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Characterization of the Hematopoietic Target Cells of Defective Avian Leukemia Viruses by Velocity Sedimentation and Density Gradient Centrifugation Analyses

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A. Introduction

As in man, several distinct types of leukemia occur in animals, e.g., lymphoid, myeloid, and erythroid leukemia. In the three best examined animal systems, i.e., chickens, mice, and cats, these neoplasms are caused in the majority by the infection or activation of C-type retroviruses. Two main categories of leukemia viruses can be distinguished: (1) replication competent viruses which have a long period of latency and cause predominatly lymphatic leukemia and (2) replication-defective viruses (DLV), which cause various types of acute leukemia within a short period of latency and are capable of inducing an in vitro transformation in both hematopoietic and nonhematopoietic tissues (Hanafusa 1977; Graf and Beug 1978).

In the avian system a large number of nondefective viruses causing lymphoid leukosis have been isolated, the prototype of which is represented by the Rous associated viruses (RAV). These viruses lack a detectable oncogene and are nontransforming in vitro. Therefore, new techniques had to be developed to study their mechanism of transformation, as is discussed by Hayward et al. elsewhere in this volume.

About 30 to 50 isolates of DLVs from chickens have been described over the past 50 years (for review see Graf and Beug 1978). Because of their ability to transform in vitro, the comparatively few surviving strains have been intensively investigated during recent years. As shown in Table 1, the eight DLV strains studied in more detail can be subdivided into three categories according to the types of neoplasms they predominantly induce and the types of transforming sequences (oncogenes) they harbor (Roussel et al. 1979). These oncogenes differ from the *src* gene of Rous sarcoma virus (Stéhelin and Graf 1978).

As described previously the hematopoietic cells transformed by the above DLV strains (Table 1) can be grouped into the same three categories according to the phenotype of differentiation they express: AEV-type viruses induce transformed cells with the phenotype of erythroblasts, MC29-type viruses give rise to transformed cells resembling macrophages, and AMV-type strains induce myeloblast-like transformed cells (Beug et al. 1979).

The hematopoietic target cells of these viruses have been partially characterized using a number of functional (adherence, phagocytosis) and antigenic markers (differentiation-specific cell surface antigens). By these means they were shown to correspond to immature erythroid cells for AEV and to immature myeloid cells for MC29 and AMV (Graf et al. 1976a,b; Graf et al. 1981).

The present study was undertaken to characterize the hematopoietic target cells of DLVs according to physical parameters, that is, sedimentation velocity at unit gravity (providing a measure of their relative size) and centrifugation in density gradients using Percoll (to determine their relative buoyant density). Included is a comparison of the DLV target cells to the normal granulocyte/macrophage colony-forming cells (CFU-C) which develop together with the DLV-transformed cells under our conditions of culturing.

B. Materials and Methods

I. Viruses

The origin and properties of the DLV strains used (AEV-ES4, MC29, and AMV BAI-A) have been described elsewhere (Graf et al. 1980b).

Table 1. Oncogenic potential of avian leukemia viruses in vitro and in vivo

Virus	Strain	Type of neoplasms predominantly induced	Type of hematopoietic cell transformed in vitro and in vivo[a]	Transforming sequences[b]
RAV		Lymphoid leukosis, osteopetrosis, erythroblastosis[c]	B-lymphoblast (only in vivo)	–
AEV	R ES4	Erythroblastosis, sarcomas, carcinomas?[d]	Erythroblast	*erb*
MC29	MC29 CMII OK10 MH2	Myelocytomatosis, sarcomas carcinomas[d]	Macrophage	*mac*
AMV	BAI/A E26	Myeloblastosis, carcinomas?[d]	Myeloblast	*myb*

[a] Beug and Graf 1978; Beug et al. 1979
[b] Roussel et al. 1979; Bister and Duesberg 1980
[c] Neoplasms induced within a relatively long period of latency (several months to years)
[d] Neoplasms induced within weeks after infection

II. Target Cell Assay

This assay, which was shown to give a reliable (although possibly minimum) estimate of the proportion of DLV target cells in a given test tissue, has been described elsewhere (Graf et al. 1981). Briefly, bone marrow cells were prepared from 1–3-week-old white leghorn chicks of the Spafas flock. Different dilutions of bone marrow cell preparations or of fractions thereof were infected with an excess of virus, mixed with methylcellulose (Methocel)-containing medium and seeded in 35 mm dishes containing a macrophage feeder layer. Colonies were evaluated 8–12 days after infection.

III. Velocity Sedimentation at Unit Gravity

The method used was essentially that described by Miller and Phillips (1969). The apparatus used consisted of a cylindrical chamber (diameter 11 cm, height 8 cm) with a conical base. Turbulence occuring upon filling or emptying of the chamber was reduced by a stainless steel flow deflector. All operations were carried out at 4°C. The chamber was loaded first with 100 ml of 7.2 phosphate buffered saline (PBS) containing 5% serum and then with a linear gradient prepared from 400 ml 15% serum mixture in PBS and 400 ml of 24.5% serum mixture in PBS. The serum mixture consisted of 60% fetal calf serum, 35% calf serum, and 5% chicken serum. In this gradient the cells retained full viability during the experimental period, which was 12 h for each run. The chamber was then loaded with 2×10^8 cells in 30 ml PBS containing 3% fetal calf serum. After the run, 14 fractions were collected at a rate of 25 ml/min. The first and last 50 ml were discarded. Cells from each fraction were counted, sedimented for 5 min at $800 \times g$, and resuspended in 2 ml target cell assay medium (Graf et al. 1981); aliquots corresponding to 6 parts for AEV, 1 part for MC29, and 3 parts for AMV were infected with undiluted stocks of the corresponding viruses as described for the target cell assay. In fractions with high cell numbers cells were diluted before testing. Each cell aliquot was adjusted to a volume of 1 ml before infection.

IV. Density Centrifugation

The method used was basically that described by Pertoft and Laurent (1977). A stock solution of Percoll (Pharmacia, Uppsala, Sweden) was first mixed at a ratio of 10:1 with $10 \times$PBS and then diluted with growth medium to yield a final density of 1.080 g/cm^3. Then, 2×10^7 bone marrow cells in 5 ml growth medium were layered on top of 30 ml Percoll suspension and centrifuged in the rotor R30 of Beckman Instruments at 19,000 rpm for 30 min at 4°C (no brakes). Cells were fractioned from the top of the gradient with a bent Pasteur pipette into 15 fractions per tube and aliquots separated for counting and for determination of density using a refractometer. After adding 5 ml of growth medium to each fraction, cells were sedimented at $800 \times g$ for 5 min, resuspended in 2 ml of target cell assay medium, and handled as described above for the sedimentation velocity studies.

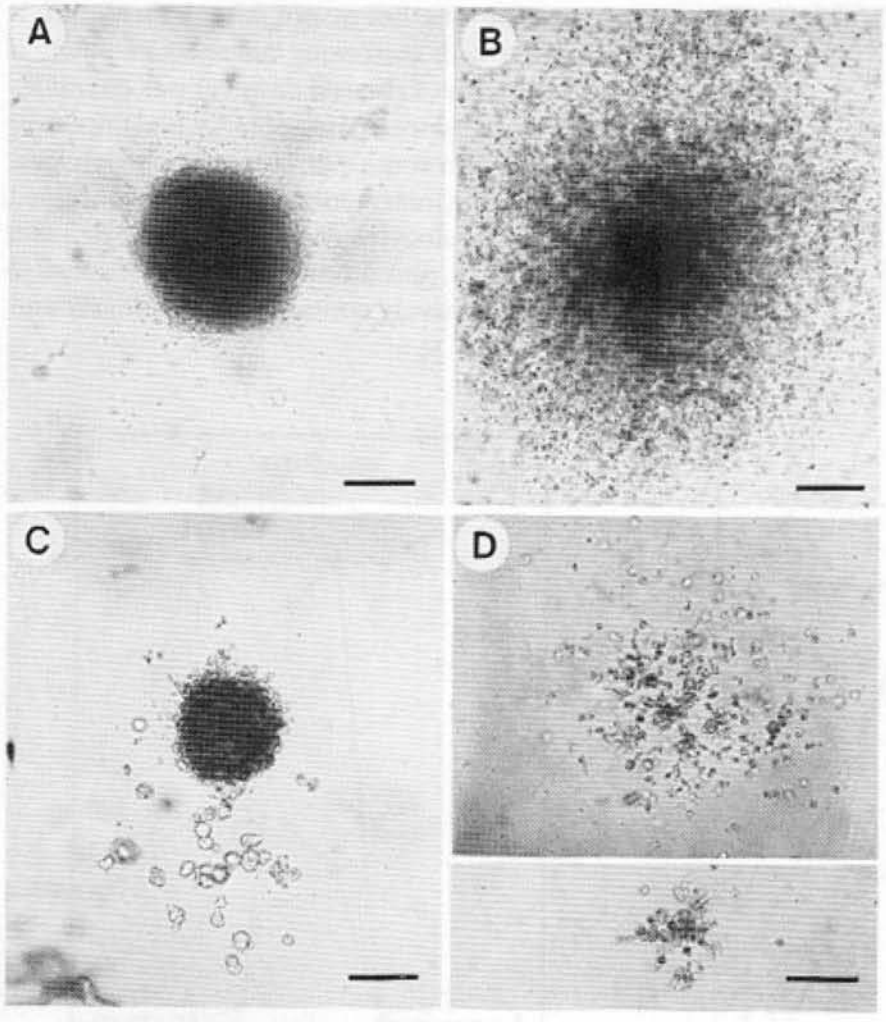

Fig. 1. Colonies of bone marrow cells transformed by AEV **(A)**; MC29 **(B)** and AMV **(C)** viruses photographed 9–12 days after infection. **D** Normal macrophage/granulocyte colonies (CFU-C) photographed 10 days after seeding bone marrow cells in Methocel-containing medium. *Bars* in **A, C,** and **D** 100 μm and in **B** 250 μm

C. Results

Colonies of bone marrow cells transformed by the three prototype DLV strains, i.e., by AEV, MC29 and AMV, are shown in Fig. 1 in comparison to a CFU-C colony. The morphology of each type of colony is characteristic for the type of infecting virus. Furthermore, cells of the DLV-induced colonies can be isolated and grown in tissue culture for up to about 30–40 generations, whereas cells isolated from CFU-C colonies cannot be grown to a significant extent. The characterization of the transformed cells thus isolated has been reported previously (Beug et al. 1979).

In order to characterize DLV target cells and CFU-C on the basis of size, normal bone marrow cells from 2-week-old chicks were subjected to sedimentation through a serum gradient at unit gravity. After sedimentation, fractions were collected and aliquots thereof tested for their content of transformable DLV target cells as well as of CFU-C cells using a newly developed target cell assay (Graf et al. 1981). The results of a typical separation experiment are shown in Fig. 2 A and B. As can be seen, colony forming target cells of all three DLVs exhibited a modal sedimentation velocity of about 5 mm/h. In several experiments the AEV target cells appeared to be slightly smaller than those of MC29 and AMV, and the distribution of AMV target cells was broader than that of MC29. In contrast, the CFU-C cells sedimented significantly more slowly, with a modal distribution of about 4.3 mm/h. Results obtained in several independent experiments are in agreement with the data shown except that in some experiments the distribution of MC29 target cells was broader than that of AMV target cells.

Next, attempts were made to separate DLV target cells on the basis of their density, using gradients containing Percoll. The results obtained after separating normal bone marrow cells on a Percoll gradient and infecting aliquots of each fraction with the three DLV virus strains are shown in Fig. 3. As can be seen, again it was not possible to obtain a clear separation of the

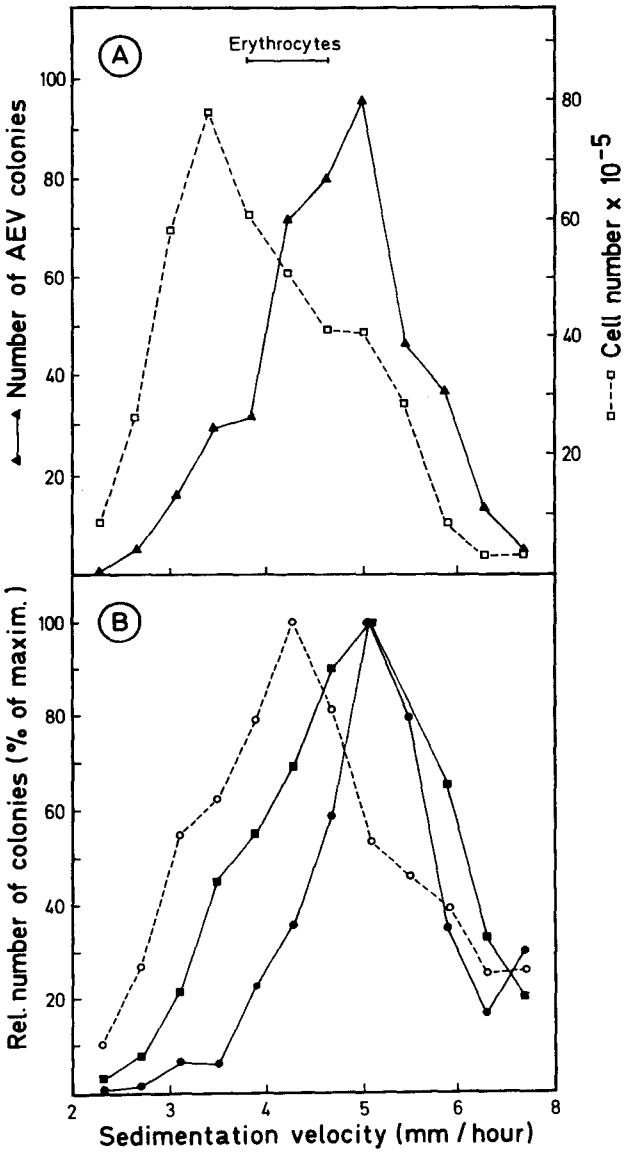

Fig. 2. Unit gravity sedimentation of chick bone marrow cells. Values shown in **A** and **B** were obtained in the same experiments using bone marrow cells of a 9-day-old chick. Maximum number of colonies per dish obtained in **B** corresponded to 442 for MC 29 (●——●), 405 for AMV (■——■), and for CFU-C (●---●)

DLV target cells nor of the CFU-C cells. However, as reproducibly seen in several experiments, there was a fraction of MC29 target cells which appeared to be of higher density than those of AMV. Since this difference might be due to the presence of cells sedimenting at high density which inhibit the colony formation of AMV target cells, as is indeed suggested by mixing experiments (data not shown), we pooled cells from three defined regions of the Percoll gradient. By this procedure a sufficient number of cells could be obtained to perform a dilution series and to determine whether or not colony formation is inhibited with increasing numbers of infected cells. Fraction I (lowest density) was characterized by the appearance of large blastlike cells together with some (immature) macrophages. Fraction II (intermediate density) contained mainly reticulocytes and some myelocytes and granulocytes, and fraction III (highest density) almost exclusively contained erythrocytes and

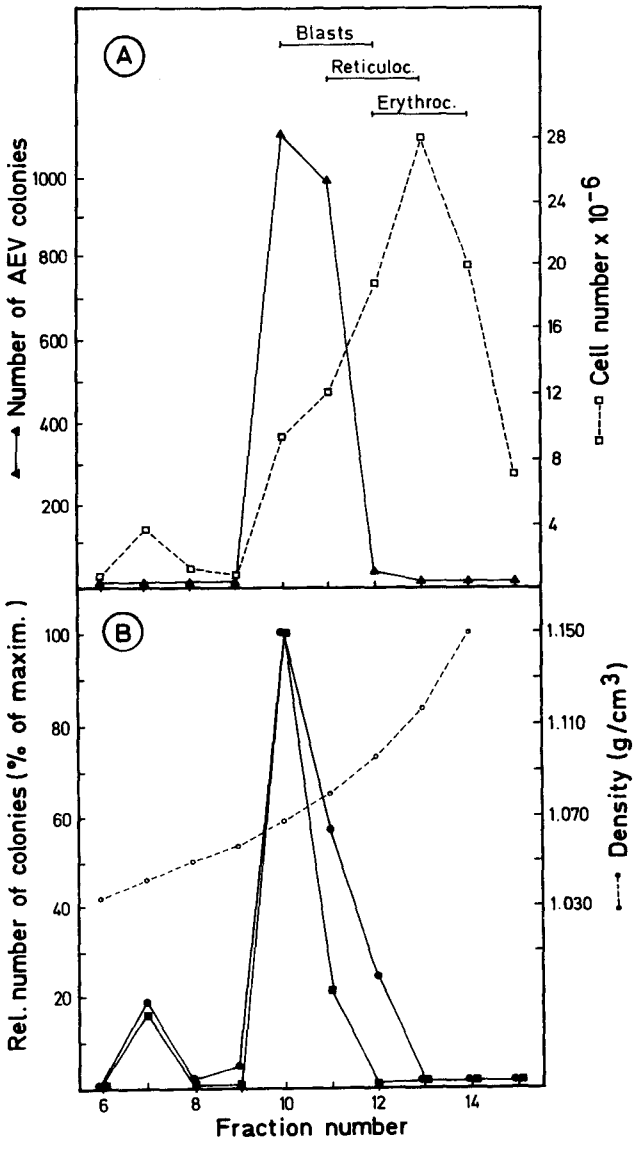

Fig. 3. Percoll density gradient centrifugation of chick bone marrow cells. Values shown in **A** and **B** were obtained in the same experiment using a pool of bone marrow cells from two 7-day-old chicks. Fractions number 1 to 5 were ommitted from the drawing since they contained negligible amounts of cells and target cells. Maximum number of colonies per dish obtained in **B** were 1900 for MC29 (●---●) and 510 for AMV (■——■). The values shown for AEV, MC29, and AMV correspond to 1/2, 1/15, and 1/5 of the total number of cells in each fraction, respectively

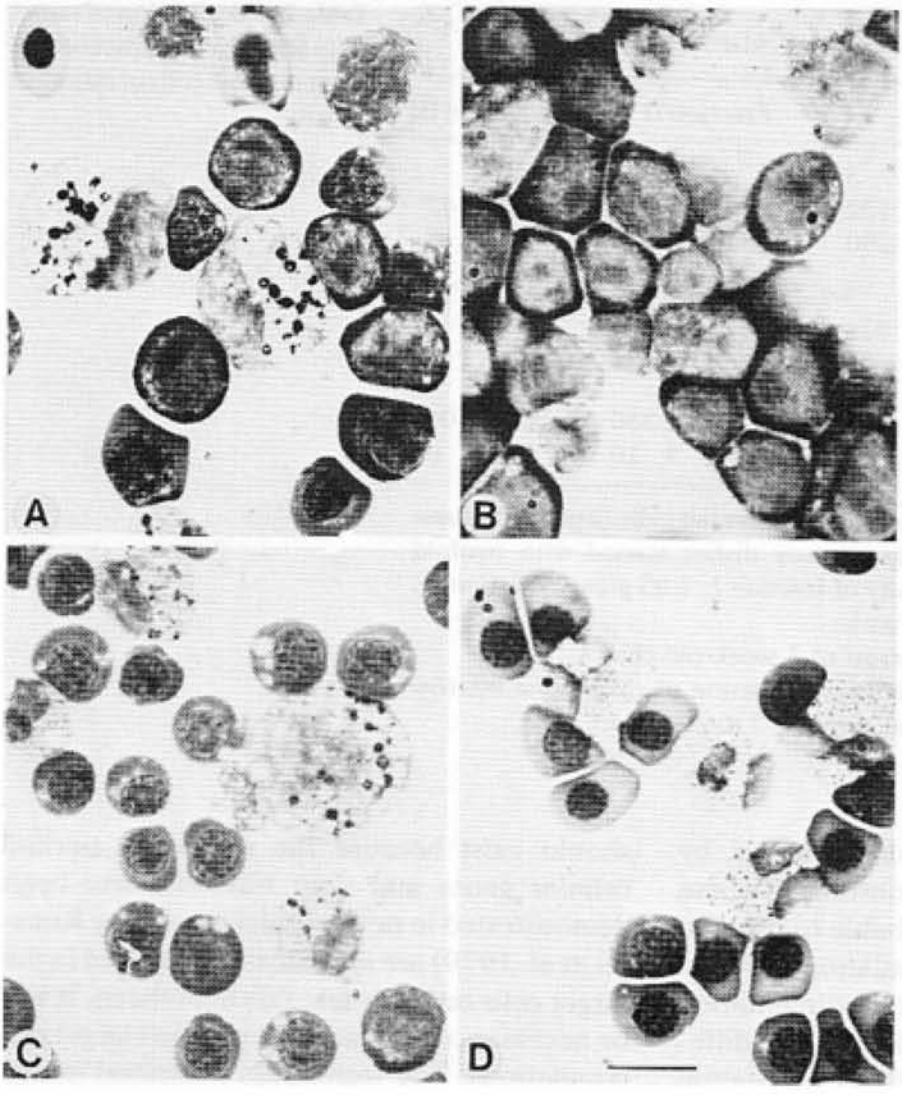

Fig. 4. Smears of bone marrow cells obtained from fractions of different density of a gradient containing Percoll. **A** Cells before centrifugation, **B** Cells from fraction I; **C** Cells from fraction II; **D** Cells from fraction III (see methods).Smears were stained with an eosine-methylene blue stain (Diff-Quik, Harleco, Herstal, Belgium). *Bar:* 10 μm

mature eosinophilic granulocytes (Fig. 4). As shown by the results in Table 2, the dense fractions II and III contained few or no target cells of AMV and AEV. In contrast, fraction II and to a lesser extent fraction III still contained MC29 target cells. Within the normal range of standard deviation, the dose-response curve obtained was in all cases linear. This indicates that the differences in the density distribution of the various types of target cells seen were not (or not only) caused by inhibitory cells.

D. Discussion

Our results show that in general target cells for all three DLV types are most abundant in those fractions which consist mainly of blast-like, immature bone marrow cells. They are similar to each other with respect to both size and density. In comparison to erythrocytes, granulocytes, and CFU-C cells they are relatively large. They are also much lighter than erythrocytes and granulocytes. Together with results described elsewhere (Graf et al. 1976a,b; Gazzolo et al. 1979; Graf et al. 1981) these results suggest that DLV target cells are rather immature but already committed to the erythroid or myeloid lineages. They are also in accord with the notion supported by earlier work (Graf et al. 1978, 1981) that DLVs upon transformation block the differentiation of their target cells.

Our finding that a fraction of the MC29 target cells differs from those of AEV and AMV with respect to density is in accord with results described elsewhere which show that about half of the MC29 target cells in bone marrow are phagocytic and adherent, suggesting that these MC29 target cells are more mature than those of AMV, which do not express such functional markers (Graf et al. 1981).

Our data concerning the relationship between normal macrophage/granulocyte colony forming cells (CFU-C) and the target cells of MC29 and AMV are still very incomplete but suggest that they are not identical. This is also

Expt. No.	Infecting virus	Proportion of target cells[a]		
		Fraction I	Fraction II	Fraction III
1[b]	AEV	17 ± 3.5	6 ± 5	1
	MC29	3470 ± 832	2600 ± 915	353 ± 47
	AMV	1167 ± 290	356 ± 32	52 ± 8
2[b]	AEV	12.8 ± 3.9	4.3 ± 1.5	NT[d]
	MC29	1058 ± 316	1321 ± 22	NT
	AMV	370 ± 26	150 ± 14	NT
3[c]	MC29	7100 ± 415	950 ± 350	355 ± 106
	AMV	5000 ± 1300	42 ± 10	5

Table 2. Distribution of target cells for DLVs in fractions of bone marrow cells of different density

[a] Number of transformed colonies per 10^6 infected cells (average values ± standard deviation from 4 to 6 assay dishes seeded with twofold dilutions of cells). Average density of fraction I: 1.05 g/cm^3; fraction II: 1.09 g/cm^3; fraction III: 1.13 g/cm^3.

[b] Cells tested were from bone marrow of 2-week-old chicks

[c] Cells tested were from the nonadherent fraction of chick bone marrow cultures maintained in growth medium for 7 days

[d] NT = Not tested

in agreement with conclusions reached by Gazzolo et al. (1979). In contrast to these authors, however, we were unable to observe a rapidly sedimenting subpopulation of AMV target cells in our unit gravity sedimentation studies. This could be due to technical differences in both the unit gravity sedimentation and target cell assay techniques employed by the two laboratories.

An important question concerning the target cell specificity of DLVs is whether or not these viruses specifically infect and replicate in their target cells only. Recent studies performed on this question showed that this is clearly not the case. AEV is capable of infecting normal bone marrow macrophages without inducing any detectable transformation, while it replicates in them to almost the same titer as found in target cells. Similarly, MC29 and AMV are capable of replicating in ts34 AEV-transformed nonproducer erythroblasts without affecting their capacity to differentiate after shift to the nonpermissive temperature (Graf et al. 1980). On the basis of these observations and our earlier work (for review, see Beug and Graf 1978) we have proposed that the observed restriction of DLV-transforming capacity to specific cell types in the bone marrow may operate at the posttranslational level (Beug and Graf 1978; Graf et al. 1978; Graf et al. 1979). In addition, we have proposed that cellular proteins homologous to the transforming proteins of DLVs (which should exist because the respective normal cellular genes and their mRNAs have been demonstrated in normal chicken cells by Roussel et al. 1979) are specifically expressed in the target cells only. To test this hypothesis, it will be necessary to characterize purified target cell populations. The techniques described in this paper could be useful as a first step in such a purification.

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