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Acute leukemia viruses E26 and avian myeloblastosis virus have related transformation-specific RNA sequences but different genetic structures, gene products, and oncogenic properties

(erythroid and myeloid leukemia/transforming genes and proteins/RNA and protein gel electrophoresis/nucleic acid hybridization)

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ABSTRACT **Replication-defective acute leukemia viruses E26 and myeloblastosis virus (AMV) cause distinct leukemias although they belong to the same subgroup of oncogenic avian tumor viruses based on shared transformation-specific (*onc*) RNA sequences. E26 causes predominantly erythroblastosis in chicken and in quail, whereas AMV induces a myeloid leukemia. However, upon cultivation *in vitro* for >1 month, a majority of surviving hemopoietic cells of E26-infected animals bear myeloid markers similar to those of AMV-transformed cells. We have analyzed the genetic structure and gene products of E26 virus for a comparison with those of AMV. An E26/helper virus complex was found to contain two RNA species: a 5.7-kilobase (kb) RNA that hybridizes with cloned AMV-specific proviral DNA and hence is probably the E26 genome; and an 8.5-kb RNA that is unrelated to AMV and represents helper virus RNA. Thus, E26 RNA is smaller than 7.5-kb AMV RNA. Hybridization of size-selected poly(A)-terminating E26 RNA fragments with AMV-specific DNA indicated that the shared specific sequences are located in the 5′ half of the E26 genome as opposed to a 3′ location in AMV RNA. In nonproducer cells transformed *in vitro* by E26, a *gag*-related nonstructural 135,000-dalton protein (p135) was found. No *gag* (Pr76) or *gag–pol* (Pr180) precursors of essential virion proteins, which are present in AMV nonproducer cells, were observed. p135 was also found in cultured E26 virus producing cells of several leukemic chickens, and its intracellular concentration relative to that of the essential virion proteins encoded by the helper virus correlates with the ratio of E26 to helper RNA in virions released by these cells. p135 is phosphorylated but not glycosylated; antigenically it is not related to the *pol* or *env* gene products. It appears to be coded for by a partial *gag* gene and by E26-specific RNA sequences, presumably including those shared with AMV. Hence, AMV and E26 appear to use different strategies for the expression of related *onc* sequences: AMV is thought to encode a transforming protein via a subgenomic mRNA, whereas E26 codes for a *gag*-related polyprotein via genomic RNA. It is speculated that differences in the oncogenic properties of E26 and AMV are due to differences in their genetic structures and gene products.**

E26, a replication-defective avian acute leukemia virus isolated in Bulgaria in 1962 from a field case of erythroblastosis in a chicken, was initially classified as an erythroblastosis (E) virus (1). Subsequently, on the basis of *in vitro* transformation assays and *in vivo* studies it was proposed to reclassify E26 as a myeloblastosis virus (2–4). However, recent analyses of differentiation parameters of leukemic cells from chicken and quail clearly indicated that, *in vivo*, the primary hematopoietic target cell for E26 belongs to the erythroid lineage but also includes some myeloid cells (5, 6). This directly confirmed earlier reports that E26 induces the proliferation of predominantly erythroid cells in various avian species including chicken, turkey, guinea fowl, and Japanese quail (1, 7, 8). It has also recently been reported that E26 can transform cultured quail fibroblasts (4), but this has not been confirmed in other laboratories (unpublished data).

Acutely transforming retroviruses of the avian leukosis/sarcoma group can be classified into seven subgroups on the basis of helper virus-unrelated, transformation-specific sequences present in the genomic viral RNA (9–11). Four subgroups of avian sarcoma viruses and three subgroups of acute leukemia viruses have been distinguished to date. On this basis, E26 and avian myeloblastosis virus (AMV) were classified as members of the same subgroup of acute leukemia viruses (9, 12–14). Different members of a given oncogenic subgroup regularly have similar genetic structures and induce similar forms of neoplasia in the animal (9–11). However, the oncogenic spectra of E26 and AMV are distinct: cloned stocks of AMV exclusively induce myeloblastic leukemia, whereas E26 causes erythroid or mixed erythroid and myeloid leukemia (6,15). Both viruses have in common that they do not appear to transform fibroblasts. Additional oncogenic properties reported for the original isolate of AMV may reflect activities of the associated helper viruses (16).

In the present communication we demonstrate that, despite the homology between their specific RNA sequences, the genetic structure and gene products of E26 RNA differ significantly from the structure and protein products of AMV RNA reported recently (12, 17). These differences are consistent with the distinct oncogenic properties of these two viruses.

MATERIALS AND METHODS

Cells and Viruses. E26 [E26-associated virus (E26AV)] was originally obtained from A. Therwath and K. Scherrer (Paris). Erythroblastosis was induced by intravenous injection of 0.1 ml of undiluted E26 virus stock (i.e., 10^2 focus-forming units, determined in a yolk sac culture assay) into 1-day-old SPAFAS (Storrs, CT) chickens. Blood smears were prepared twice weekly, and leukemia was usually apparent after 1–2 weeks. Blood was obtained by heart puncture, heparinized, and centrifuged for separation of blood cells. Cells from the buffy coat were cultured *in vitro* as described (12). *In vitro* transformation

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Abbreviations: NaCl/Cit, standard saline citrate (0.15 M NaCl/0.015 M sodium citrate, pH 7); kb, kilobase(s); RPV, ring-necked pheasant virus.

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of chicken bone marrow cells by infection with E26 at low multiplicity generated virus-producing and some nonproducing cell colonies in agar or methocel suspension culture (18). Viruses used as sources for marker RNAs were: avian myelocytomatosis virus MC29 (MCAV)-I (19), MC29 deletion mutant viruses MC29-10C and MC29-10H as pseudotypes with ring-necked pheasant virus (RPV) (20), and MAV-2 (12).

Analysis of Viral RNA. Radiolabeling, purification, and polyacrylamide gel electrophoresis of viral RNA were done essentially as described (12, 19–21). Hybridization of viral RNA, fractionated by preparative gel electrophoresis (21) or gradient sedimentation (10, 22), with AMV-specific proviral DNA (23) was carried out as follows. RNA samples were suspended in 25 μl of H_2O and dotted onto 20× NaCl/Cit-treated (1× NaCl/Cit is 0.15 M NaCl/0.015 M Na citrate, pH 7) nitrocellulose sheets (Schleicher & Schuell), baked, and washed as described (24). Prehybridization (20 hr) and hybridization (20 hr) of filters was carried out at 68°C in 20 ml of 6× NaCl/Cit containing 0.1% $NaDodSO_4$, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, and 0.2% bovine serum albumin. A pBR322 plasmid containing the 1-kilobase (kb) *Hae* II–*Xba* fragment of proviral AMV DNA, which represents most of the helper virus-unrelated transformation-specific sequence of AMV (23), was used here as a probe of shared AMV- and E26-specific RNA sequences (12). Plasmid DNA was labeled by nick-translation (25) to a specific activity of 6×10^7 cpm/μg of DNA by using [α-^{32}P]dCTP (Amersham) with a specific activity of 410 Ci/mmol (1 Ci = 3.7×10^{10} becquerels). The probe was added to the hybridization mixture after denaturation at 95°C for 5 min. After hybridization, filters were washed four times with 2× NaCl/Cit containing 0.1% $NaDodSO_4$ at 20°C for 5 min and then two times with 0.1× NaCl/Cit containing 0.1% $NaDodSO_4$ at 68°C for 30 min. Dots were visualized by autoradiography and quantitated by scintillation counting.

Analysis of Virus-Specific Protein Synthesis. Labeling of cells with [^{35}S]methionine, $H_3{}^{32}PO_4$, or a mixture of [^{3}H]glucosamine, [^{3}H]mannose, and [^{3}H]fucose, preparation of cell lysates, immunoprecipitation, antisera, and $NaDodSO_4$/polyacrylamide gel electrophoresis have been described (10, 12, 21, 26, 27), with the addition here of 2% dialyzed calf serum to the labeling medium intended to limit adherence of virus to infected cells.

RESULTS

Cell Transformation by E26. Intravenous injection of E26 into 1-day-old chickens leads to erythroid leukemia within 1–2 weeks. The buffy coat of blood cell preparations from birds with E26 leukemia appears to be pink as opposed to the white buffy coat obtained from the blood of chickens with AMV-induced leukemia. Furthermore, blood smear analysis shows a preponderance of immature cells of erythroid origin (5, 6). Cells from the buffy coat were cultured *in vitro* and grew within a period of 3–8 weeks into cultures in which about half of the cells were in suspension and the other half were adherent with a low percentage of spindle-shaped and a majority of rounded cells. The appearances of E26 transformed cells and of 5YS cells, an AMV-transformed nonproducer chicken myeloblast line (12, 18), are similar (Fig. 1), and it has been observed that the small number of immature myeloid cells in the buffy coat from the blood of chickens with E26 leukemia can proliferate *in vitro* to represent 20–60% of the entire culture (6). The biochemical results described below were obtained by using such leukemic cells.

Identification of E26 RNA. Virus-producing cell cultures from different experimentally induced E26 leukemias were labeled with [^{3}H]uridine, and RNA present in released virions was analyzed by polyacrylamide gel electrophoresis. It was found that, in addition to 8.5-kb helper viral (E26AV) RNA, a smaller RNA component of about 5.7 kb was present in viral RNA preparations (Fig. 2). The molar ratio of 8.5- and 5.7-kb RNAs was usually >5, and in virus preparations of some leukemic chickens the smaller RNA component was barely detectable. This unfavorably high ratio of helper to apparently E26-specific RNA, as well as the low metabolic rate and virus production of these cell cultures and the poor uptake of $H_3{}^{32}PO_4$ by these cells, prevented direct sequence analysis of this RNA component by cell culture labeling with $H_3{}^{32}PO_4$ and analysis of viral RNA by patterns of RNase T1-resistant oligonucleotides.

In order to determine whether the 5.7-kb RNA was related to AMV, E26 (E26AV) RNA was fractionated by preparative

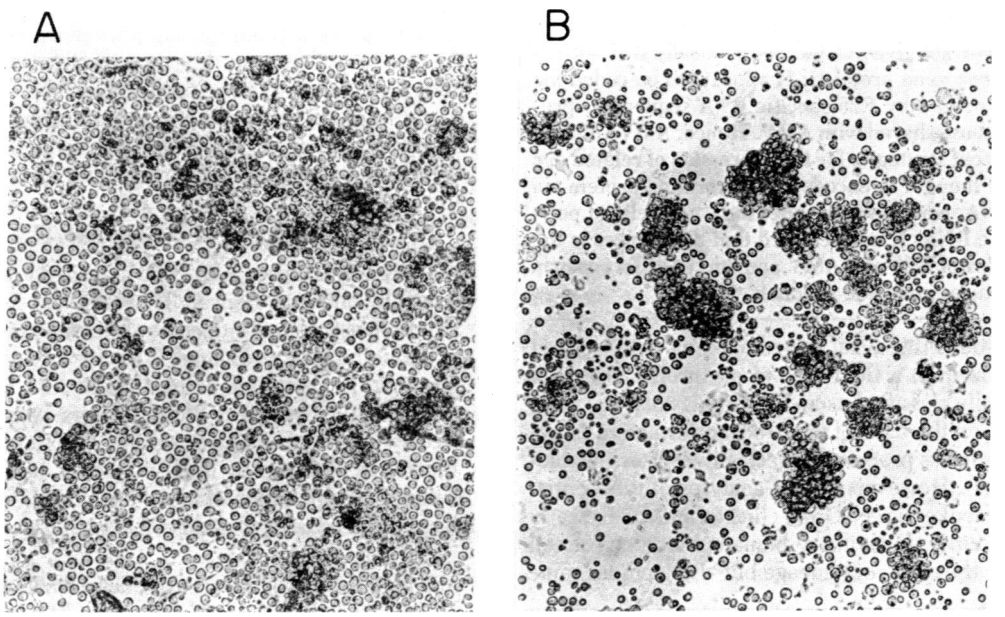

FIG. 1. (*A*) Cell culture from the buffy coat of the blood of a chicken (no. 344) with E26-induced erythroblastosis; (*B*) cells from the AMV-transformed myeloblast line 5YS (12). (×163.)

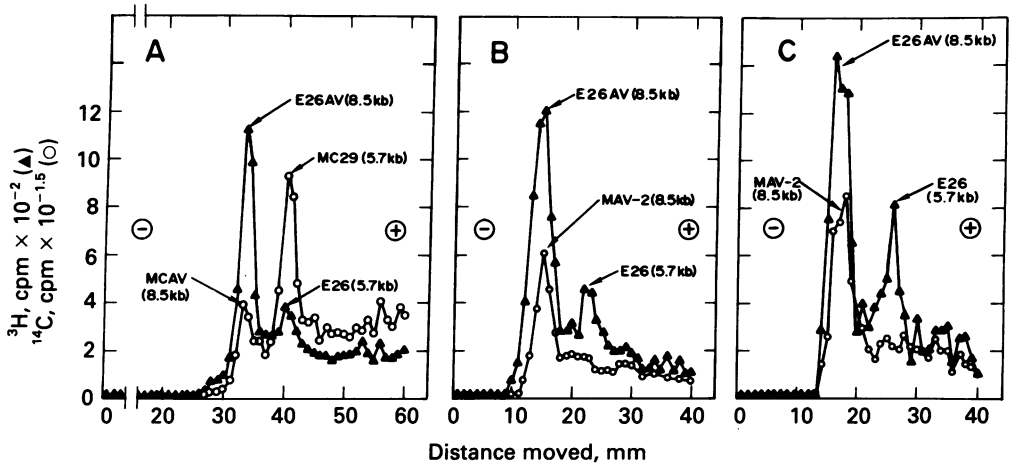

FIG. 2. Electrophoresis in 2.1% polyacrylamide gels (10, 12, 21) of the [^{3}H]RNA monomers of E26 (E26AV) from cell cultures 79 (*A*), 342 (*B*), and 348 (*C*) in the presence of [^{14}C]RNA of MC29(MCAV)-I (*A*) or MAV-2 (*B* and *C*).

electrophoresis and RNA fractions were hybridized to cloned AMV-specific proviral DNA. Specific hybridization was observed with the 5.7-kb RNA component and smaller degradation products but not with the 8.5-kb E26AV RNA (Fig. 3). AMV (MAV-2) RNA, electrophoresed in a similar manner, showed hybridization of the same probe to a RNA species of 7.5 kb, the genome size of AMV described previously (12, 17).

Mapping of E26 RNA Sequences Specifically Shared with AMV. The AMV genome contains helper virus-unrelated transformation-specific sequences immediately adjacent to regulatory sequences at the 3′ end of viral RNA (12, 17). These sequences are at least partially present in E26 RNA (12–14). To determine their location in E26 RNA, poly(A)-tagged RNA fractions, selected by chromatography on oligo(dT)-cellulose (21) and ranging in size from 35 to 10 S, were hybridized with molecularly cloned AMV-specific proviral DNA. To ensure that the concentration of AMV-related sequences, if present, would be as high (or higher) in RNA fragments of low as in fragments of high molecular weight, approximately equal amounts (in A_{260} units) of poly(A)-containing RNA fragments of 35–23 S (8.5–4 kb) and 22–10 S (3.5–0.8 kb) were pooled to prepare the gradient. Low-level hybridization started at fraction 8 (32 S), reached a maximum at fraction 11 (26 S), and sharply decreased after fraction 13 (22 S) (Fig. 4). These results imply that the specific sequences shared by AMV and E26 RNAs map in the 5′ half, between about 4.5 and 3.5 kb from the 3′ end, of the 5.7-kb E26 RNA. Hence, homologous helper virus-unrelated RNA sequences are located in different map positions in AMV and E26 RNAs. A control experiment (not shown) proved that this method located the specific sequence of AMV RNA near the 3′ end as defined previously by another method (12).

E26-Specific Viral Protein Synthesis. A clonal culture of chicken bone marrow cells transformed by E26 in the absence of E26AV (i.e., nonproducer cells) was labeled with [^{35}S]methionine and cellular lysates were analyzed for the presence of virus-specific proteins. The only protein detectable by immunoprecipitation with antiserum against whole disrupted Rous

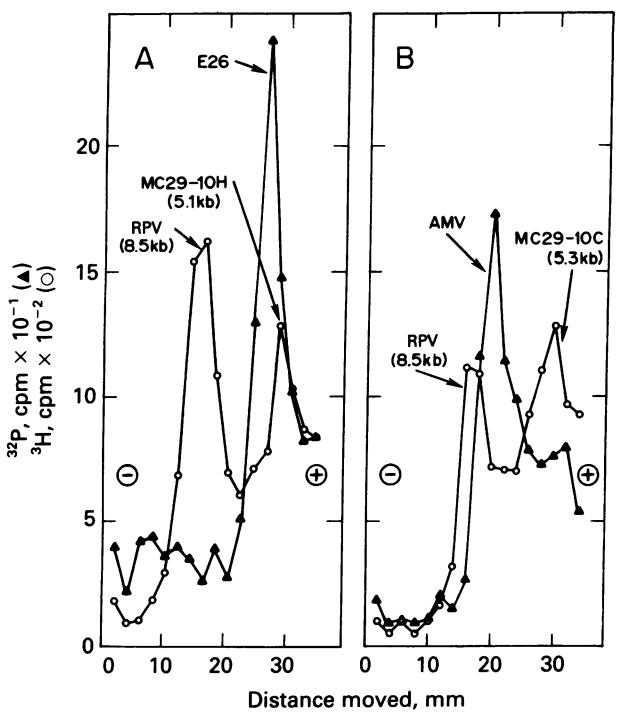

FIG. 3. Coelectrophoresis of [^{3}H]RNA of MC29-10H (RPV) (*A*) or MC29-10C (RPV) (*B*) with unlabeled RNA of E26 (E26AV) (*A*) or AMV (MAV-2) (*B*) in 2.1% polyacrylamide gels. RNA of two consecutive 1-mm gel slices was eluted, and [^{3}H]radioactivity was determined on aliquots (10, 21, 22). The blot hybridization of eluted RNA with a ^{32}P-labeled molecularly cloned fragment of AMV-specific proviral DNA is described in *Materials and Methods* and in ref. 24.

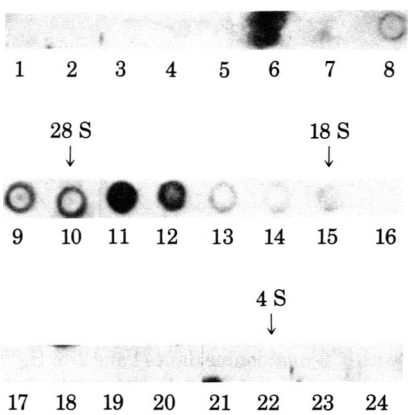

FIG. 4. Autoradiogram of ^{32}P-labeled AMV-specific DNA hybridized to poly(A)-containing E26 (E26AV) RNA size-selected on a sucrose gradient (21). RNA from fractions 1 (bottom) to 24 (top) of the gradient was ethanol-precipitated, spotted onto nitrocellulose filter, and hybridized. The positions of ribosomal RNA markers sedimented in a parallel gradient are indicated.

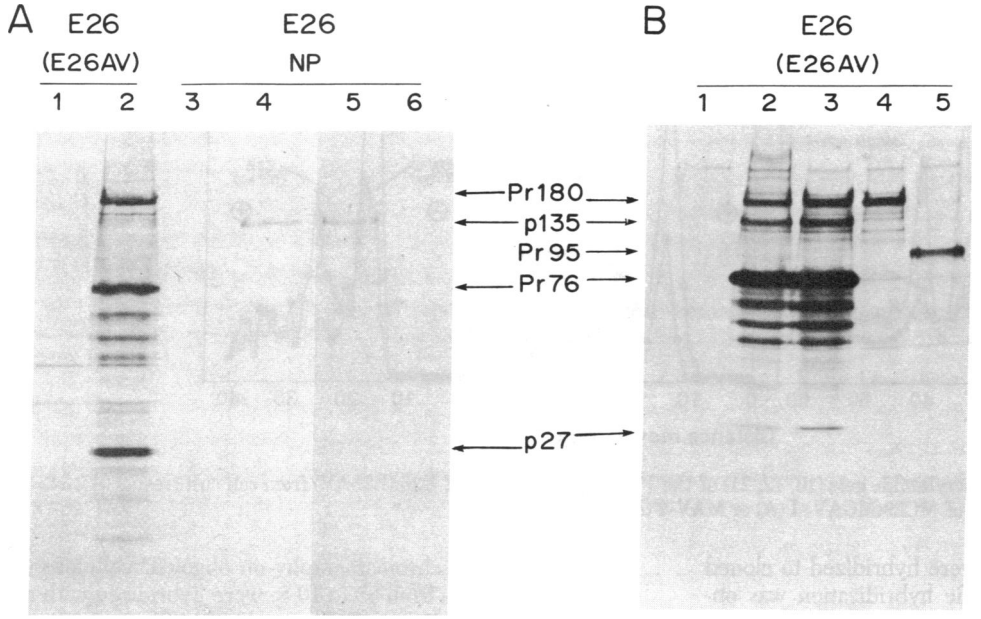

FIG. 5. E26-specific protein synthesis. Cells transformed by E26 (E26AV) (culture 1794) (*A*), E26-transformed nonproducer cells (*A*), or cells from producer culture 348 (*B*) were pulse-labeled with [^{35}S]methionine (75 μCi/ml of labeling medium), and immunoprecipitates from cellular detergent extracts were prepared with the following rabbit sera: preimmune serum (*A*, lanes 1 and 3; *B*, lane 1), anti-disrupted Rous sarcoma virus serum (*A*, lanes 2 and 4; *B*, lane 2), anti-p27/p19 serum (*A*, lane 5; *B*, lane 3), anti-reverse transcriptase serum (*A*, lane 6; *B*, lane 4), and anti-gp85 (of Prague Rous sarcoma virus, subgroup C) serum (*B*, lane 5). Gel electrophoresis in a NaDodSO$_4$/6–18% gradient polyacrylamide gel and fluorography were as described (21, 26).

sarcoma virus was a polyprotein of 135,000 daltons (p135) (Fig. 5*A*). This protein could also be precipitated by antiserum specific for gag proteins p27 and p19 (anti-gag serum) but not by antiserum directed against retroviral DNA polymerase (anti-pol serum). The same p135 was present in E-26-producing cell culture 348 and was not precipitated by antiserum directed against group-specific determinants of the viral envelope glycoprotein (12) (Fig. 5*B*).

Initial analysis of virus-producing E26-transformed cell cultures revealed very little of this protein, as in culture 1794 (Fig. 5*A*). However, cultures 348 (Fig. 5*B*), 342 (Fig. 6), and 344 (not shown) contained higher amounts of p135 in addition to helper viral proteins Pr76 (gag), Pr180 (gag–pol), and Pr95 (env). Moreover, the relative intracellular amount of p135 correlated well with the ratio of E26 to helper viral RNA in virions released from the same cells. As shown in Fig. 2, more E26 RNA per E26AV RNA correlated with more p135 proteins per Pr76 and Pr180 helper viral proteins (Figs. 5 and 6). Similar correlations between the relative concentrations of the RNAs and proteins of defective transforming and helper viruses have been observed previously (9, 19, 28).

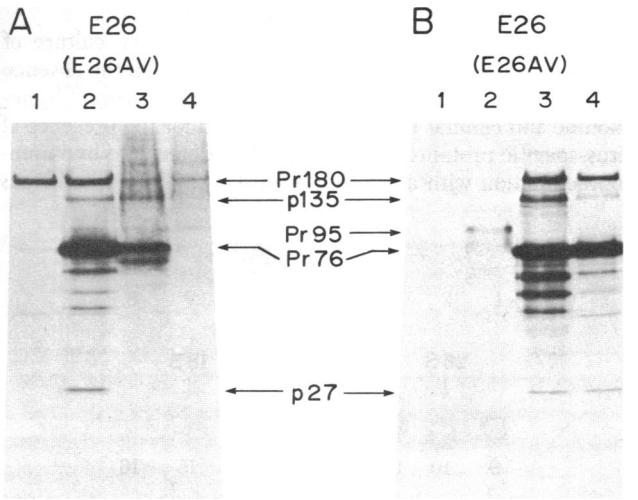

FIG. 6. Phosphorylation and glycosylation of virus-specific proteins in E26(E26AV)-transformed cells. (*A*) Cells from culture 342 were labeled with [^{35}S]methionine (lanes 1 and 2) or $H_3{}^{32}PO_4$ (500 μCi/ml of labeling medium) (lanes 3 and 4), and immunoprecipitates from cellular detergent extracts were prepared with anti-p27/p19 serum (lanes 2 and 3) or anti-reverse transcriptase serum (lanes 1 and 4). (*B*) Cells from culture 348 (lane 3) or 342 (lanes 1, 2, and 4) were labeled with [^{35}S]methionine (lanes 3 and 4) or a mixture of [^{3}H]glucosamine, [^{3}H]mannose, and [^{3}H]fucose (140 μCi of each per ml of labeling medium) (lanes 1 and 2). Immunoprecipitates from cellular detergent extracts were prepared with anti-p27/p19 serum (lanes 1, 3, and 4) or anti-gp85 serum (lane 2). Electrophoresis was as in Fig. 5.

Labeling of E26(E26AV)-producing cells with $H_3{}^{32}PO_4$ and immunoprecipitation with anti-gag serum showed that p135 is a *gag*-related phosphoprotein. The relative specific radioactivity of ^{35}S compared to ^{32}P-labeled proteins suggests that p135 may have additional phosphorylation sites not shared with the *gag* sequences of Pr76 and Pr180 (Fig. 6*A*). p135 could not be labeled with a mixture of [^{3}H]glucosamine, [^{3}H]fucose, and [^{3}H]mannose, and therefore does not appear to be glycosylated (Fig. 6*B*).

DISCUSSION

We have shown here that the genetic structure and the gene products of E26 RNA are basically different from those determined before for AMV RNA (12, 17). This is surprising in view of the observation that E26 and AMV belong to the same subgroup of oncogenic avian tumor viruses based on shared transformation-specific RNA sequences (see above and refs. 12–14). In all other subgroups of acutely transforming avian retroviruses, the general genetic structure is similar for all members of a given subgroup sharing one of two basic types of *onc* genes: (*i*) in the Rous sarcoma virus subgroup, the transformation-specific *src* RNA sequences are expressed independently of replicative genes via a subgenomic mRNA, as is also thought to be the case in AMV (9, 11, 12, 29, 30); (*ii*) in all other subgroups, such as the MC29 acute leukemia virus or Fujinami sarcoma virus subgroups, transformation-specific RNA sequences are part of hybrid genes consisting of specific sequences and partial complements of replicative genes, typically the *gag* gene (9–11).

Analysis of the AMV genome structure and protein products and of intracellular mRNAs in cells transformed by AMV has suggested that AMV codes for a transforming protein of about 30,000–40,000 daltons unrelated to viral structural proteins (12,

17, 29–31). *In vitro* translation of AMV RNA has indeed shown that AMV-specific proteins of that size class are synthesized (ref. 32; unpublished data), but an AMV-specific protein in transformed cells has yet to be identified. In AMV-transformed nonproducer cells, Pr76 and Pr180 proteins are observed, indistinguishable from the proteins synthesized by replication-competent helper viruses (12).

In contrast to the AMV genome, E26 RNA appears to be principally structured like the genome of an MC29-like virus (9–11). The absence of Pr76, the product of a complete *gag* gene, in E26 nonproducer cells indicates that, like MC29 (10, 26), E26 lacks a complete *gag* gene. Instead, a partial *gag* gene and E26-specific sequences, which include those shared with AMV, appear to code for a nonstructural, presumably transforming, polyprotein. Although our data do not exclude that the p135 protein is generated via a spliced mRNA, this possibility appears unlikely by analogy to other retroviruses with hybrid *onc* genes and based on preliminary *in vitro* translation studies in which p135 was generated from E26 RNA (unpublished data). It has been shown that the complexity of the transformation-specific sequences in AMV RNA is about 1 kb, and that E26 RNA contains sequences related to about 70% of the AMV-specific sequences (12, 14, 17). Because p135 requires about 3.6 kb of coding RNA capacity, it appears certain that, in addition to *gag* and possibly AMV-related sequences, other genetic information of E26 RNA is also expressed in the P135 polyprotein.

It would appear, then, that a related transformation-specific RNA sequence is part of a different genetic structure in E26 and AMV. The shared specific sequence appears to be expressed as a *gag*-related nonstructural protein in E26 and as a protein that is not linked to structural proteins in AMV. This different mode of expression, and presumably unique E26 sequences, might be the basis for the differences between the oncogenic properties of these two viruses. In addition, differences among the envelope genes of the helper viruses of E26 and AMV (4, 15) and of *c*-regions of E26 and AMV (12) may also contribute to their oncogenic differences.

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