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Mutant of avian erythroblastosis virus defective for erythroblast transformation: Deletion in the *erb* portion of p75 suggests function of the protein in leukemogenesis

(target cells/cell transformation/*gag*-related proteins)

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ABSTRACT **Previous studies have shown that *td*359 AEV, a mutant of avian erythroblastosis virus (AEV), is unable to transform erythroblasts *in vitro* or *in vivo* but is capable of transforming fibroblasts *in vitro* and of causing sarcomas in chicks. In this paper we show that the mutant synthesizes a *gag*-gene related protein (Δp75) which is about 1000 daltons smaller than the protein, p75, induced by wild-type AEV. The mutant protein lacks 3 of the approximately 53 lysine-arginine tryptic peptides resolved in p75 and also contains an additional peptide. By cleavage of Δp75 with p15 protease and analysis of the fragments for size and peptide composition, the deletion in Δp75 could be located in the non-*gag* region of the molecule. In contrast, with p40 AEV, a second AEV-specific protein synthesized in *in vitro* translation experiments, there is no change in size of translation products obtained from *td*359 AEV RNA. Our data provide direct evidence that p75 is required for erythroblast transformation.**

Avian erythroblastosis virus (AEV), an acute avian leukemia virus, requires a helper virus for replication (1). *In vivo*, it induces both erythroleukemia and sarcomas after inoculation into susceptible chicks (2, 3). *In vitro*, it is capable of transforming erythroblasts (4, 5) as well as cloned chicken embryo fibroblasts (1). This dual transforming capacity of AEV was shown to reside in a single transforming virus and to be independent of the type of helper virus used (1). In transformed cells, AEV synthesizes a 75,000-dalton protein (p75) which consists of part of the *gag* gene product pr76 and a unique portion unrelated to virus structural proteins which is presumably derived from the *erb* gene (6–11). In addition, AEV RNA directs the synthesis of a 40,000-dalton protein (p40 AEV) in *in vitro* translation experiments (9, 12–14). It is not known which of these two proteins is responsible for the transformation of hematopoietic and of fibroblastic cells although p75 has been implicated in leukemogenesis (10, 11). Recently, a mutant, designated *td*359 AEV, which had lost its ability to transform erythroblasts *in vitro* and *in vivo* but still was capable of transforming fibroblasts and of inducing sarcomas was isolated (15). In the present paper we will show that Δp75 synthesized by *td*359 AEV carries a small deletion in the *erb* region of the molecule whereas p40 AEV is unaltered in size.

MATERIALS AND METHODS

Viruses and Cells. The origins of RAV-2, *td*SR-D, and the ES4 strain of AEV (in short, AEV) have been described (1). The isolation of *td*359 AEV and pseudotypes thereof has also been described (15). All cells used were derived from SPAFAS chickens, a White Leghorn flock originally obtained from R. Luginbuhl. Fibroblast nonproducer clones 5 and B5 and producer clone A1 transformed by *td*359 AEV were isolated from colonies in Methocel-containing medium after infection of chicken embryo cells with *td*359 AEV (RAV-2). *td*359 AEV-transformed tumor cells were obtained by cultivating finely minced pieces of a *td*359 AEV (RAV-1)-induced sarcoma (15). NP75 cells, an AEV-transformed nonproducer fibroblast strain (6), a continuous nonproducer AEV-erythroblast line (LSCC HD3), and the same line superinfected with RAV-2 served as wild-type AEV-containing control cells.

Cell Labeling and Immunoprecipitation. Cells were labeled for 1–2 hr at 37°C with [^{35}S]methionine (Amersham-Buchler; 900 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) and extracts were prepared according to published procedures (6). For immunoprecipitation, a rabbit antiserum prepared against the structural proteins of avian myeloblastosis virus (BAI-A) and B77 virus [R α virus (6)] and a chicken antiserum directed against the non-*gag* part of p75 (Ch α p75 AEV) were used. Immunoprecipitation with the rabbit antiserum was as described (6) with protein A-containing *Staphylococcus aureus* (16). For immunoprecipitation of the chicken antiserum, affinity-purified goat anti-chicken IgG coupled to Sepharose was used as a means to immobilize the immune complex. Samples were analyzed by discontinuous gradient 6–15% $NaDodSO_4$/polyacrylamide gel electrophoresis and prepared for fluorography as described (6). Procedures described by Oppermann *et al.* (17) were used to reduce the background during immunoprecipitation.

Tryptic Peptide Analysis. Cell extracts labeled with either [^{35}S]methionine or [^{14}C]lysine plus [^{14}C]arginine (11) were immunoprecipitated, treated with trypsin, and subjected to two-dimensional peptide analysis according to described procedures (11).

Cleavage of p75 AEV with p15 Protease. This method was performed as described by Vogt *et al.* (18). p15 protease was prepared from sucrose density gradient purified avian myeloblastosis virus (a kind gift of J. W. Beard, Life Sciences, St. Petersburg, FL) by detergent treatment (18). p75 immunoprecipitated from approximately 2 × 10^6 labeled cells was incubated for 30 min with 5 μg of crude p15 protease. The cleavage products were then analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorography as described above.

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Abbreviations: AEV, avian erythroblastosis virus; td359 AEV, a mutant of AEV defective for erythroblast transformation; p75 and Δp75, *gag*-related proteins coded for by AEV and *td*359 AEV, respectively.

***In Vitro* Translation of Viral RNA.** RNA was prepared from purified [^{3}H]uridine-labeled virus and translated *in vitro* as described (19).

RESULTS

To analyze the Δp75 induced by *td*359 for possible changes in molecular weight, four different clones of mutant-transformed fibroblasts obtained from single Methocel colonies as well as a fibroblast strain obtained from a *td*359 AEV-induced sarcoma were labeled with [^{35}S]methionine. They were then immunoprecipitated with a chicken antiserum predominantly directed against the non-*gag* part of p75 and with a rabbit antiserum against virus structural proteins. In all cells tested, *td*359 AEV directed the synthesis of a protein that migrated slightly faster than p75 induced by AEV (Fig. 1). The difference seen in apparent size was on the order of 1000 daltons.

To demonstrate that the small change seen was indeed due to a deletion, Δp75 was compared with p75 by tryptic peptide analysis. Both proteins were labeled with a mixture of [^{14}C]-lysine and [^{14}C]arginine. Of about 53 lysine-arginine peptides resolved from p75, Δp75 lacked 3 tryptic peptides and had acquired 1 additional peptide (Fig. 2). The same result was obtained in three independent experiments, two of which were done with different *td*359 AEV-transformed fibroblast clones.

In order to locate the deletion within p75 we made use of the recent observation by Vogt *et al.* (18) that p75 can be cleaved by the p15 protease of avian retroviruses into two discrete fragments of approximately 45,000 and 30,000 daltons, designated F45 and F30 in the following.

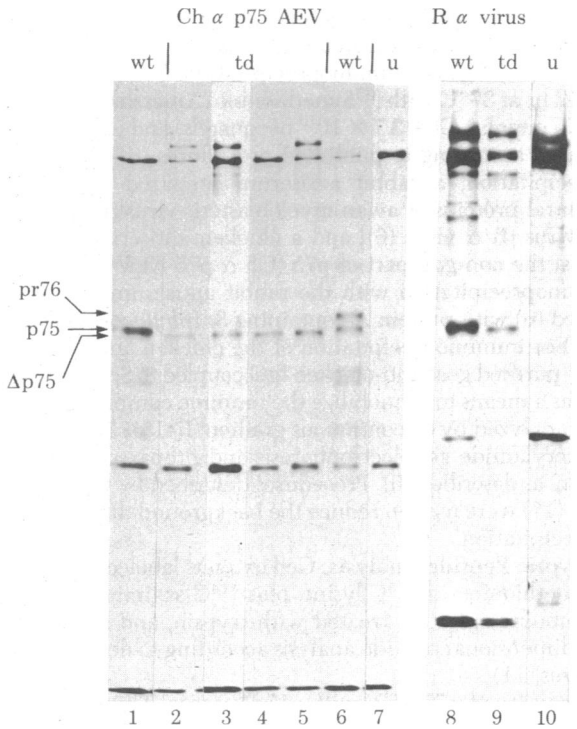

FIG. 1. Expression of p75 and Δp75 in chicken cells transformed with AEV and *td*359 AEV. [^{35}S]Methionine-labeled cell extracts of wild-type AEV (wt)-, *td*359 AEV (td)-transformed cells or uninfected cells (u) were immunoprecipitated with rabbit antiserum against whole virus (R α virus) or chicken antiserum against p75 AEV-serum (Ch α p75 AEV) and subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography. Lanes: 1, AEV-transformed nonproducer erythroblasts; 2, *td*359 AEV-transformed nonproducer fibroblast clone 5; 3, clone B5; 4, fibroblasts grown from a *td*359 AEV-induced sarcoma; 5, *td*359 AEV-transformed producer fibroblast clone A1; 6, same cells as in lane 1 superinfected with RAV-2; 7, uninfected chicken embryo fibroblasts; 8, *wt* AEV-transformed nonproducer fibroblast clone 75; 9, same as lane 2; 10, same as lane 7. Pr76 is not detected in lanes 4 and 5 because of the low residual anti-*gag* activity of the Ch α p75 AEV antiserum batch used.

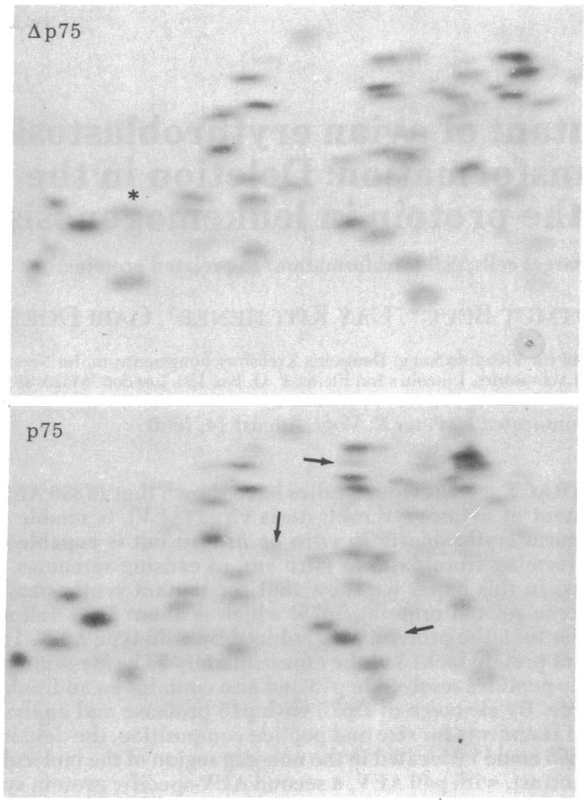

FIG. 2. Tryptic peptide mapping of p75 and Δp75. *td*359 AEV and AEV-transformed nonproducer fibroblasts were labeled with [^{14}C]lysine plus [^{14}C]arginine. Cell extracts were immunoprecipitated with rabbit anti-virus antiserum and subjected to two-dimensional peptide analysis. Peptides consistently differing between p75 (*Lower*) and Δp75 (*Upper*) in separate experiments (using *td*359-AEV-transformed nonproducer clones 5 and B5) are marked: arrows, peptides missing in Δp75; asterisk, additional peptide in Δp75.

After partial p15 digestion of Δp75 and p75, the mobility of the F45 fragment was increased in the mutant-transformed clones compared to F45 from AEV (Fig. 3). In contrast, the mobility of F30 was unchanged in the mutant. Identical results were obtained with rabbit anti-virus antiserum (Fig. 3) and chicken α anti-p75 antiserum (data not shown). This indicates that the deletion in Δp75 is located in the F45 fragment.

The location of the F45 and F30 fragments within the p75 molecule was studied by peptide analysis of methionine-labeled p75 partially digested with p15 protease. As shown in Fig. 4, F30 contained all p19-related peptides known to correspond to the *gag* region of p75 (Fig. 4; cf. figure 1*C* in ref. 11) whereas F45 contained all non-*gag* peptides of p75. This indicates that F45 corresponds to the *erb* portion and F30 to the *gag* portion of p75 and that the deletion in Δp75 is located in the *erb* region. This was confirmed by the observation (20) that a chicken antiserum specific for the non-*gag* determinants of p75 reacted with F45, whereas anti-*gag* antiserum reacted with the F30 fragment.

In vitro translation of subgenomic fragments of the AEV genome suggests that p40 AEV is coded for by the 3′ half of the AEV-specific sequences of the virus (10, 21). We therefore determined whether or not the deletion in *td*359 AEV spans into the coding region for p40 AEV, thus resulting in a size reduction of this protein. Analysis of the *in vitro* translation

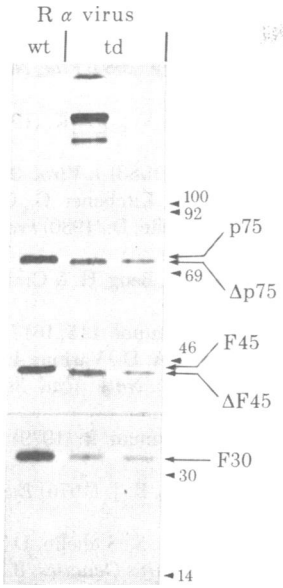

FIG. 3. Mapping of the deletion in Δp75 by partial digestion with p15 protease. Nonproducer *wt* AEV-transformed fibroblasts (clone 75, left lane) and two *td*359 AEV-transformed nonproducer clones (5 and B5, middle and right lanes) were labeled with [^{35}S]methionine, immunoprecipitated with rabbit anti-virus antiserum, and digested with p15 protease. This figure is a composite of the same gel exposed for 2 days (top, showing p75 and F45) and 6 days (bottom, showing F30) to compensate for the differences in radioactivity of the F45 and F30 bands. Small numerals: position of ^{14}C-labeled molecular weight standards.

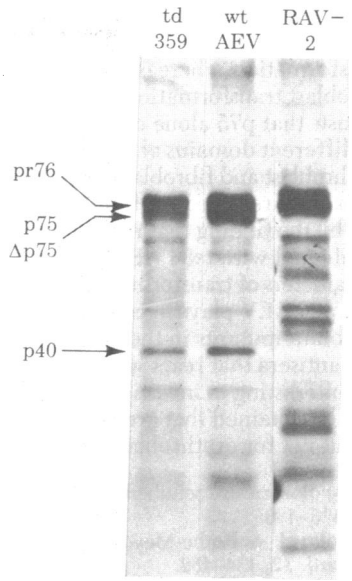

FIG. 5. Comparison of p40 AEV translated *in vitro* from *td*359 AEV and AEV RNA. AEV (RAV-2) was harvested from erythroblasts, *td*359 AEV (*td*-SR-D) was obtained from nonproducer fibroblasts freshly superinfected with *td*-SR-D, and RAV-2 virus was harvested from infected chicken embryo fibroblasts. RNA extracted from these viruses was translated *in vitro* and the products were run on a 10% NaDodSO$_4$ gel (lanes shown were from the same gel). This gel system, used to obtain maximal resolution in the 40,000-dalton region, did not resolve the size difference between p75 and Δp75.

products of *td*359 AEV RNA on a high-resolution NaDodSO$_4$/polyacrylamide gel revealed no difference in the mobility of *td*359 AEV- and AEV-directed p40 proteins (Fig. 5). This result, in conjunction with the observation that the genomic 28S RNA of *td*359 AEV is indistinguishable from the respective AEV RNA by both liquid hybridization (20) and reverse Southern blotting analysis (S. Saule and D. Stéhelin, personal communication), indicates that in *td*359 AEV the region coding for p40 AEV does not contain a detectable deletion.

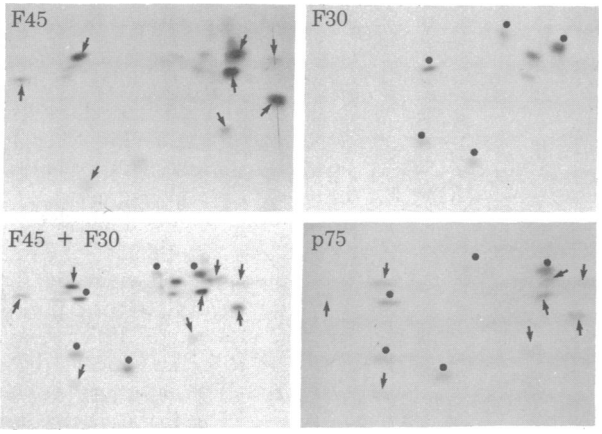

FIG. 4. Characterization by peptide mapping of the fragments generated by p15 digestion of p75. p75 was immunoprecipitated from [^{35}S]methionine-labeled AEV-transformed nonproducer erythroblasts, partially digested with p15 protease, and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. p75, F45, F30, and a mixture of the last two were then subjected to tryptic peptide analysis. Black dots, *gag*-specific peptides of p75; arrows, peptides of the non-*gag* region of p75. Identification of peptides was based on earlier analyses (11). Unmarked peptides in F45 and F30 are probably derived from contaminating background proteins because they were absent from p75 in this and earlier experiments (6, 11).

DISCUSSION

The results described in this paper provide direct evidence for the idea that p75 is the protein responsible for erythroblast transformation. They are in accord with previous lines of indirect evidence showing (*a*) that the 5′ part of the transformation specific sequences in the RNA of AEV [also designated as the *erb* gene (22)] correlates with the non *gag* part of p75 (10, 21, 22), (*b*) that a viral gene product is required for maintenance of AEV-induced cell transformation (23), and (*c*) that the *gag*-related fusion proteins of DLV strains with the same transformation specificity contain similar non-*gag* peptides, whereas strains with different biological specificities contain fusion proteins with different sets of non-*gag* peptides (11).

There still exists a slight possibility that the sequences deleted in Δp75 and ΔF45 correspond to *gag* peptides rather than to peptides derived from the *erb* portion if it is assumed that they are located close to the boundary between the *gag* and the *erb* region. However, this would be incompatible with the size of the *gag* region in the AEV genome as determined by oligonucleotide mapping [0.7–1 kilobase (10, 24)] as well as with the fact that a 30,000-dalton fragment would be more than large enough to accommodate all the *gag*-protein detected so far in p75 (8, 11).

In this regard it would be desirable to isolate and to study deletion mutants of AEV similar to *td*359 AEV, in particular to determine if they exhibit a larger deletion in the *erb* gene. Such mutants would allow a more precise definition of the genomic region required for erythroblast transformation. Another approach to prove the idea that p75 is responsible for erythroblast transformation would consist of demonstrating that p75 synthesized by a temperature-sensitive mutant of AEV such as *ts*34 AEV (23) is thermolabile itself. With regard to its antibody-binding activity this is not the case (unpublished observations). Unfortunately, because no detectable enzyme activity of p75 has been found so far, the possibility that p75 from *ts*34 AEV is thermolabile in a functional parameter could not yet be determined.

At first sight, our results suggest that there are two separate transforming genes in AEV and that p75 is responsible for erythroblast transformation whereas p40 AEV might be responsible for fibroblast transformation. However, the alternative possibility exists that p75 alone can transform both types of cells and that different domains of the p75 molecule are responsible for erythroblast and fibroblast transformation or that less functional protein is required for fibroblast transformation. This is supported by the finding that not only erythroblasts but also fibroblasts infected with *ts*34 AEV are temperature-sensitive in some parameters of transformation (25). To determine what role, if any, p40 AEV plays in cell transformation it will be necessary to obtain mutants in the genomic region coding for p40 as well as antisera that react with this protein. In addition, it would be interesting to investigate whether or not mutants of AEV can be obtained that are defective for fibroblast transformation but not for erythroblast transformation.

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