than the AMV *Hin*dIII 2.6-Md fragment by isolating from the latter its 3′ *Kpn*I-*Xba*I segment. This probe still contains 100 to 200 base pairs of viral sequences on either side of the cellular substitution but lacks any part of the AMV terminal repeat. This new probe can no longer detect the chicken endogenous proviral *Eco*RI 13.8-Md fragment, and the intensity of the endogenous proviral *Eco*RI 2.6-Md band is greatly reduced, whereas the intensity of the *Eco*RI 5.8-Md band remains approximately the same and that of the *Eco*RI 3.7- and 1.5-Md bands is increased (B. Perbal and M. A. Baluda, unpublished data). The H&N C/O chicken DNA used in these experiments contains the endogenous *ev-1* provirus as mapped by Astrin (1). The detection by the AMV *Hin*dIII 2.6-Md probe of the endogenous proviral *Eco*RI 13.8-Md fragment which contains the 3′ end of the *ev-1* provirus (12) is apparently due to the presence of part of the AMV terminal repeat in that probe. Also, on the basis of nucleotide sequencing data (K. E. Rushlow, J. A. Lautenberger, P. E. Reddy, L. M. Souza, M. A. Baluda, J. G. Chirikjiom, and T. S. Papas, Proc. Natl. Acad. Sci. U.S.A., in press) the AMV *Hin*dIII 2.6-Md probe should not detect the endogenous proviral *Eco*RI 5.8-Md fragment which contains the 5′ end of *ev-1* (12). In addition, the MAV *Eco*RI 2.6-Md probe detects only the *ev-1* *Eco*RI 2.6-Md fragment (Fig. 3, lane c). Therefore, it appears that a chicken DNA *Eco*RI fragment having the same size (5.8 Md) as the one which

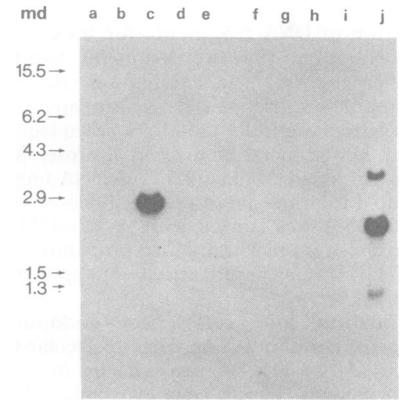

FIG. 3. *Southern blot analysis of vertebrate DNAs hybridized to the MAV-1-like EcoRI 2.6-Md probe. DNAs of fish and frogs were isolated from erythrocytes. Mammalian DNAs were isolated from normal mouse embryos, cat spleens, and human leukocytes. The purified high-molecular-weight DNA was digested with either EcoRI or HindIII, electrophoresed, and Southern blotted. The blots were hybridized with the [α-^{32}P]dCTP-labeled MAV EcoRI 2.6-Md probe under the same conditions as in Fig. 2. Lanes a through i are EcoRI digests of DNA from: a, fish (Japanese koi); b, bullfrogs; c, H&N C/O chickens; d, Japanese quails; e, Bronze turkeys; f, pigeons; g, NIH Swiss mice; h, cats; and i, humans. Lane j is H&N C/O chicken DNA digested with HindIII. Molecular weight standards are as in Fig. 2.*

contains the 5′ end of the *ev-1* provirus has homology to the *amv* sequence. A similar situation occurs with *Hin*dIII-digested chicken DNA. An endogenous proviral fragment of 3.6 Md is detected by both the AMV *Hin*dIII 2.6 Md and the MAV *Eco*RI 2.6-Md probes. However, the intensity of this band and of the 1.0-Md band is substantially increased if the AMV *Kpn*I-*Xba*I probe is used (Perbal and Baluda, unpublished data). This suggests that a *Hin*dIII fragment of 3.6 Md not containing endogenous proviral DNA is homologous to the *amv* sequence. Additional evidence for this conclusion comes from the observation that several other avian DNAs also contain a fragment of approximately 3.6 Md with homology to *amv* (Fig. 2). Thus it appears that normal H&N C/O chicken DNA contains three *Eco*RI fragments and two *Hin*dIII fragments containing *proto-amv* sequences.

Since endogenous proviral sequences were not detected with the MAV probe in the other avian DNAs tested (Fig. 3), the bands which had hybridized to the AMV *Hin*dIII 2.6-Md probe (Fig. 2) should represent sequences identical to or related to the *amv* sequences of the AMV genome. The more specific *amv* probe (*Kpn*I-*Xba*I fragment) detects the same fragments as those shown in Fig. 2. Prior studies had also shown that normal DNA from Japanese quails, turkeys, ducks, mice, cats, and humans does not share homology with chicken endogenous proviral DNA closely related to RAV-0 (18). The data (Fig. 2) suggest that the cellular *proto-amv* analogs are present at one or very few locations within the avian genomes. Also, common bands, e.g., the *Eco*RI 1.5-Md band and the *Hin*dIII 3.6- and 1.0-Md bands, are present in widely separated species belonging to two different avian orders, Galliformes and Anseriformes. Even the more distantly related pigeon (Columbiformes) shares an *Eco*RI band (5 Md) and a *Hin*dIII band (1.5 Md) with the goose (Anseriformes). This suggests that the normal cellular analogs of the viral leukemogenic sequences are highly conserved and may play an important role in cellular metabolism, perhaps in hematopoiesis.

Presence of normal cellular analogs to *amv* sequences in all vertebrates above fish. High-molecular-weight (greater than 30×10^6) DNA from several invertebrate and vertebrate species was digested with either *Eco*RI or *Hin*dIII, electrophoresed, Southern blotted, and hybridized with either the AMV *Hin*dIII 2.6-Md probe or the MAV *Eco*RI 2.6-Md probe as described for the avian DNAs. DNA from *E. coli*, sea urchins, or the various vertebrates tested did not hybridize with the MAV probe (Fig. 3 and 4). However, if these various DNAs were hybridized to the AMV *Hin*dIII 2.6-Md probe, homology to the *amv* sequences was detected in DNA from bullfrogs, mice, rabbits, cats, macaques, baboons, and humans (Fig. 4). Sequences related to the AMV *Hin*dIII 2.6-Md probe were not detected in DNA from *E. coli*, sea urchins, or fish (Japanese koi) (Fig. 4). Thus, cellular sequences analogous to the *amv* gene are present in the genome of vertebrates ranging from amphibians to humans representing an evolutionary period of approximately 300 million years (29). From the number of visible bands, these sequences appear to be located in at least one or very few genomic loci or to represent common internal fragments. As observed with the avian DNAs, common *Eco*RI bands appeared in DNA from widely separated species, such as rabbits, macaques, baboons, and humans (Fig. 4, lanes f, h, i, and j). A *Hin*dIII band was common to mouse and rabbit DNAs, and another *Hin*dIII band was shared by all of the primate DNAs.

Southern blot analysis of DNA from leukemic patients. Since sequences related to *amv* are present in DNA from normal human leukocytes, we tested whether leukemic leukocytes from leukemic patients show different *Eco*RI

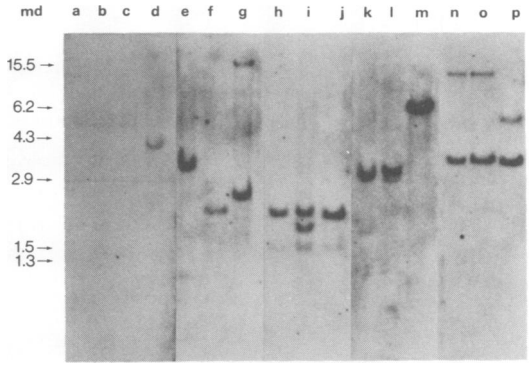

FIG. 4. *Southern blot analysis of nonavian DNAs hybridized to the AMV HindIII 2.6-Md probe containing the amv sequences. High-molecular-weight DNA was purified, digested with either EcoRI or HindIII, electrophoresed, and Southern blotted. The blots were hybridized with the [α-^{32}P]dCTP-labeled AMV HindIII 2.6-Md fragment. The autoradiographs were exposed two to three times longer than those in Fig. 2 and 3. Lanes a through j are EcoRI digests and lanes k through p are HindIII digests of DNA from: a, E. coli; b, sea urchins; c, fish (Japanese koi) erythrocytes; d, bullfrog erythrocytes; e and k, NIH Swiss mouse embryos; f and l, rabbit spleens; g and m, cat spleens; h and n, Japanese macaque spleens; i and o, baboon thymuses; and j and p, normal human leukocytes. Molecular weight standards are as in Fig. 2.*

and *Hin*dIII band patterns. Our probe did not detect any qualitative difference between leukemic DNA from two patients with acute myeloblastic leukemia or from one patient with chronic myelogenous leukemia and DNA from normal adult leukocytes (Fig. 5). However, studies with more restriction enzymes and leukemic patients are needed to detect more subtle structural differences. Also, the level of expression of this gene(s) in normal and leukemic cells should be investigated.

DISCUSSION

Recent evidence indicates that the genetic information required to induce acute myeloblastic leukemia in chickens is contained within the cellular sequence substituted for the viral envelope gene in the AMV genome (10, 22–24). Presumably, this substituted sequence was derived from the chicken, which is the natural host of AMV; at least it is the only avian species tested in which AMV induces acute myelogenous leukemia (4). As shown in this study, homology with the putative AMV leukemogenic sequences can be detected in the DNA of other avian species and in the DNA of all vertebrate species from amphibians to humans. These findings are analogous to those found for the transforming gene (*src*) of avian sarcoma virus.

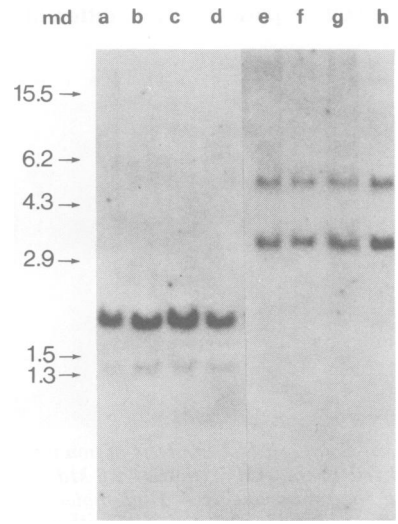

FIG. 5. *Southern blot analysis of human DNA from normal or leukemic leukocytes hybridized to the AMV HindIII 2.6-Md probe. High-molecular-weight DNA was purified, digested with either EcoRI or HindIII, electrophoresed, Southern blotted, and hybridized with the [α-^{32}P]dCTP-labeled amv probe. Lanes a through d are EcoRI digests and lanes e through f are HindIII digests of leukocyte DNA from: a and e, normal human donor different from that in Fig. 4; b and f, acute myelogenous leukemia patient 1; c and g, acute myelogenous leukemia patient 2; and d and h, chronic myelogenous leukemia patient. Molecular weight standards are as in Fig. 2.*

With the probe used, the complex structure of eucaryotic genes, which includes exons, introns, and other regulatory sequences (27), does not permit us to determine the exact level of homology between the viral *amv* sequence and the chicken cellular *proto-amv* sequence(s). Nearly all eucaryotic genes studied to date contain intervening sequences separating coding regions (7). One of two *amv* probes generated by cleavage of the viral *amv Eco*RI site detects one chicken *proto-amv Eco*RI fragment of 3.7 Md, whereas the other one detects *proto-amv* fragments of 5.8 and 1.5 Md (22). This finding suggests that the cellular *Eco*RI 3.7-Md fragment is adjacent to either the 5.8-Md or the 1.5-Md fragment in the chicken genome. The remaining fragment could be either separated from the other two by an intron or present at another homologous genetic locus. Data obtained with *Hin*dIII-digested cellular DNA is compatible with either alternative. Two cellular fragments of 3.6 and 1.0 Md with homology to *amv* were detected in *Hin*dIII-digested chicken DNA, whereas the viral *amv* sequence does not contain any *Hin*dIII site. If the cellular *Hin*dIII *proto-amv* fragments of 3.6 and 1.0 Md are near each other in the chicken genome, they could possibly represent only one cellular gene which contains an intron with a *Hin*dIII site. The implication of this model would be that the *amv* gene was generated through a cDNA copy of the normal chicken *proto-amv* mRNA, which would not contain the genomic intron.

The endogenous provirus *ev-1* is present in our H&N chickens at approximately one copy per haploid genome (1, 2, 11). By comparing the autoradiographic intensity of the endogenous proviral *Eco*RI 2.6-Md fragment with that of the cellular *proto-amv* fragments, one to two copies of the *proto-amv* sequences seem to be present per haploid chicken genome. Also, by taking into account the number, size, and hybridization intensity of the different restriction endonuclease-generated DNA fragments which are homologous to *amv* in avian species other than the chicken, it appears that only one or very few cellular homologs exist. In general, there is some loss of homology between *amv* and the various cellular *proto-amv* sequences of divergent species. This difference in homology could be accompanied by the creation of new *Eco*RI and *Hin*dIII sites, giving the impression of an increase in the number of cellular genes homologous to *amv* which actually exist. Finally, since the *proto-amv* cellular sequences are highly con-

served in evolution, they probably perform an essential function in the normal cellular state.

ACKNOWLEDGMENTS

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