Transformation of Brown Leghorn Chicken Embryo Fibroblasts by Avian Myeloblastosis Virus Proval DNA

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Brown Leghorn chicken embryo fibroblasts were transfected with a mixture of avian myeloblastosis virus (AMV) and myeloblastosis-associated virus type 1 (MAV1) proviral DNA purified from λ-Charon 4A recombinant clones. A transformed cell line (T1AM) able to grow without anchorage in semisolid medium was obtained. The presence of both proviral AMV and MAV sequences was detected in T1AM DNA by hybridization with v-myb- and MAV1-specific probes. Altered AMV and MAV1 proviral genomes were found in T1AM genome. Characterization of the RNA species expressed in transformed cells showed that in addition to a 2.5-kilobase (kb) putative subgenomic v-myb-specific RNA, three other myb-containing RNAs (9.4, 8.4, and 7.0 kb) were present in T1AM cells. No AMV genomic RNA was detected. Also, a new 5.0-kb MAV1-specific RNA species was expressed in transformed cells in addition to MAV1 genomic RNA species (7.8 kb). No infectious AMV virions are released by T1AM cells. Chicken embryo fibroblasts infected by T1AM-released virions contained and expressed all MAV1 sequences detected in T1AM transformed cells but did not express any transformation parameter. These results indicated that the presence of AMV proviral sequences in T1AM cells is responsible for their transformed phenotype.

Avian myeloblastosis virus (AMV) is a replication-defective retrovirus that transforms hematopoietic cells in vitro (3, 39) and induces myeloblastic leukemia in vivo (6, 35, 55). The tumorigenicity of AMV has been attributed to a nucleotide sequence, designated v-myb (16), which is located at the 3’ end of the viral genome (18, 31, 49, 58) and probably arose by the integration of several exons of a cellular gene known as c-myb (4, 20, 30, 46–48, 58). Expression of v-myb proceeds through a spliced subgenomic messenger RNA of 2.0 kilobases (kb) (14, 22) which is translated into a 45,000- to 48,000-Mr protein (13, 32).

The helper myeloblastosis-associated virus (MAV) is also known to induce tumors in chickens (5, 37, 52, 53, 55, 62) but is unable to induce cellular transformation in vitro.

The in vitro transforming potential of AMV has been reported to be highly restricted to hematopoietic cells since this virus is the only acute avian leukemogenic virus which failed to transform fibroblasts in cultures (23). Previous experiments showed that susceptible cell populations contain myeloid cells at various stages of differentiation or maturation (2) and established that replicating differentiated macrophages are likely to represent target cells (19, 38, 40). More recent studies demonstrated that cells committed to the macrophage lineage at all stages of differentiation may serve as target cells for AMV infection (10).

The inability of AMV to transform fibroblasts does not result from a block in the expression of the v-myb-specific polypeptide, since it was found to accumulate in the nuclei of infected fibroblasts (33) as well as in those of leukemic myeloblasts (33, 41). Klempnauer et al. (33) suggested that either (i) larger doses of the transforming protein could be required for fibroblast transformation or (ii) subtle modifications of the v-myb-specific product or the absence of a crucial cellular target might prevent AMV from transforming fibroblasts.

In our approach to understanding the restricted transforming ability of AMV, we analyzed the properties of chicken embryo fibroblasts transfected with cloned proviral AMV and MAV type 1 (MAV1) DNAs. This procedure allowed us to isolate a fibroblastic transformed cell line expressing both AMV and MAV information. The results presented in this report show that the presence of AMV proviral DNA in the cellular genome is responsible for the transformed phenotype observed.

MATERIALS AND METHODS

Chicken strains and cell cultures. Our fertile chicken eggs were CE Brown Leghorn, Edinburgh strain (gs⁺ chf⁺ V⁻) from Station de Pathologie Aviaire et de Parasitologie, Institut National de la Recherche Agronomique, Nouzilly, France. Chicken embryo fibroblasts (CEF) were prepared from 13-day-old embryos by standard techniques (34). Before being used, trypsinized fibroblasts were passaged three times and grown for about five to eight generations in Eagle medium supplemented with 5% calf serum (GIBCO Laboratories, Paisley, Scotland) and 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) to eliminate all contaminating hematopoietic cells. Yolk sac cells (3) from the same embryos were used as a source of AMV target cells (2, 19, 38, 40). Cloning efficiency without anchorage in semisolid medium was tested by seeding 1 × 10⁴ to 3 × 10⁵ cells per 50-mm plate in 0.5% Bacto-Agar as previously described (36).

Interference assay. Filtered (0.22-mm pore) supernatants from cultures to be assayed for the presence of infectious virus were applied to susceptible CEF which were then subcultured twice and superinfected with Rous sarcoma virus (Schmidt-Ruppin A strain). After 18 h, the cultures were overlaid with 5 ml of agarose (0.4%) containing regular culture medium. Foci of transformed cells were counted 7 days later (60).

Transfection of CEF with purified DNA. The DNA-calcium phosphate coprecipitation technique (24) was used to transfect monolayers of CEF. Salmon sperm DNA (Sigma

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Chemical Co., St. Louis, Mo.) was used as a carrier. Before being used, this DNA was extracted once with phenol mixed 1:1 with chloroform-isooamylalcohol (24:1) and four to five times with chloroform-isooamylalcohol until no interface was visible. The recombinant proviral DNAs were purified as described before (46).

The DNA-calcium phosphate coprecipitates were left in contact with the cells for 4 h at 37°C with occasional shaking. The medium was then removed, and the cells were washed twice with fresh prewarmed medium containing 10% calf serum. After an overnight incubation at 37°C, the transfected cells were trypsinized, seeded at a density of 2 × 10⁶ cells per plate, and returned to 37°C. Two days later, the content of each plate was split in four, and the cells were incubated for three more days at 37°C before being seeded in soft agar (10⁵ cells per plate).

**DNA purification and analysis.** High-molecular-weight DNA from normal and transformed cells in culture was purified and digested with restriction endonucleases as already described (44). Southern blotting (54) of DNA fragments and hybridization to 32P-labeled probes were performed as described before (45). A mixture of HindIII-digested λ DNA and HaeIII-digested φX174RF DNA was used as size markers in gels.

**RNA purification and analysis.** Total RNA from normal and transformed cells in culture was purified by guanidine-thiocyanate extraction (15) and analyzed by electrophoresis in 1% agarose-formaldehyde gels, transfer to nitrocellulose, and hybridization to radioactive probes (59). Chicken rRNAs (4.4 and 2.1 kb) were run in all gels as molecular weight markers. Polyadenylated RNA species were selected on oligodeoxynucleotidylic acid-cellulose columns (44).

**Proviral DNA recombinant clones.** The AMV proviral DNA clone A11A1-1 was isolated from a λ-Charon 4A library of leukemic chicken DNA partially digested with EcoRI (57). Restriction endonuclease analysis performed on this cloned DNA (56) allowed us to recognize three AMV-related EcoRI fragments: a 3.3-kb internal AMV-specific fragment containing v-myb sequences, a 2.0-kb 3' proximal junction fragment containing v-myb sequences and flanking cellular sequences, and a 4.3-kb 5' proximal junction fragment containing viral sequences and flanking cellular sequences. Digestion with HindIII allowed the recognition of two AMV-specific fragments: a 2.8-kb 5' proximal internal fragment and a 4.1-kb 3' proximal internal fragment which contains the entire v-myb sequences (56). This clone was reported to be leukemogenic when tested in vivo (51).

The MAV1 proviral DNA clone λ311411 was obtained after screening of the same library (B. Perbal et al., unpublished data). This clone contains the entire MAV1 proviral DNA (7.8 kb) flanked by adjacent cellular sequences. A partial restriction map of this proviral DNA is represented in Fig. 1. Digestion of this DNA with EcoRI revealed three MAV1-related fragments: a 3.9-kb internal MAV1-specific fragment (8) which contains the 3' portion of the gag gene, the entire pol gene, and the 5' portion of the env gene; a 3.2-kb 5' proximal junction fragment containing gag, 5' long terminal repeat (LTR), and flanking cellular sequences; and a 2.3-kb 3' proximal junction fragment which contains env, 3' LTR, and flanking cellular sequences. Digestion of λ311411 DNA with HindIII showed three MAV1-specific fragments: a 3.4-kb internal fragment, a 2.8-kb 5' proximal fragment corresponding to that detected with A11A1-1 DNA, and a 1.20-kb 3' proximal fragment.

Transfection of CEF with purified λ311411 DNA leads to the production of infectious MAV1 virus, detected by an interference assay performed with the Schmidt-Ruppin A strain of Rous sarcoma virus (B. Perbal et al., unpublished data).

**Origin and derivation of probes.** Molecular cloning of the different probes was carried out by standard techniques (44). *Escherichia coli* HB101 (pro leu thi lac4 hasR endA recA rpsL20 ara-14 galK2 xyl1-5 mtl1 supE44) was the recipient in transformation experiments (12). The vector used was pBR322 (11). (i) MAV1-derived probes. The ME probe corresponded to the 3.9-kb internal EcoRI fragment from MAV1 cloned in pBR322 (B. Perbal, unpublished data). After digestion of this DNA with EcoRI and BglII, two fragments of 1.9 and 2.0 kb containing the 5' and 3' proximal sequences of ME, respectively, were generated. These fragments were purified and cloned in pBR322 previously digested by EcoRI and BamHI (the cohesive ends generated by BglII and BamHI are compatible). Clone pBR/EBG contains the 3' proximal sequences of the gag gene and the 5' proximal sequences of the pol gene, whereas clone pBR/BGE contains 3' proximal sequences of the pol gene and 5' proximal sequences of the env gene.

Purified DNA from pBR/ME clone was digested with XbaI.
and KpnI endonuclease to generate a probe (XK) specific for the 3’-proximal sequences of the pol gene.

Clone pBR/B3H192 was obtained after BamHI digestion of pBR/H1H91 clone which contains the 5’-proximal HindIII fragment of MAV1 (B. Perbal, unpublished data).

This subclone contains 110 base pairs from the U5 region of MAV1-LTR, the leader sequences (L) detected in viral-specific transcripts (14, 22), and 150 base pairs from the gag gene (U5L-S-gag).

DNA from the λ311411 recombinant was digested with EcoRI, and the two purified junction fragments were cloned at the EcoRI site of pBR322. One of the resulting clones (pBR/M35) contains env, 3’ LTR, and cellular sequences, whereas the other (pBR/M28) contains gag, 5’ LTR, and cellular sequences. The DNA obtained from the pBR/M35 clone was digested with EcoRI and XbaI. The resulting 0.7-kb fragment generated was used as an env-specific probe. DNA purified from the pBR/M28 clone was digested by BamHI, and the 1.2-kb fragment containing gag-specific sequences obtained was cloned at the BamHI site of pBR322 (pBR/B2H193).

The location of these different probes on λ311411 recombinant DNA is shown in Fig. 1, and their sizes and specificities are reported in Table 1.

(ii) v-myb specific probes. The isolation and characterization of the v-myb probes used in this study were described previously (45, 46). Clones pBR322/HAX4 and pBR322/SX12 both also contain the 33 base pairs homologous to the env gene of MAV1 located at the 3’ terminus of the v-myb gene (31, 49). Clone pBR322/SES3 contains only 350 base pairs of v-myb-specific sequences.

Physical and biological containment. This work was carried out at the P2 containment level. Biohazards associated with the experiments described in this publication have been examined previously by the French National Control Committee.

RESULTS

Isolation of TlAM transformed cells. CEF obtained as described above were transfected with purified DNA from cloned proviral recombinants λ11A1-1 and λ311411. Three different sets of transfections were performed. The first set of two plates (7 × 10^6 cells per plate) was transfected with 5 μg of proviral MAV1 DNA in the presence of carrier salmon sperm DNA. The second set of two plates was transfected with 10 μg of proviral AMV DNA in the presence of carrier DNA, and the third set of two plates was transfected with a mixture of MAV1 and AMV proviral DNA (5 and 10 μg, respectively) in the presence of salmon sperm DNA. A control experiment with carrier DNA alone was conducted in the same way, and a mock transfection which consisted of an incubation of CEF without any DNA was also performed. All cells used in this study derived from the same embryo.

After 15 days of incubation, four colonies were obtained in the set of CEF cotransfected with AMV and MAV1 proviral DNAs. No colony was obtained with cells transfected with AMV DNA or MAV DNA alone.

One of the colonies picked was selected because it grew well in agar and could be easily propagated, whereas the others eventually degenerated. The cells from this clone (TlAM) could be propagated for up to 5 months after their isolation. Microscopic examination of stained TlAM cells did not reveal any contaminant hematopoietic cell in the cultures.

Transformation parameters expressed by TlAM cells. TlAM cells in culture express three of the transformation parameters generally associated with fibroblast transformation induced by tumor viruses (43).

(i) Altered morphology and anarchic proliferation. Tumor-virus-transformed cells are characterized by a great number of changes at the level of their external morphology and by a high pleomorphism (numerous different shapes) in culture (43).

One of the most striking features of TlAM cells is the dual morphology that they exhibit in culture. At low cell density, they form mainly patches of oriented polygonal cells (Fig. 2A) which become progressively disorganized as the number of cells increases. At higher cell density, TlAM cells acquire a fusiform morphology very similar to that of MC29- and avian erythroblastosis virus-transformed chicken fibroblasts or to that of papovavirus-transformed rodent fibroblasts. At this stage of growth, cells begin to pile up and form multilayers (Fig. 2B), conferring a crisscross aspect to semiconfluent TlAM cultures. We checked by subcloning that patches and fusiform cells do not result from a mixture of two distinct cell populations but rather derive from each other.

(ii) Ability to grow in the presence of low serum concentration. It is now well established that transformed cells have reduced serum requirements and can grow in the presence of low serum concentrations (43). TlAM cells and corresponding normal CEF were grown in the presence of 5 and 0.2% calf serum in Eagle liquid medium. The number of growing cells at different times after seeding was counted in the presence of trypsin blue (Fig. 3). In the presence of 5% calf serum, both TlAM and control CEF grew with very similar growth rates, their generation times being 41 and 32 h, respectively. On the contrary, only TlAM cells were able to grow in the presence of 0.2% serum, with a slightly increased generation time (57 h). No significant growth was obtained with CEF under similar conditions.

(iii) Ability to grow without anchorage in semisolid medium. Loss of anchorage dependence has long been considered an absolute criterion for cell transformation, although it might require profound alterations of cell physiology (43). TlAM cells, derived from a colony originally isolated in soft agar (see above), acquired the capacity to grow without anchor-

age. The cloning efficiency of TlAM cells was measured by seeding plates with 3 × 10^4 cells in a soft agar overlay. The number of colonies was scored after 15 days of incubation at 37°C. About 0.5 to 1% of the cells gave rise to colonies (0.25- to 0.5-mm diameter), whereas no colony was obtained with normal CEF under the conditions used.

Endogenous proviral and c-myb sequences in the DNA of Brown Leghorn CEF. Since all chicken strains do not contain identical endogenous viral genetic information (1, 29), it was necessary to examine the endogenous proviral pattern of the

Table 1. Specificity and size of the different probes used

<table>
<thead>
<tr>
<th>Probe</th>
<th>Insert size (kb)</th>
<th>Viral sequences detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR/ME</td>
<td>3.9</td>
<td>gag, pol, env</td>
</tr>
<tr>
<td>pBR/EBG</td>
<td>1.9</td>
<td>gag, pol</td>
</tr>
<tr>
<td>pBR/BGE</td>
<td>2.0</td>
<td>pol, env</td>
</tr>
<tr>
<td>pBR/M28</td>
<td>3.2</td>
<td>U3, R, U5L'S-gag</td>
</tr>
<tr>
<td>pBR/M35</td>
<td>2.3</td>
<td>env, U3, R</td>
</tr>
<tr>
<td>pBR/B3H192</td>
<td>0.56</td>
<td>U5L'S-gag</td>
</tr>
<tr>
<td>pBR/B2H193</td>
<td>1.2</td>
<td>gag</td>
</tr>
<tr>
<td>EXE</td>
<td>0.7</td>
<td>env</td>
</tr>
<tr>
<td>XK</td>
<td>1.5</td>
<td>pol</td>
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</table>
FIG. 2. Altered morphology and crisscross aspect of T1AM-transformed cells. T1AM cultures at low cell density (A) and reaching confluence (B). Normal cells reaching confluence (C).
CEF used in our transfection experiments. The results obtained in several independent experiments are compiled in Table 2.

Hybridization of EcoRI-digested CEF DNA with B2HI93, XK, and EXE probes allowed us to recognize DNA fragments containing gag-specific sequences (3.7 and 2.6 kb), pol-specific sequences (7.4 and 3.9 kb), and env-specific sequences (7.0, 5.2, 5.0, and 3.4 kb), respectively, while comparison of the results obtained with ME, EBG, BGE, and XK probes enabled us to establish that the 2.6-kb fragment detected by BGE contained sequences homologous to the 5' proximal sequences of the MAV1 env gene. A fragment of the same size was also detected by B3HI92 probe. These observations suggest that this 2.6-kb EcoRI fragment contains both env and U5 or gag sequences. Since it is not detected by the EXE probe, this fragment could contain a deletion in the gag-pol genes, as suggested by the expression of the 4.6- and 3.9-kb RNA transcripts detected in the same cells (see below). Similarly, the 7.0- and 5.2-kb fragments detected with both B3HI92 and EXE probes contain U5- or gag-related sequences and env-related sequences.

Hybridization of HindIII-digested CEF DNA with EXE probe revealed five endogenous env-specific fragments (9.6, 5.4, 4.5, 4.2, and 3.2 kb). Three fragments (4.2, 3.4, and 1.4 kb) were detected after hybridization with ME and EBG probes. Since the 1.4-kb fragment was not detected after hybridization with BGE probe, it contained gag- or pol-related sequences. This fragment, not being detected by B3HI92 probe, did not contain U5 sequences. The 4.2-kb fragment, detected by all probes used, contained sequences homologous to gag, pol, env, and U5, whereas the 3.4-kb fragment contained only gag and pol sequences, since it did not hybridize to EXE and B3HI92 probes.

Hybridization of EcoRI- and HindIII-digested CEF DNA with v-myb-specific probes (HAX4, SES3, and SX12) led to the detection of the DNA fragments previously detected in White Leghorn Spafas (Spafas Inc., Storrs, Conn.) cellular DNA with the same probes (45, 46), indicating that the gross organization of c-myb sequences in these chicken strains was similar (data not shown).

Analysis of proviral MAV1 DNA in CEF transfected with λ311411 recombinant DNA. To determine whether transformed cells contained all the MAV1-specific DNA fragments detected in MAV1-infected cells, we first examined the proviral DNA pattern of CEF transfected with the λ-MAV1 recombinant DNA.

CEF were transfected with λ311411 recombinant DNA under the conditions described above, and the production of MAV1 infectious virus was checked by interference assay with the Schmidt-Ruppin A strain of Rous sarcoma virus. Cellular DNA was purified from cells passaged three times to allow reinfections. These cells did not express any transformation parameter. EcoRI-digested DNA was hybridized with ME probe. The intensity of hybridization obtained from the 3.9-kb fragment in MAV1 transfected cells (Fig. 4) revealed the presence of the internal MAV1-specific fragment in addition to the cellular DNA fragment of the same size containing proviral endogenous sequences.

Hybridization of HindIII-digested DNA with the EBG probe led to the detection of both MAV1-specific 2.8-kb 5' proximal and 3.4-kb internal fragments, while the BGE probe only hybridized with the 3.4-kb internal fragment. The 5' proximal 2.8-kb fragment was also detected by the gag-specific probe (B2HI93), and hybridization of HindIII-digested DNA with the env-specific probe (EXE) allowed the detection of the 1.20-kb, 3' proximal, MAV1-specific fragment (Fig. 4).

Analysis of proviral DNA in TlAM cells. (i) Hybridization with v-myb-specific probes. In addition to the EcoRI c-myb-specific fragments (8.7, 5.4, 2.1, and 2.0 kb), two fragments (1.45 and 3.0 kb) containing v-myb-related sequences were detected after hybridization of EcoRI-digested TlAM DNA with the HAX4 probe (Fig. 5A). Hybridization with SES3 and SX12 probes, which contain v-myb sequences downstream to the internal AMV EcoRI site (46) allowed the detection of the 1.45-kb fragment. This observation suggested that the 1.45-kb fragment corresponded to a 3' proximal junction fragment being detected because of the clonal origin of TlAM cells. The 3.0-kb fragment detected with HAX4 might therefore correspond to the internal EcoRI fragment of a proviral AMV DNA integrated in TlAM genome. This DNA fragment is shorter than the internal EcoRI AMV-specific fragment obtained after digestion of

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**TABLE 2. Endogenous proviral sequences in Brown Leghorn chicken DNA**

<table>
<thead>
<tr>
<th>DNA probe</th>
<th>ME</th>
<th>EBG</th>
<th>BGE</th>
<th>B3HI92</th>
<th>EXE</th>
<th>XK</th>
<th>B2HI93</th>
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<td>Cellular</td>
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<tr>
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<tr>
<td>EcoRI</td>
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<tr>
<td>7.4</td>
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<td>3.9</td>
<td>2.6</td>
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<td></td>
</tr>
<tr>
<td>7.0</td>
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<td>3.9</td>
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<td></td>
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<tr>
<td>7.0</td>
<td>5.2</td>
<td>5.0</td>
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<td>7.4</td>
<td>3.9</td>
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<tr>
<td>3.7</td>
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</tr>
<tr>
<td>HindIII</td>
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<tr>
<td>4.2</td>
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</tr>
<tr>
<td>9.6</td>
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</table>

* DNA (10 µg) prepared from three different chicken embryos was digested with EcoRI and HindIII. Blots were prepared and hybridized with the indicated probes. Data are a compilation of the results obtained.

* These fragments were not detected in all chicken strains.

ND, Not determined.

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**FIG. 3. Growth of TlAM transformed cells in low serum concentration.** Cultures were performed in the presence of 5% calf serum with TlAM transfected cells (○) and normal fibroblasts (△) and in the presence of 0.2% calf serum with TlAM transfected cells (●) and normal fibroblasts (▲).
\[\text{X11A1-1 proviral DNA (see above). We checked that EcoRI-digested proviral AMV recombinant DNA run in the same gels gave rise to a 3.3-kb internal AMV fragment and to the 2.0- and 4.3-kb junction fragments already described (56).}

\[\text{Hybridization of HindIII-digested T1AM DNA with the HAX4 and SES probes (Fig. 5A) led to the detection of a single 3.8-kb fragment in addition to the c-myb-specific fragments (5.4 and 1.2 kb) previously described (45, 46). The internal AMV-specific HindIII fragment was found to have a size of 4.1 kb.}

\[\text{Therefore, it appeared that proviral AMV sequences integrated in T1AM DNA did not correspond to an intact AMV proviral genome. The same conclusion was reached in the following set of hybridizations.}

\[\text{Hybridization with MAV1-specific probes. Hybridization of EcoRI-digested T1AM DNA with the ME probe (Fig. 5B) allowed the detection of two new MAV-related fragments (3.0 and 1.0 kb) in addition to the proviral endogenous specific fragments described above. The 3.0-kb fragment corresponds to the AMV internal fragment detected with the c-myb probes (see above). The nature of the 1.0-kb fragment will be discussed in more detail below.}

\[\text{A careful analysis of the autoradiograms obtained after a short-term exposure of EcoRI-digested T1AM DNA hybrid-}

\[\text{FIG. 4. MAV1-specific restriction fragments in CEF transfected with \(\lambda\)S11411 proviral DNA. High-molecular-weight DNA (10 \(\mu\)g) from MAV1-infected fibroblasts (lane b) was digested with EcoRI and HindIII endonucleases, electrophoresed in 0.8% agarose gels and hybridized to the indicated \(^{32}\)P-labeled probes. An equivalent amount of DNA from the corresponding uninfected fibroblasts (lane a) was treated in the same way.}

\[\text{FIG. 5. Proviral sequences detected in EcoRI- and HindIII-digested T1AM DNA. High-molecular-weight DNA (10 \(\mu\)g) from (lane a) uninfected fibroblasts and (lane b) T1AM transformed cells (10 \(\mu\)g per lane) was digested with EcoRI and HindIII, electrophoresed on 0.8% agarose gels, and hybridized to the indicated \(^{32}\)P-labeled probes. (A) c-myb-specific probes, (B) MAV1-derived probes. A mixture of HindIII-digested \(\lambda\) DNA and HaeIII-digested \(\phi X174\)RF DNA was run as size markers.}

\[\text{FIG. 6. Endogenous proviral RNA species expressed in Brown Leghorn CEF. Samples of RNA (10 \(\mu\)g) purified from embryo fibroblasts were run on formalddehyde-agarose (1%) gels and hybridized to MAV1-derived probes B2H193 (A), ME (B), EXE (C), and B3H192 (D) and to myb-specific probe HAX4 (F). Yolk sac cell RNA (10 \(\mu\)g) (E) purified from the same embryo was used as a positive control for HAX4 hybridization. Size markers are chicken rRNA species.}
ized with the gag-specific probe (B2H193) revealed the presence of three bands (Fig. 5B) aside from the proviral endogenous pattern already characterized (see above). Two of the corresponding fragments had very closely related sizes (9.0 and 8.6 kb) and migrated as a doublet. The third fragment had a size of 3.5 kb. These three fragments are likely to represent 5′-proximal junction fragments since they are not detected in CEF infected with T1AM-released virus (CEF/T1) (see below). This result suggested that three partial or intact proviral genomes are integrated in T1AM cellular DNA. This hypothesis was confirmed by analysis of proviral information contained in CEF/T1 cells (see below).

In addition to the DNA fragments containing proviral endogenous sequences, two fragments (3.8 and 2.8 kb) were detected in HindIII-digested T1AM DNA hybridized with the ME probe. The 2.8-kb fragment corresponded to the 5′-proximal MAV1-specific fragment detected in λ311411 DNA and in MAV1-infected fibroblasts (see above), whereas the 3.8-kb fragment corresponded to the internal AMV-related fragment also detected with v-myc probes. The very dark band obtained with the ME probe for the 3.4-kb fragment (Fig. 5B) revealed the presence of the internal MAV1-specific fragment which comigrates with endogenous proviral sequences. Hybridization of HindIII-digested T1AM DNA with the env-specific (EXE) probe allowed the detection of the 1.20-kb, 3′-proximal, MAV1-specific HindIII fragment, while hybridization with the gag-specific probe (B2H193) led to the detection of a new 3.4-kb fragment in addition to the expected 2.8-kb 5′-proximal fragment (Fig. 5B). Hybridization with the B2H192 probe enabled the detection of both the 2.8- and 3.4-kb fragments in addition to the proviral endogenous sequence (Fig. 5B). The 3.4-kb fragment is thought to result from the presence of a rearranged MAV1-like proviral genome in T1AM DNA. The intensity of hybridization obtained for the 2.8-kb fragment with the two probes suggested that it corresponded to a doublet resulting from the presence of both MAV1 and AMV 5′-proximal proviral sequences.

Expression of endogenous proviral sequences in normal Brown Leghorn chicken cells. The expression of endogenous proviral sequences in chicken cells has been well documented and was shown to vary with the origin of different chicken strains (26). To allow a comparison of viral transcripts expressed in normal and transformed cells, the pattern of endogenous proviral transcripts expressed in Brown Leghorn CEF was examined by hybridization with MAV1-derived probes.

Previous studies (61) showed that White Leghorn chicken cells expressing the ev3-specific gs+ chf+ phenotype contained two polyadenylated RNA species with sedimentation coefficients of 31S and 19S (corresponding to 6.5 and 2.3 kb, respectively) as well as the 21S (3.2 kb) RNA species found in avian leukosis virus-infected cells. The amount of viral RNA in these cells was higher than in gs− chf− cells but was less than 5% of that detected in avian leukosis virus-infected cells (26).

Hybridization of total polyadenylated RNA from normal Brown Leghorn CEF with the ME, EXE, and B3H192 probes permitted the detection of four main RNA species (6.2, 4.6, 3.9, and 3.2 kb) (Fig. 6). The 3.2-kb RNA species was not detected by the B2H193 (gag) or by the EBG (gag, pol) probes (data not shown) but was detected after hybridization with the EXE (env) probe. It therefore corresponded to an env-specific RNA species. The 4.6- and 3.9-kb RNA species were detected by B2H193 (gag), EXE (env), ME (gag, pol, env), and B3H192 (U5, L, gag) probes (Fig. 6). Since these RNA species were detected by the BGE (pol, env) probe and not by the EBG (gag, pol) probe (data not shown), they might therefore correspond to transcripts containing internal deletions within the gag-pol region. The 4.6-kb RNA species gave rise to a broad light band when it was hybridized to B3H192 (U5, L, gag) and ME (gag, pol, env) probes and to a dark band when it was hybridized to B2H193 (gag) and EXE (env) probes (Fig. 6). This indicates that the 4.6-kb RNA species contains mainly sequences homologous to env and gag and is not transcribed at a high level in chicken fibroblasts.

The 6.2-kb RNA species which was detected by all probes used might correspond to the 31S ev3-specific RNA species described in the gs+ chf+ White Leghorn chicken. Both a 7.2- and a 7.8-kb RNA species were also detected in some chicken strains (data not shown). The presence of an RNA species with a size and a genetic composition similar to viral RNA detected in productively infected cells was already reported in White Leghorn chickens as being a transcriptional product of ev1 locus (26). Since the size of MAV1 genomic RNA in infected cells was 7.8 kb (see below), we checked that the fibroblasts being used in our transfection experiments did not express detectable amounts of the 7.8-kb endogenous RNA species.

Expression of myb-related sequences in normal Brown Leghorn chicken cells. After hybridization of total RNA with the v-myb-specific probe HAX4, no RNA species containing myb-related sequences in Brown Leghorn chicken fibroblasts was found, while the 4.0-kb c-myb-specific RNA species (20) was detected in yolk sac cells from the same chicken (Fig. 6). This result is in agreement with the previous observations that c-myb sequences are not expressed in normal chicken fibroblasts (21).

Expression of viral RNA species in CEF transfected with λ311411 recombinant DNA. The virus-specific RNAs detectable in cells infected with avian leukosis or sarcoma viruses...
have been characterized previously (25). Two major species of viral RNA were described in Rous-associated-virus-infected cells: a 35S (7.8-kb) genomic RNA species and a 21S (3.2-kb) subgenomic env-specific RNA species. RNA from cells transfected with A31411 MAV1 proviral recombinant DNA was hybridized with ME, B2HI93, B3HI92, and EXE probes. They detected a full-length 7.8-kb genomic MAV1 RNA species and a 3.3-kb subgenomic env-specific RNA species (Fig. 7). The high intensity of hybridization observed for the 3.3-kb species in MAV1-transfected cells is in agreement with the fact that the amount of viral transcripts in infected gs" cchf" cells may reach 3,000 to 20,000 copies per cell (25).

Expression of proviral and myb-related RNA species in TlAM transformed cells. To determine whether the dual morphology of TlAM cells observed at low and high cell density (see above) could be related to any difference at the level of RNA transcripts expressed in TlAM cells, RNAs from exponentially growing cells and from cells reaching confluence were purified and analyzed in the same way. No difference in the patterns obtained after hybridization with MAV1- or v-myb-specific probes was ever observed (data not shown).

After hybridization with MAV1-derived probes (B3HI92, ME, B2HI93, and EXE), we detected an elevated expression of a full-length 7.8-kb MAV1-specific RNA species and of an env-specific 3.3-kb RNA species in TlAM cells (Fig. 8A). The high level of hybridization obtained for these two classes of transcripts with all probes used was correlated with the fact that TlAM cells produced subgroup A infectious particles, as revealed by the interference assay (data not shown).

Interestingly, a novel 5.0-kb RNA species was detected after hybridization with EXE, B2HI93, and B3HI92 probes in TlAM RNA (Fig. 8A). The high intensity of hybridization obtained with B2HI93, EXE, and B3HI92 probes enabled us to determine that the size of these transcripts was quite different from that of the 4.6-kb species detected in uninfected parental cells (Fig. 8A). The new abundant 5.0-kb species detected in transfected cells might correspond to the expression of a rearranged MAV1-like provirus in these cells. Analysis of CEF/T1 cells (see below) confirmed this hypothesis.

Hybridization of TlAM RNA with v-myb-specific probes allowed us to detect several RNA species containing myb-related sequences. Again, no difference was ever observed in the patterns obtained with RNA purified from either exponentially growing or confluent cells (data not shown).

Four RNA species (9.4, 8.4, 7.0, and 2.5 kb) were detected after hybridization with HAX4 and SES3 probes. (Fig. 8B). The 8.4- and 9.4-kb RNA species were not detected in all preparations and appeared to be minor species. The very low amount of these species has not yet allowed us to determine whether they correspond to polyadenylated RNA species. They might therefore correspond to unprocessed c-myc RNA transcripts similar to those described in avian erythroblastosis virus-transformed erythroblasts (32). Since they were not detected by US-containing probe (B3HI92), they do not represent super transcripts resulting from a reading through of termination signals, as proposed for OK10-specific large transcripts in transformed cells (50). Both the 7.0- and 2.5-kb RNA species were detected among polyadenylated RNA (Fig. 8B). The intensity of hybridization obtained for the 2.5-kb RNA species indicated that they were expressed at a high level in transfected cells (Fig. 8B).

The 2.5-kb RNA species which was also detected by B3HI92 probe (US, L, gag) corresponded most probably to v-myb-specific transcripts slightly larger than those characterized in leukemic myeloblasts (14, 22).

Since the 7.0-kb RNA species was not detected by hybridization with ME, B2HI93, EXE, or B3HI92 probes (Fig. 8A), it did not correspond to the 7.2-kb AMV-specific genomic RNA species expressed in AMV-transformed myeloblasts (18, 22) and infected fibroblasts (unpublished observations). This RNA species might therefore correspond either to v-myb-related RNA species arising as a
result of the rearrangement described above for the AMV proviral genome in T1AM cells or to c-myb-related RNA species.

**Biological properties of T1AM-released virions.** Production of infectious MAV1-like particles by T1AM cells was detected by the interference assay performed as described above with the Schmidt-Ruppin A (SRA) strain of Rous sarcoma virus (data not shown). Since no AMV-specific genomic RNA species could be detected in T1AM cells, we could reasonably assume that no AMV virion would be released by these transformed cells. Three pieces of evidence supported this hypothesis. (i) No morphological (myeloblastic) transformation of myeloid cells could be obtained after infection of 13-day-old embryo yolk sac cells (which are known to contain target cells for AMV transformation) by pooled T1AM culture medium. (ii) T1AM-released virions were collected by ultracentrifugation (90 min at 30,000 rpm in a Beckman rotor 30) and resuspended in culture medium so as to obtain a 100-fold concentrated stock. Chickens (1 day old) were injected with either the concentrated or the diluted T1AM virion stocks. No leukemia was induced in injected birds, which all developed MAV-associated neoplasms (53). This observation revealed that MAV1 released by T1AM transformed cells is fully tumorigenic. (iii) CEF infected with T1AM-released virions did not contain or express any viral information other than the MAV1-related sequences detected in transformed cells (see below).

We could therefore take advantage of this situation to determine whether the presence and expression of the rearranged MAV1 sequences detected in T1AM cells was responsible for their transformed phenotype.

**Biological properties of CEF/T1 cells.** Brown Leghorn CEF were prepared as described above and infected with T1AM-released virus. These cells were designated as CEF/T1. The titer of MAV-related infectious virus contained in T1AM culture medium was estimated by the interference assay.

Fibroblasts were infected at either a high (10 interfering particles per cell) or a low (0.1 particle per cell) multiplicity of infection and grown either continuously in T1AM-conditioned culture medium or in regular medium after infection.

In all cases, CEF/T1 cells expressed none of the T1AM transformation parameters. They did not give rise to any colonies when seeded in soft agar, they exhibited the same morphology as that of normal and MAV1-infected CEF cells (or MAV1-infected cells), and they were not able to grow in the presence of a low serum concentration. These cells differed from normal cells only by their proviral DNA and RNA contents.

**Analysis of proviral DNA in CEF/T1 cells.**

(i) Hybridization with *v-myb*-specific probes. Hybridization of EcoRI- and HindIII-digested CEF/T1 DNA with the *v-myb*-specific probes HAX4, S653, and SX12 enabled us to detect the c-myb-specific fragments but did not allow us to detect any of the AMV-specific fragments (Fig. 9). Under similar conditions, the internal EcoRI and HindIII AMV-specific fragments were detected in AMV-S-infected fibroblasts (55). No junction fragments are expected in these cases since the cellular population is not clonal.

(ii) Hybridization with MAV1-derived probes. Both the 3.9-kb internal MAV1-specific fragment and the new 1.0-kb MAV1-related fragment were detected in EcoRI-digested CEF/T1 DNA hybridized with ME, EBG, and BGE probes (Fig. 10A). The presence of the internal MAV1 fragment was revealed by the high level of hybridization obtained for the 3.9-kb band as compared with the level of hybridization detected in uninfected cells. The 1.0-kb fragment was not detected after hybridization with the XK probe, which contains the 3′-proximal sequences (delimited by the *XbaI* and *KpnI* restriction sites) of the *pol* gene. Since it was detected by EBG (gag, pol) and BGE (pol, env) probes (Fig. 10A), this 1.0-kb fragment is likely to contain sequences homologous to either the 3′-proximal sequences of the *pol* gene or the 5′-proximal sequences of the *env* gene (detected by BGE) and to either the 5′-proximal sequences of the *pol* or the 3′-proximal sequences of the *gag* gene (both detected by EBG). In any case, it corresponded to the deletion of at least 1.5 kb of *pol* sequences corresponding to XK probe.

Since the cell population was not clonal, hybridization of EcoRI-digested CEF/T1 DNA with B3H192 and EXE probes did not permit the detection of any junction fragment. Only endogenous proviral specific fragments were detected with these probes (data not shown).

Two fragments (2.8 and 3.4 kb) were detected with the same intensity after hybridization of HindIII-digested CEF/T1 DNA with the *gag*-specific probe B2H93 (Fig. 10B). These two fragments were also revealed by the B3H192 probe. They corresponded to the 5′-proximal sequences of the intact and rearranged MAV1 proviral genomes characterized in T1AM transformed cells. Since the EBG probe contains a small amount of *gag* sequences, it also hybridized with the 2.8- and 3.4-kb fragments. A higher degree of hybridization was observed for the 3.4-kb fragment because the digestion of proviral MAV1 DNA by HindIII generates an internal fragment of the same size detected by both the EBG and BGE probes. The 1.2-kb fragment revealed by the EXE probe corresponded to the MAV1-specific 3′-proximal sequences. The results obtained showed that both normal and rearranged MAV1 genomes found in T1AM transformed cells were also present in CEF/T1 cells without inducing the expression of a transformed phenotype. The same conclusion was drawn from the analysis of proviral sequences expressed in CEF/T1 cells.

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FIG. 9. *myb*-related sequences in EcoRI- and HindIII-digested CEF/T1 DNA. High-molecular-weight DNA from CEF/T1 (10-μg samples) was digested with EcoRI and HindIII endonucleases and hybridized, after electrophoresis, to HAX4 (*myb*-specific) probe. Lanes: a, uninfected CEF; b, CEF/T1. A mixture of HindIII-digested λ DNA and *Hae*III-digested φX174RF DNA was run as size markers.
Expression of proviral sequences in CEF/T1 cells. (i) Hybridization with \( v\)-myb-specific probes. Neither \( c\)-myb nor \( v\)-myb-related RNA species were detected in the RNA purified from CEF/T1 cells and hybridized with the HAX4 probe (Fig. 11A). This observation was in agreement with the results obtained in the analysis of proviral DNAs contained in CEF/T1 and confirmed that no infectious AMV particles were released by T1AM transformed cells.

(ii) Hybridization with MAV1-derived probes. Aside from the endogenous RNA species characterized in normal fibroblasts, the 7.8-kb MAV1-specific genomic RNA and the 3.3-kb \( env\)-specific RNA species were detected in the RNA purified from CEF/T1 and hybridized with B3H192, ME, and EXE probes (Fig. 11B). This result correlated with the production of MAV1 infectious particles by T1AM cells and with the presence of MAV-specific DNA fragments in the genome of CEF/T1 cells (see above).

Interestingly, the novel 5.0-kb RNA species initially detected in T1AM cells was also detected at a high level in the RNA purified from CEF/T1 cells with B2H193, EXE, and B3H192 probes (Fig. 11B).

Therefore, it appeared that all MAV-related information present and expressed in T1AM cells was also present and expressed in the same way in CEF/T1 cells, without inducing phenotypic transformation.

**DISCUSSION**

Several unsuccessful attempts have been made in the past to obtain CEF transformed by AMV. Since the expression of the proviral AMV genome integrated in the DNA of infected fibroblasts results in the production of the 48,000-Mr, \( v\)-myb-specific polypeptide (13, 32) which is thought to be responsible for myeloblastic transformation, the restricted transforming ability of AMV is not due to a block in the expression of a \( v\)-myb-specific product; rather, transformation of fibroblasts by AMV might require either larger quantities of the normal \( v\)-myb-specific product or production of an altered \( v\)-myb-related polypeptide. It is interesting that another acute leukemia virus (E26) able to induce in vitro transformation of fibroblasts (9) expresses \( v\)-myb sequences in the form of a fusion polypeptide in which neither the 5' nor the 3' \( v\)-myb sequences are represented (35, 42), reinforcing the possibility that fibroblastic transformation by AMV might require the expression of an altered \( v\)-myb product.

Our approach to understanding the restricted transforming
properties of AMV relied on the use of cloned intact infectious proviral AMV and MAV DNA (containing both 5' and 3' LTR sequences flanked by cellular sequences) to transfect Brown Leghorn CEF. This strategy was chosen because it allowed the use of well-defined proportions of proviral AMV and MAV genomes and, possibly, direct integration of the proviral DNA in the host cell genome.

A transformed fibroblastic cell line (T1AM) expressing three of the most common transformation parameters used to characterize tumor-virus-transformed cells (43) was obtained when both AMV and MAV proviral DNA were used. Aside from their ability to grow in the presence of low serum concentration and without anchorage in semisolid medium, T1AM cells displayed a fibroblastic morphology very similar to that of myelocytomatosis virus (MC29)- or avian erythroblastosis virus-transformed fibroblasts and were in this respect different from the typical leukemic cells which were obtained, in other hands, after infection of muscle tissue by AMV (41). Since only one cell line of this type could be obtained, its occurrence probably involved rare events. No transformant was obtained when AMV or MAV proviral DNA was used alone in transfections. Therefore, it is possible that transformation of chicken cells required the production of infectious virus particles, as already suggested by others (17).

In an attempt to determine whether AMV or MAV1 or both were responsible for the transformation of CEF, we determined which part of the AMV and MAV1 proviral information was retained and expressed in transformed cells.

In addition to the endogenous proviral sequences present in the DNA of normal chicken embryo cells and to the MAV1-specific fragments, two new MAV1-related DNA fragments and three EcoRI junction fragments were identified in the transformed cells, suggesting that three proviral genomes were integrated in T1AM DNA. Two of them would be MAV1 related, the third one being an altered AMV proviral genome.

The presence of the two new MAV1-related DNA fragments in the genome of T1AM transformed cells was associated with the expression in these cells of a new 5.0-kb RNA species containing gag-, pol-, and env-related sequences.

We took advantage of the fact that only MAV1-like infectious virions were released by T1AM transformed cells to examine whether there was any relationship between the expression of this new MAV1-related information and the T1AM-transformed phenotype. Virions released by T1AM cells were used to infect CEF. The resulting cells (CEF/T1), which were found to contain and express MAV1-related proviral genomes and specific RNA identical to those of T1AM-transformed cells, did not show any of the transformation parameters characteristic of these cells.

These observations demonstrated clearly that the expression of T1AM-transformed phenotype did not result from the presence and expression of MAV1 information in these cells. Rather, the presence of proviral AMV in the genome of T1AM cells appeared to be responsible for transformation.

Analysis of AMV proviral sequences integrated in the genome of T1AM transformed fibroblasts revealed that no complete AMV proviral genome was integrated in the DNA of these cells. The internal EcoRI AMV-specific fragment which was shown to be present in AMV-transformed myeloblasts and infected fibroblasts (54, 56, 57) was not detected in transformed cells.

By hybridization of HindIII-digested T1AM DNA with the v-myb probes, we established that the 3'-proximal AMV sequences are present in the DNA of T1AM cells and confirmed that no normal internal AMV sequences are integrated in the T1AM genome. Since AMV and MAV share about 80% homology in their genome constitution, restriction endonuclease analysis does not allow us to distinguish between the AMV and MAV sequences located upstream to the internal EcoRI site present in both proviral genomes. Therefore we could not, at this stage, determine unambiguously whether the 5'-proximal sequences of proviral AMV were present in the genome of transformed cells.

The analysis of v-myb-specific RNA species expressed in T1AM cells confirmed that altered AMV proviral sequences were integrated in the genome of transformed cells, since no full-length viral genomic AMV RNA could be detected in these cells.

The most abundant RNA species detected after hybridization with v-myb probes was found to have a size of 2.5 kb,
which is slightly larger than the previously reported 2.0-kb size for v-myb-specific mRNA expressed in transformed myeloblasts (14, 22). Recent studies performed to characterize the transforming product of AMV in leukemic myeloblasts (13, 32) led to the conclusion that v-myb-specific RNA was a spliced message containing, in addition to the leader sequence common to all viral messengers, a portion of the 5′-proximal sequences of the gag gene. Since we have found that the 5′ half of the AMV proviral genome integrated in T1AM transformed cells was altered, experiments are in progress to determine whether the abundant 2.5-kb RNA species has the same spliced structure and corresponds to a true v-myb subgenomic mRNA.

The results reported in this paper strongly suggest that expression of the altered AMV proviral sequences integrated in the genome of T1AM cells was responsible for the expression of their transformation phenotype. We cannot decide yet whether transformation of these CEF by AMV resulted from the production of high levels of an intact v-myb product or from the expression of an abnormal v-myb-related polypeptide. Alternatively, transformation might be due to the expression of a myb-related product encoded by the 7.0-kb RNA species also detected in T1AM cells. The use of antibodies against v-myb, such as those which were previously described by others (13, 32), should help to answer this question.

Since no transcript other than those corresponding to the expression of v-myb, MAV1, and endogenous proviral sequences, were detected by the 5′-containing probe (B3H192), it is unlikely that transformation of CEF by AMV resulted from a downstream promotion of oncogene sequences as reported with avian leukemia virus-induced lymphomas (27, 28). Experiments are in progress to determine whether integration of the altered AMV proviral genome in host cell DNA resulted in the activation of unrelated c-onc sequences.

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