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Post-transcriptional suppression of globin gene expression in cells transformed by avian erythroblastosis virus*

(oncornavirus/hemoglobin genes/mRNA formation/post-transcriptional regulation)

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ABSTRACT **Cells transformed by avian erythroblastosis virus were grown *in vitro* for up to 5 months. After a few days in culture, synthesis of hemoglobin was undetectable and could not be induced by dimethyl sulfoxide. As shown by globin cDNA hybridization to nuclear and cytoplasmic RNA carried to Cr_0t values of 10^5 moles of nucleotide per liter × sec, globin genes in these cells are transcribed into pre-mRNA, but no trace of globin mRNA appears in the cytoplasm. The implications of this observation for schemes of post-transcriptional regulation and viral transformation are discussed.**

One of the major problems relating to the regulation of eukaryotic gene expression, and its defects in transformed cells, is the question of the subcellular and molecular localization of the biochemical mechanisms involved in control.

Recent research interest has concentrated on transcriptional regulation on the assumption that chromatin is the site of action of the major mechanisms involved in the control of the expression of specific genes such as globin (1, 2), histone (3), or ovalbumin (4) and, thus, also of those regulating cellular differentiation.

However, while studying the general pattern of mRNA and protein synthesis in avian erythroblasts, cells that, at the translational level, express globin genes to the extent of 80–90% of total protein synthesis, we became interested in the evidence for regulation of the formation and expression of globin mRNA at *post-transcriptional* levels. The possibility of the existence of a multistep regulative system involving both transcriptional and post-transcriptional controls at several levels was proposed (5–9).

Cytoplasmic mRNA storage is known to exist (10–12); however, there is no example known so far of nuclear mRNA storage, although the considerable amount of high molecular weight RNA retained in the nucleus of animal cells (9, 13, 14) led to the assumption that at least some of these molecules contain mRNA sequences (7).

It is evident that the very scheme of a possible retention of information in the nucleus in the form of (pre-)mRNA and, thus of post-transcriptional regulation at this level, depends on the demonstration of the restriction to the nucleus of a specific transcript, in at least one system.

Looking for such a system, we adopted the working hypothesis that the transforming leukosis virus, e.g., the Friend virus or the avian erythroblastosis virus (AEV), blocking the normal pattern of cellular differentiation might not necessarily act at transcriptional level but might also interfere in a specific fashion with the post-transcriptional expression of specific genes normally transcribed in specialized cells. The availability of globin cDNA as a probe (8, 15) made it possible to test such a hypothesis biochemically in the case of a gene specific to erythroid terminal differentiation.

In the following we will show that in AEV-transformed erythroblasts globin genes are transcribed but no globin mRNA appears in the cytoplasm. A preliminary report of this work was published previously (16).

AEV-Induced Leukemia. Chicken erythroleukemia or erythroblastosis is a disorder of the hemopoietic system induced by the avian erythroblastosis virus, which belongs to the avian leukosis group (17). The disease is almost always fatal and is characterized by the appearance, a few days after infection, of immature "erythroblasts" in the peripheral blood. These young cells, some of them in mitosis, can be isolated from the blood of birds in the terminal phase of the disease and grown *in vitro* in cell culture, where they continue to divide for many generations (18).

These "erythroblasts" were tentatively identified as such for some time on morphological grounds, and later, biochemically, by the presence of histone F2 C typical of terminal erythroid differentiation (19).

However, the transformed cells do not undergo terminal red cell differentiation and, unlike the Friend virus-induced erythroleukemia cells, they fail to respond to any of the known inducers of red cell differentiation (20). The AEV-induced erythroblasts are totally devoid of hemoglobin synthesis and, thus, represent a "clean" population of cells showing no leakiness in respect to either terminal differentiation or hemoglobin synthesis.

The several variations of the Friend virus-induced cell show to some extent signs of terminal differentiation leading, in low percentage, to spontaneous induction of hemoglobin synthesis. In contrast, the AEV-induced disorder resembles one of the forms of acute leukemia in which any signs typical of and necessary for terminal differentiation seem to be missing, possibly due to block at some level of cell regulation (cf. ref. 21).

METHODS

Induction of Erythroblastosis in Chicken, and Tissue Culture of the AEV-Transformed Erythroblasts. Details of the experimental induction of erythroleukemia with AEV in inbred leukosis-free chickens and of the *in vitro* culture of the transformed erythroblasts will be described in detail elsewhere. The disease was almost always fatal. Within an hour or two prior to death the maximum number of erythroblasts had begun circulating in the peripheral blood. At this stage, blood from the birds was collected either from the jugular vein or by heart puncture. A pure population of erythroblasts for *in vitro* culture was prepared by fractionating the total blood cells on bovine serum albumin density gradients according to Sortirov *et al.* (19).

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Abbreviations: AEV, avian erythroblastosis virus; Cr_0t, product of RNA concentration in moles of nucleotide per liter and incubation time in seconds.

* *Dedicated to the memory of Ingrid Blank.*

Labeling with radioactive ^{55}Fe, benzidine staining (22), and induction experiments in the presence of 2% (vol/vol) dimethyl sulfoxide were carried out on these cells grown in tissue culture. In the experiments reported here, erythroblasts were grown in tissue culture for 3 weeks, after which the medium was supplemented with hemin (10 μM) for a further period of 24 hr and the cells were then harvested for RNA extraction.

Chicken embryo fibroblasts were prepared from 7-day-old chick embryos according to Vogt (23) and grown as monolayers in plastic petri dishes for three weeks. The fibroblasts were detached from the petri dishes by a 0.2% trypsin solution.

Preparation of RNA. Total cell RNA from the chicken embryo fibroblasts and the AEV-transformed erythroblasts, grown *in vitro*, were prepared by lysing the cells with sodium dodecyl sulfate (final concentration 1%), followed by hot phenol extraction at pH 5 as described by Scherrer (24). The extraction yield was routinely of the order of 75–85%.

Cytoplasmic and nuclear RNAs from erythroblasts were prepared as follows: The cells were subjected to a hypotonic shock for 150 sec in 4 vol of hypotonic lysis buffer at 4°, and then isotonicity was restored by addition of sucrose to a final concentration of 0.25 M. The nuclei were sedimented for 10 min at 3000 rpm, 4°, in the HG-4L bucket rotor of a Sorvall RC-3 centrifuge. The supernatant after centrifugation was pipetted off and called "cytoplasmic fraction."

To purify the nuclei, the pellet was resuspended in 6 vol of suspension buffer containing 0.02% deoxycholate and centrifuged as before.

The supernatant obtained after deoxycholate treatment was called "cytoplasmic membrane fraction." The nuclear pellet was further cleaned by diluting the pellet again in 6 vol of suspension buffer and 0.2% Cemulsol NP-12, and by suspension–homogenization in a loose-fitting Dounce glass homogenizer. The resulting suspension of nuclei was centrifuged as before for 10 min at 3000 rpm. The supernatant of the NP-12 wash was pooled with the cytoplasmic membrane fraction. The pelleted nuclei were examined under a light microscope and verified to be intact and free of cytoplasmic membranes.

The nuclear, cytoplasmic, and cytoplasmic membrane fractions were treated with DNase (10 μg/ml, 30 min, 0°) and extracted with hot phenol (24) at pH 5. The RNA was alcohol precipitated, dissolved in buffer, and subjected again to extensive DNase treatment (20 μg/ml, 60 min, 20°). Each sample was reextracted with phenol at pH 7.8 at room temperature, and RNA was precipitated by addition of ethanol. The RNA was taken up in hybridization buffer for the annealing reaction. The final RNA preparation was almost free of DNA: labeled phage DNA, added to the nuclei prior to extraction as an internal control, was reduced to 0.1%.

Preparation of cDNA. Pure chicken globin 9S mRNA prepared from acetylphenylhydrazine-induced anemic chicken reticulocytes was transcribed *in vitro* into complementary globin cDNA with RNA-dependent DNA polymerase from avian myeloblastosis virus according to Imaizumi *et al.* (8).

Single-strand specific S_1 nuclease was prepared from commercially available crude preparations of *Aspergillus oryzae* according to the method of Vogt (25).

RNA·DNA Hybridization. Hybridization was carried out as described before (8), except that the hybridization buffer was 355 mM ionic strength, and as specified in the figure legends.

Buffers. Lysis buffer: 10 mM triethanolamine·HCl/20 mM KCl/2 mM $MgCl_2$/5 mM 2-mercaptoethanol, pH 7.4; suspension buffer: 250 mM sucrose/10 mM triethanolamine·HCl/10 mM NaCl/1 mM $MgCl_2$/5 mM 2-mercaptoethanol, pH 7.4.

RESULTS

Intravenous injection of AEV in leukosis-free chicken aged 8–12 weeks provoked in the majority of the birds an acute and fatal erythroblastosis which was monitored by differential blood cell count as shown in Fig. 1.

Possibly, the virus-induced erythroid cells found in the peripheral blood of the birds (cf. Fig. 2 *left*) were simply erythrocyte precursor cells, still in a very early stage of terminal differentiation and hence capable of cell division and, eventually, of hemoglobin synthesis. To test this hypothesis, an *in vitro* system for long-term and mass cultivation of the erythroblasts was developed in which cells were grown for up to 5 months; after an initial period of 6 days, they neither incorporated radioactive iron nor stained positive with Ralph's benzidine staining technique (22), indicating that they did not synthesize any detectable amount of hemoglobin (data not shown). Under culture conditions that allowed bone marrow erythroblasts (26) and embryonic erythroid precursor cells (27) to differentiate *in vitro*, no morphological or biochemical signs of terminal differentiation could be detected in the AEV-transformed cells.

The cultured cells (cf. Fig. 2 *right*), however, continued to produce and shed infectious virus particles into the tissue culture medium. Fig. 3 is the electron micrograph of a typical AEV-transformed erythroblast after 22 days in tissue culture, demonstrating the presence of intracytoplasmic virus particles and budding virus.

Thus, the most marked effect of AEV transformation was, apart from the presence of virus particles and the absence of morphological differentiation, the failure of the phenotypic expression of the globin genes. Such a failure could have occurred at any one of a number of levels of mRNA formation or function (7).

To test whether the globin genes were activated in these virus-transformed cells, we analyzed the transcriptional products of the erythroblast genome for the ability to hybridize radioactive cDNA prepared against purified globin mRNA. In control experiments, about 90% of the globin cDNA reannealed to its template, i.e., to purified chicken globin mRNA, with a $Cr_0t_{1/2}$ of 1.122×10^{-3} moles of nucleotide per liter × sec, confirming the value reported previously (29) and comparable to that obtained for a pure duck globin mRNA·cDNA hybrid-

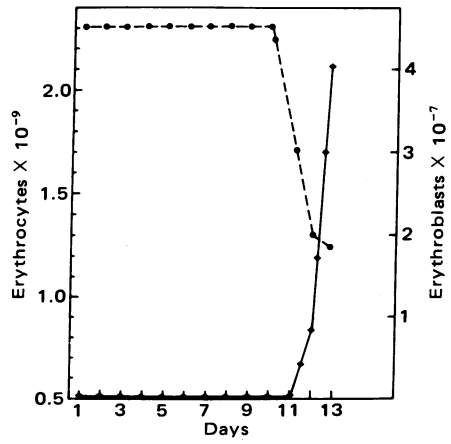

FIG. 1. The course of development of erythroblastosis in leukosis-free chickens. Viremic plasma (0.5 ml), from serial passage *in vivo*, was injected intravenously into 1- to 3-month-old leukosis-free chickens. The development of erythroblastosis was monitored by daily examination of blood smears and total blood cell count of the infected birds. ●, Total erythrocyte count; ♦, immature erythroblasts in peripheral blood.

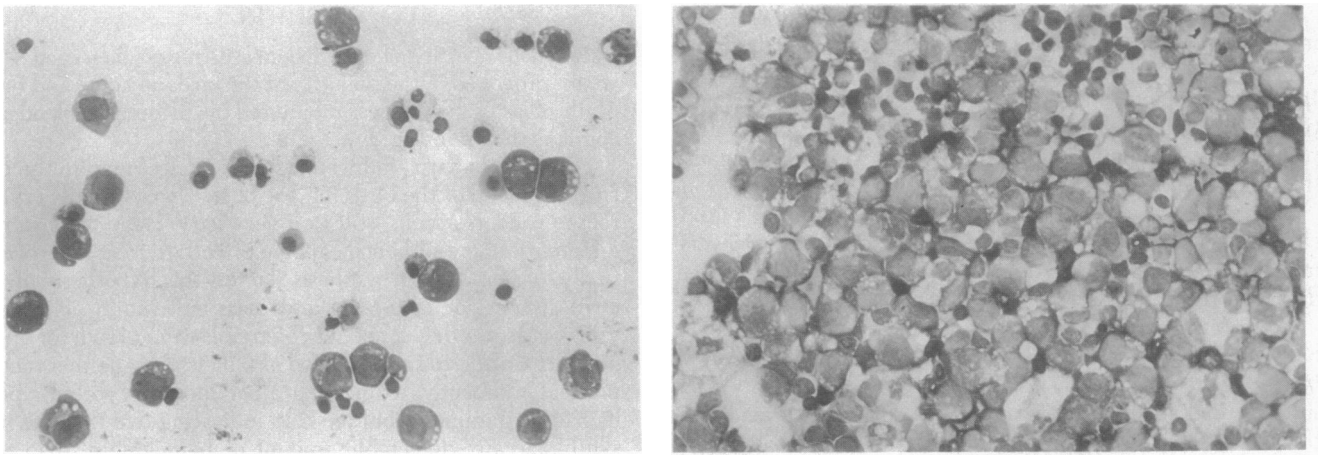

FIG. 2. (×500.) (*Left*) Blood picture of infected birds at the terminal phase of erythroblastosis, showing a predominance of immature erythroblasts in peripheral circulation. At this point, blood was drawn for obtaining the erythroblasts for the *in vitro* tissue culture. (*Right*) Cytocentrifuge preparation of erythroblasts grown in tissue culture for 3 weeks. Erythroblasts were separated from other blood cell types on bovine serum albumin gradients (19). The cells could then be grown up to 5 months in suspension culture using GIBCO NCTC 135 medium supplemented with 30% fetal calf serum, 3% anemic chicken plasma (to provide erythropoietin), and penicillin and streptomycin at 50 units/ml.

ization (8). Total polysomal RNA from normal anemic chicken reticulocytes reannealed to globin cDNA at a $Cr_0t_{1/2}$ of 0.1 M·sec (Fig. 4), indicating that about 1% of the total polyribosomal RNA was globin mRNA.

Other mammalian RNA (HeLa cells) and insect RNA (*Bombyx mori*) failed to form hybrids with globin cDNA even at Cr_0t 5000 (Fig. 4), indicating that the hybridization method was highly specific for molecules with globin mRNA nucleotide sequence complementarity. With cDNA of a specific activity of about 10^7 cpm/μg the sensitivity of the method allowed the detection of one globin mRNA molecule per 10^5–10^6 molecules.

Fig. 4 shows the results of two experiments in which the hybridization of RNA from different batches of cells, cultured for 22 days, was compared to the hybridization of the highly purified chicken globin mRNA. The Cr_0t curves indicate that the total RNA of the AEV-induced cells contains globin mRNA sequences, though at a level far below that found in normal differentiating chicken reticulocytes (8). At Cr_0t values of 100 and 500 M·sec the maximum hybridization obtained in the two different experiments of Fig. 4 was 35% and 24%, respectively. In these experiments, we could not quantitate with precision the amount of globin mRNA sequences; limited by the amount of RNA available, we could not obtain in the annealing reaction the very high Cr_0t values desirable.

However, if in these two experiments, plateau Cr_0t values have been reached, then we must conclude that only a part of the different adult globin mRNA sequences has been represented in the cellular RNA. A conservative interpretation of these results is that very low but nevertheless measurable

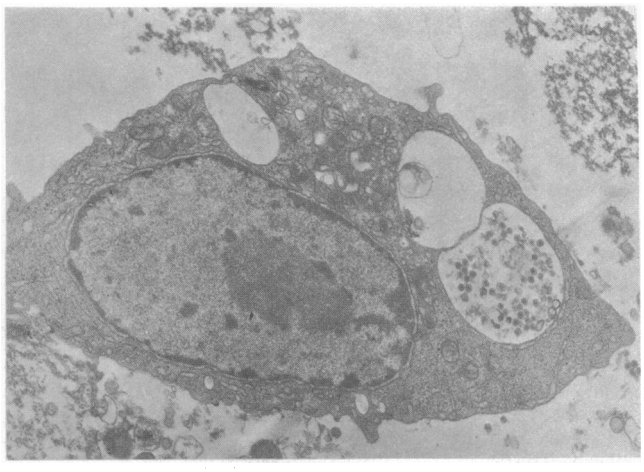

FIG. 3. Electron micrograph of an erythroblast grown in tissue culture. One of the vacuoles contains numerous C type virus particles; the outer cytoplasmic membrane shows virus budding. (×10,000.)

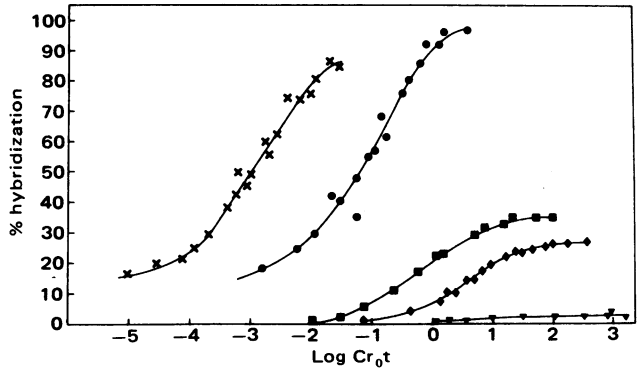

FIG. 4. Hybridization kinetics of globin-specific cDNA with various RNA populations. Reaction mixture: RNA, 0.01–4.51 mg/ml (where present, globin gene products were in 5- to 53-fold excess over cDNA); [^{3}H]deoxycytidine-labeled globin-specific cDNA at 5–10 ng/ml (250–650 cpm/sample); 0.3 M NaCl; 50 mM Tris·HCl, pH 7.4; 1 mM EDTA; and 0.1% sodium dodecyl sulfate. Hybridization: denaturation (100°, 5 min) and incubation at 65° in sealed sterile glass capillaries for time periods ranging from 3 sec to 213 hr. Assay of hybrid: capillaries were frozen at −20° to arrest the reaction, the contents were then injected into 200-μl digestion mixtures containing 0.15 M NaCl, 30 mM Na acetate (pH 4.5), 1.5 mM $ZnSO_4$, 5 μg of sonicated and denatured salmon sperm DNA, and 1.0 unit of S_1 nuclease. Incubation was for 40 min at 45°; trichloroacetic acid-precipitable radioactivity was determined. Percent hybridization was determined relative to acid-precipitable cDNA at time 0 after denaturation. cDNA stability and background (after S_1 digestion) were monitored at the end of the incubation period and varied in general from 95 to 97% (90% in the experiment shown by ♦) and from 1 to 5%, respectively. RNA stability was monitored by checking acid-precipitable cpm of labeled nuclear RNA in analogous experiments; chicken polysomal RNA hybridized for 240 hr with globin cDNA led to 90–95% of the cDNA in hybrid. The percentage of hybridized cDNA is plotted according to Britten and Kohne (28) ×, Chicken globin mRNA; ●, chicken reticulocyte total polysomal RNA; ♦ and ■, AEV cell total RNA from two different batches of cells; ▼, HeLa cell RNA.

amounts of globin mRNA sequences were present in the RNA of the AEV cells.

Having found, thus, that globin genes were active, we next considered the localization of the apparent post-transcriptional suppression of the globin gene expression. In view of the reports on uninduced Friend cells (30), the hypothesis of a translational block as the primary cause of the absence of globin synthesis had to be considered. Such a mechanism was, however, unlikely *a priori*. Indeed, the very low level of globin mRNA sequences found in the AEV-induced erythroblasts ruled out the possibility of an accumulation of untranslated globin mRNA in the AEV cell cytoplasm, not associated with polysomes, in amounts comparable to those present in normal cells.

Nuclear and cytoplasmic RNA from AEV-transformed cells were separately prepared and purified. Each of these RNAs was then hybridized with radioactive globin cDNA and the Cr_0t curve was determined. Fig. 5 gives results representative of this set of experiments. Total cytoplasmic RNA from the AEV-transformed cells failed to form hybrids with globin cDNA even at Cr_0t 3000 M·sec, indicating that it contains less than 1 globin mRNA per 3×10^5 mRNA molecules of equal size. The total nuclear RNA, on the other hand, reannealed to the cDNA with a $Cr_0t_{1/2}$ value of 39 M·sec, showing about 0.003% of this RNA to be globin mRNA sequences. The half melting point of the hybrids thus formed was 95° as compared to 97° for the globin mRNA·cDNA duplex, showing that genuine hybrids with less than 2% mismatch (31) were formed (data not shown).

It was thus evident that in the earlier experiments involving total AEV cell RNA the hybridization obtained was with the nuclear component alone.

In the results of Fig. 5, the nuclear RNA protected only 50% of the globin cDNA even when the Cr_0t value was as high as 5000 M·sec and a seemingly genuine saturation plateau had been reached. A possible explanation might be that, in transformed state, the erythroblasts transcribe only one of the two major globin genes represented in adult hemoglobin. This view is strengthened by the fact that the identical cDNA produced 95% plateau values when hybridized to nuclear RNA of erythroblasts transformed *in vitro* by another strain of AEV; on the other hand, RNA from AEV-infected and transformed fibroblasts did not allow more than 5% of the cDNA to hybridize (unpublished data). To resolve the problem it is desirable to prepare globin chain-specific probes; unfortunately, the successful separation of mammalian α and β globin mRNA by electrophoresis was not feasible in the case of avian mRNA.

In a control experiment (Fig. 5A), we confirmed, as reported earlier (29), the total absence of globin gene transcripts in the chicken embryo fibroblast RNA.

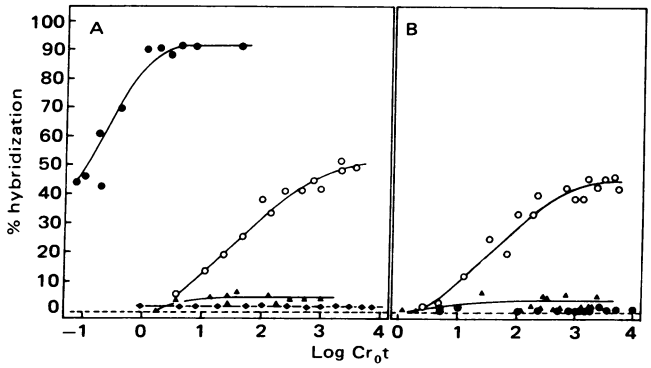

FIG. 5. Hybridization kinetics of globin-specific cDNA with AEV cell nuclear and cytoplasmic RNA. Hybridization was carried out and evaluated as described in the legend of Fig. 4 except that all reagent solutions were purified by Chelex-100 chromatography and that lead-free glass capillaries were used. RNA concentrations varied from 0.01 to 4.5 mg/ml and incubation times from 3 sec up to 168 hr for experiment *A* and 1.25 to 5.8 mg/ml and times from 3 min up to 168 hr for experiment *B*. *A* and *B* are results of hybridization experiments in which the RNA was obtained from two separate series of *in vivo* transformed AEV cells grown in tissue culture. ●, Chicken reticulocyte total polyribosomal RNA (partial curve shown); ○, AEV cell nuclear RNA; ▲, AEV cell cytoplasmic RNA; ⊗, AEV cell cytoplasmic RNA from the mild detergent wash of cell nuclei; ♦, chicken fibroblast RNA.

DISCUSSION

Restriction of globin mRNA sequences to the nuclear fraction of the AEV-transformed cell shows that a leukosis virus, while blocking the morphological and biochemical expression of erythroid differentiation, does not prevent the transcription of genes that contain information for a differentiation-specific protein, hemoglobin. The mRNA sequence for this protein, however, is confined exclusively to the nucleus and, thus, remains unexpressed. Possibly, at some step during post-transcriptional processing of the globin pre-mRNA (8, 9, 32–36), the globin mRNA sequences are blocked and eventually destroyed while other mRNAs are transferred to the cytoplasm.

Thus, provided the primary transcript of the globin gene is normal, this finding might represent an example of post-transcriptional regulation in support of our working hypothesis and the cascade regulation scheme (5, 6).

There are up to 10^3 globin mRNA sequences in the AEV cell; this is about 1/10th the amount found in the nuclear RNA of the normally differentiating red cell (8, 9), and it is up to 1/10,000th that of total cellular globin mRNA in normal reticulocytes (8, 37). Thus, it is comparable to the amount one would expect theoretically if the globin mRNA sequences were confined to the immediate primary transcript, i.e., the giant pre-mRNA, which in the normal cell represents only 10–15% of the total nuclear globin pre-mRNA population (9), or about 100 molecules per cell. It is therefore not excluded that synthesis of primary pre-mRNA is normal in the transformed cell; further work is required to determine whether the defect is transcriptional or post-transcriptional.

The absence of globin mRNA in the cytoplasm of AEV cells rules out the possibility of a translational block for globin mRNA, as encountered in some variants of Friend virus-transformed cells (30). In fact, in most Friend cell variants, prior to induction, globin mRNA is found in the cytoplasm as well as in the nucleus, either in quantities similar—in order of magnitude—to those found in the induced cell (38) or in much lower concentration (39).

It then becomes important to explore further the underlying cause and mechanism that selectively prevent the normal processing, conservation, and eventual transport to the cytoplasm of an mRNA. Indeed, these events may be involved in the mechanisms that allow a cell to become committed to a given pathway of terminal differentiation. Although such a post-transcriptional block of pre-mRNA processing and transport, for a gene characteristic of the differentiation state of a cell, may be only an indirect consequence of the transforming event brought about by the virus, the hypothesis is equally appealing that the leukemia virus, by interfering with the post-transcriptional regulation of a differentiation-specific gene, may simply hold the erythroid line-determined cell in a compartment of early differentiation characterized by globin gene activity prior to hemoglobin formation.

Although *in ovo* the embryonic development is too rapid to analyze such a phase (29), Wilt (40), and more recently Chan *et al.* (41, 42) have shown by the use of *in vivo* culture of embryonic blood island explants the existence of cells containing

globin mRNA in the nuclear compartment well before the onset of detectable hemoglobin synthesis. Such cells may represent the target for the AEV.

After viral infection, the arrest of morphological differentiation and the post-transcriptional block of the expression of differentiation-specific genes may be explained by a virus-dependent event capable of interrupting differentiation without otherwise interfering with the cell and its genome. A single event may suffice: the many biochemical mechanisms underlying differentiation may well be interlocked in a machinery providing the necessary coordination to the developing cell and organism.

An illustration of such a situation can be found in the recent description of presumptive myoblasts transformed by a temperature sensitive Rous sarcoma virus mutant (43). In this case, the inactivation of a temperature-sensitive structure primed by the virus, and hence, probably a single protein or protein–RNA complex, allows instantaneous muscle cell differentiation upon shift to a temperature that is nonpermissive to the viral gene products.

In view of the finding with the AEV-transformed cells, one may postulate that, in the transformed myoblasts, a virus-coded signal may interfere with the post-transcriptional expression of differentiation-specific genes. As a working hypothesis one might follow thus the idea of the interference of a viral product with a specific pleiotropic signal acting at an early post-transcriptional level of the postulated cascade of regulation governing gene expression in eukaryotic cells (5, 6). Indeed, such a scheme would formally satisfy the experimental results in both the virus-transformed myogenic and erythropoietic systems.

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