Molecular Mechanisms in Erythroid Differentiation

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The most striking molecular event during erythroid differentiation is the accumulation of haemoglobin but this is only one of many overt changes which occur in the maturing erythroid cell. Other proteins accumulate progressively, notably carbonic anhydrase and catalase while yet others, particularly the enzymes of haem synthesis, such as aminolaevulinic acid synthetase, accumulate during early maturation and diminish later (Freshney, R. I. and Paul, J. 1972). Membrane changes also occur. These include the accumulation of spectrin on the inner surface of the membrane, of specific antigens on the outer surface and changes in lectin binding properties. Simultaneously, there is progressive reduction of transcription leading eventually to a complete cessation of RNA synthesis and, indeed, in most mammals, to the extrusion of the nucleus itself. These changes are orchestrated in the orderly manner that we associate with many differentiating systems and, for this reason, erythropoiesis has been regarded as a very good model for investigating normal differentiation in mammals and, hopefully, therefore, for providing information about the mechanisms which are disturbed in leukaemia.

In any experimental situation, it is desirable that one should be able to initiate the process at will and to follow at least some components of it in detail. Erythroid tissues readily respond to increased demand and at least part of this response is mediated through erythropoietin which is produced in the juxtaglomerular cells of the kidney in response to anoxia and promotes the maturation of erythroblasts. It has been possible to purify erythropoietin at least partially and to demonstrate its effects on cultured erythroid tissue *in vitro* (Krantz, S. B., Gallien, Lartigue, O. and Goldwasser, E. 1963; Krantz, S. B. and Goldwasser, E. 1965; Cole, R. J. and Paul, J. 1966). Hence, part of the requirement can be met by using these techniques. Relatively recently, however, an alternative system of considerable power has emerged following the discovery that Friend erythroleukaemic cells of the mouse can be induced to synthesise large amounts of haemoglobin when treated with dimethylsulphoxide although normally they synthesise minimal amounts (Friend, C., Scher, W., Holland, J. G. and Sato, T. 1971; Scher, W., Holland, J. G. and Friend, C. 1971). Experiments with both these systems will be discussed.

In analysing the places in metabolic pathways where the accumulation of a protein can be controlled, the first principle to be appreciated is that accumulation occurs when synthesis exceeds degradation. This applies both to the final product, the protein, and also to intermediates in its synthesis such as messenger RNA. Degradation is unquestionably just as important as synthesis but here are more

technical difficulties in studying changes in rates of degradation than in studying changes in patterns of synthesis. Moreover, powerful new tools have been fashioned in recent years to enable us to study the synthesis of specific messenger RNA in cells. Accordingly, this communication will deal mainly with information concerned with the synthesis and accumulation of globin and the nucleic acids involved in its synthesis. However, it is emphasised that our knowledge will be incomplete until we have an equally detailed understanding of degradative processes.

Control of globin synthesis could, theoretically, occur at several levels. There could be an increase in the number of genes for globin chains (gene amplification): the rate at which RNA is transcribed from DNA in chromatin could alter: the processing of newly transcribed RNA into messenger RNA could be subject to control: finally, the efficiency of utilisation of messenger RNA in the translational machinery could be involved. Each of these will be discussed.

Experimental Methods

Globin messenger RNA – The greatest single technical advance in this work has been the isolation of pure messenger RNAs (Williamson, R., Morrison, M., Lanyon, W. G., Eason, R. and Paul, J. 1971). Since a messenger RNA is a direct transcript of a gene, it bears the same absolutely specific relationship to the sense strand of that gene as does a photographic print to its negative. Moreover, it is possible to form hybrid molecules between RNA and DNA and thus it can be used as an absolutely specific probe for globin gene sequences.

Globin messenger RNA was initially isolated from reticulocyte polysomes on the basis of size. More recently, other techniques have become available, especially techniques of affinity chromatography which exploit the existence of a polyadenylate tract at the 3' end of messenger RNA. These methods make it possible to isolate globin messenger RNA in large amounts. That it is a messenger RNA molecule can be proven by using it to programme a cell-free protein synthesising system to make α and β globin chains.

Complementary DNA – The second important technical advance has been the discovery of techniques to prepare DNA copies of messenger RNA with the enzyme reverse transcriptase from RNA tumour viruses (Kacian, D. L. and Spiegelman, S. 1972; Ross, J., Aviv, H., Scolnick, E. and Leder, P. 1972). Since cDNA is an exact transcript of messenger RNA, it is a precise replica of at least part of the sense strand of the globin gene. Consequently, it can be used as an absolutely specific probe both for the nonsense strand of the globin gene and also for globin messenger RNA. Since it is possible to synthesise it at very high specific activity, it is not only an absolutely specific probe but an exquisitely sensitive one.

Results

Is there a change in globin gene number during erythroid differentiation? At least three major possibilities must be entertained. The first is that every cell in an organism has the same number of globin genes. The second is that globin genes are segregated in such a way that most cells have no globin genes whereas erythroid cells do. The third possibility is that all, or most, cells contain globin genes but the

number is increased in erythroid tissues, like the ribosomal genes in amphibian oocytes. Within the past two or three years, methods have been devised for measuring the concentration of globin genes in DNA using either messenger RNA or cDNA. The kinetic analysis is very sensitive and has made it possible to determine that, on average, each globin gene occupies about 4×10^{-7} of the genome, i. e. there are 1–2 copies of each globin gene sequence in each genome. These methods are so specific and so sensitive that they have made it possible recently to show that α-thalassaemia is due to a deletion involving the two α genes in the human (Ottolenghi, S., Lanyon, W. G., Paul, J., Williamson, R., Clegg, J., Pritchard, J., Pootrakul, J. and Wong Hock Boon, 1974).

These methods were used to investigate globin gene dosage in different animal tissues (Harrison, P. R., Birnie, G. D., Hell, A., Humphries, S., Young, B. D. and Paul, J. 1974). In particular, we estimated the globin gene concentration in DNA from mouse sperm, from the total mouse embryo and from mouse foetal liver (which is an active erythropoietic tissue). We found that the concentration of globin genes was identical in all three kinds of DNA and corresponded to about one copy of each gene per genome. Hence, the gene segregation and amplification hypotheses can be discarded; all tissues in the mouse seem to have one copy of each globin gene per genome.

Is there evidence for regulation of transcription of the globin gene? It has been shown by a number of workers that it is possible to transcribe mammalian chromatin with bacterial RNA polymerase and that the transcript resembles very closely the nuclear RNA of the cell from which the chromatin was derived. We, therefore, undertook experiments to determine whether we could demonstrate differences between chromatin from erythroid and non-erythroid tissues. Chromatin from mouse foetal liver and from brain was transcribed with *E. coli* RNA polymerase and cDNA was then used to measure the concentration of globin messenger RNA in the transcript (Gilmour, R. S. and Paul, J. 1971; Paul, J., Gilmour, R. S., Affara, N., Birnie, G. D., Harrison, P. R., Hell, A., Humphries, S., Windass, J. and Young, B. 1974). These experiments revealed detectable amounts of newly synthesised globin messenger RNA in the RNA transcribed from mouse foetal liver chromatin but no detectable globin messenger RNA in the RNA transcribed from brain chromatin (Table 1). This, therefore, provided presumptive evidence for transcriptional specificity embodied in the structure of the chromatin itself.

Are there controls at other levels? In the reticulocyte, there is good evidence that

Table I: Transcription of the globin gene from chromatin by E. coli RNA-dependent RNA polymerase (see Paul et al. 1974, Cold Spring Harbour Symp. Quant. Biol. 28, 885.

Source of Chromatin	Globin mRNA as fraction of total RNA synthesised x 10^7
Mouse brain	< 3
Mouse fetal liver (erythroid)	25

a control exists at the level of translation. This evidence indicates that there is a high molecular weight diffusible repressor of globin synthesis, the effect of which is inhibited by haemin (Gross, 1974). Hence, haemin can act as an inducer of translation of globin from messenger RNA. Evidence to be cited later indicates that controls at this level may also occur in maturing erythroblasts.

Mode of Action of Erythropoietin – The most primitive erythroid cell to be identified positively in the adult mammal is the colony forming cell (CFC); it can be demonstrated by injecting bone marrow into animals which have received a lethal dose of irradiation, such as to eliminate their own erythroid capacities. If a small enough dose of bone marrow is injected, colonies form in the spleens of the recipient animals and these can give rise either to granulocyte or erythroid cells. There is evidence too that these then give rise to a cell, the erythropoietin-sensitive cell (ESC) which is capable of developing into mature erythroid cells on treatment with erythropoietin. In theory, therefore, erythropoietin could act by influencing the decision of a stem cell to form granuloid or erythroid tissue or to increase, by multiplication, the number of ESC, or again, by stimulating the erythropoietin-sensitive cells to enter into maturation rather than self-maintaining cell divisions or, more specifically, by stimulating the synthesis of globin and other messenger RNAs. There is good experimental evidence against the first and second of these hypotheses, rather good evidence to support the third and mixed evidence concerning the last. Unfortunately, it has not proved possible to culture CFC and ESC continuously. Consequently, most of these deductions are drawn from experiments in whole animals which are sometimes difficult to interpret and experiments with tissue culture material of foetal liver which relate to stages from the proerythroblast onwards. Nevertheless, in short-term cultures, it has proved possible to demonstrate quite striking effects of erythropoietin.

When mouse foetal liver from embryos of 12–14 days gestation is cultured *in vitro*, it retains some capacity to synthesise haemoglobin but this diminishes quite rapidly in the course of 48–72 hours. On the other hand, if erythropoietin is added, then after a lag of about 2 hours, there is increased synthesis of DNA, RNA and haemoglobin at rates which continue to increase for 24 hours and at that time are considerably greater than those of untreated tissue (Figure 1) (Cole, R. J. and Paul, J. 1966; Paul, J. and Hunter, J. 1969; Ortega, J. A. and Dukes, P. P. 1970; Gross, M. and Goldwasser, E. 1969; Gross, M. and Goldwasser, E. 1970; Nicol, A. G., Conkie, D., Lanyon, W. G., Drewienkiewicz, C. E., Williamson, R. and Paul, J. 1972).

Some reports about the response to erythropoietin differ. The increases in messenger RNA and protein synthesis are generally agreed by all workers. Paul and Hunter (1969) originally proposed that while there was an early increase in RNA synthesis, this was followed by an obligatory DNA synthetic step before a specific increase in globin messenger RNA and haemoglobin synthesis occurred. Similar observations have been made by Gross and Goldwasser (1969; 1970) but these have been disputed by Djaldetti, Marks and Rifkind (1972) although this group reported that a rapid decrease in DNA synthesis, which they observed in their cells in culture, was prevented by erythropoietin. Paul. J., Freshney, R. I., Conkie, D. and Burgos, H. (1971) and Harrison, P. R., Conkie, D. and Paul, J. (1973) are of the view that the entire erythropoietin effect in short-term cultures

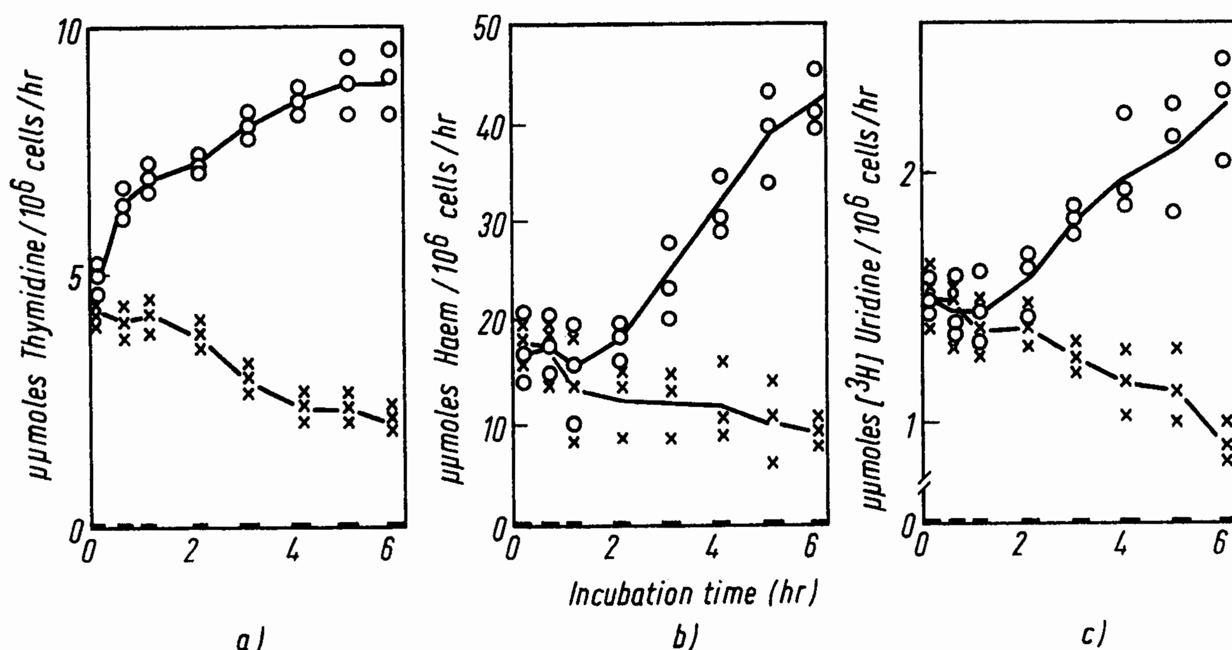

Fig. 1: Rates of synthesis of DNA (a), haemoglobin (b) and RNA (c) in primary mouse foetal liver cultures in the presence or absence of erythropoietin, -x-x-, control; -o-o-o, erythropoietin present from zero time. Labelled precursors were added during the times indicated in the time axis.
(Paul, J. and Hunter, J. A. (1969) P. N. A. S. *42*, 31)

can be explained by increased production of haemoglobin-synthesising cells, achieved by promotion of division of proerythroblasts. They claim that many of the resulting 'G2' cells are unable to divide in tissue culture and form giant cells which are double-sized and contained double the normal amount of haemoglobin. It is certainly agreed that there is no specific increase in the rate of globin synthesis per cell as a result of erythropoietin treatment although there is some disagreement concerning the rate of RNA synthesis.

In this discussion, it is obviously of importance to determine at what stage in erythroid development globin messenger RNA synthesis commences. There is difficulty about obtaining sufficient amounts of pure populations of immature erythroid cells to permit direct biochemical studies with globin cDNA. Accordingly, Harrison, P. R., Conkie, D., Affara, N. and Paul, J. (1974) applied an *in situ* hybridisation method, developed by Harrison, P. R., Conkie, D., Paul, J. and Jones, K. (1973) which permits the demonstration of the distribution of globin messenger RNA in individual cells. Using this technique, they were able to show that globin messenger RNA makes its appearance during the transition from proerythroblast to basophilic erythroblast. This observation is of considerable interest for a number of reasons. For one thing, haemoglobin synthesis is usually not detectable until the next stage in differentiation, the polychromatic erythroblast stage. Hence, the observation provides presumptive evidence for translational control. Secondly, an increase in the number of basophilic erythroblasts at the expense of proerythroblasts is produced by erythropoietin and this also results in the appearance of globin messenger RNA in a few late proerythroblasts. This observation can be considered in the context of other observations by Paul, J.,

Freshney, R. I., Conkie, D. and Burgos, H. (1971) which indicated that erythropoietin was necessary for the completion of cell division in some proerythroblastic cells. It seems possible that erythropoietin is needed to facilitate the maturation divisions which occur in erythrocyte precursors. These may be essential for the transition to occur and to permit activation of globin genes.

The Friend Cell System – Experiments with short-term primary cultures of erythroid tissues have given us some idea of the ways in which globin synthesis may be regulated but unfortunately they leave us with a very incomplete picture of mechanisms. The discovery of the induction of haemoglobin synthesis by dimethylsulphoxide in the Friend system has provided us with a means of studying some of the molecular events (Figure 2).

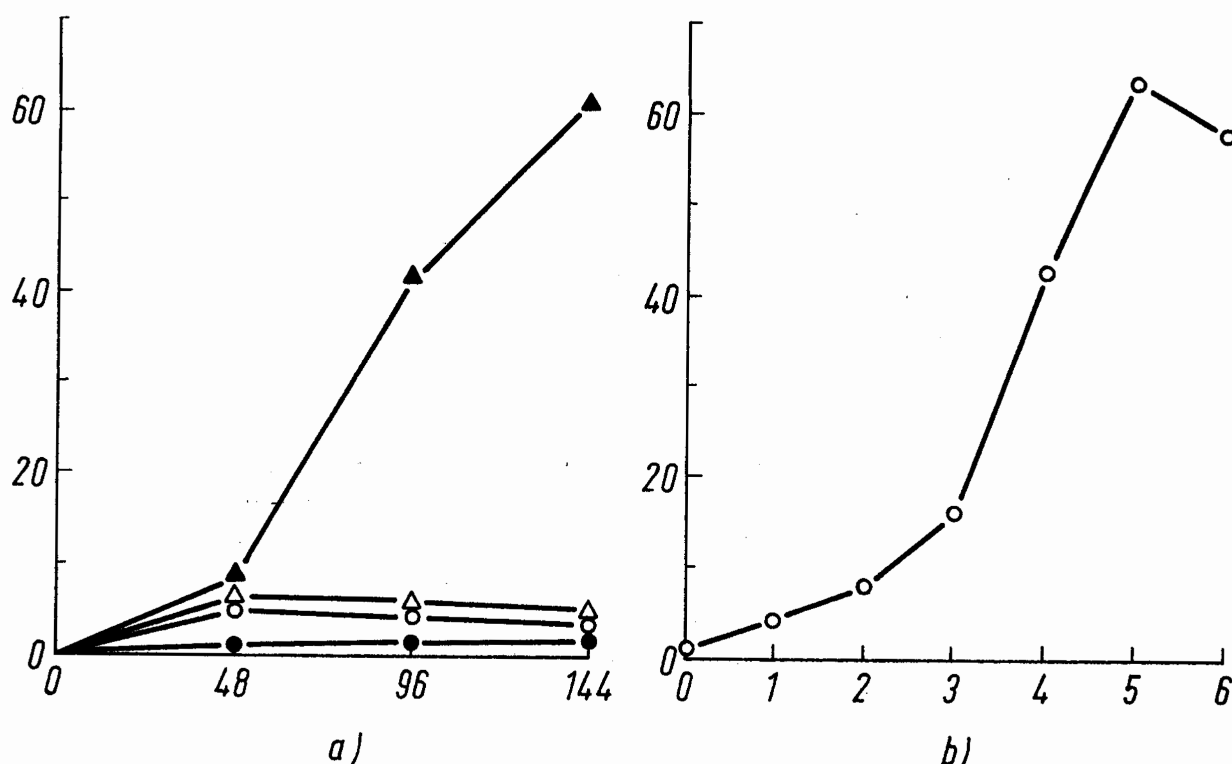

Fig. 2: Induction of haem and benzidine-stained cells following treatment of Friend cells with 2 % DMSO.
1. Abscissa: time (hours; ordinate, pmoles haem/10^6 cells.
FtID$^+$, no DMSO added (△–△); DMSO added (▲–▲).
FwID$^-$, no DMSO added (o-o); DMSO added (●-●).
2. Abscissa, time (days); ordinate, percentage of cells staining with benzidine.
FtID$^+$.
(From: Paul, J. and Hickey, I. (1974) E. C. R. *87*, 20).

When Friend cells are grown in suspension cultures, they have a doubling time of 12–14 hours and rarely show evidence of haemoglobinisation. Following treatment with dimethylsulphoxide, haemoglobin synthesis occurs at quite a high rate after a lag period of 24–48 hours. The cells rapidly become haemoglobinised until, after about 5 days, upwards of 80 % contain quite large amounts of haemoglobin. Simultaneously, they exhibit many other phenomena characteristic of erythroid differentiation; cell division diminishes and eventually ceases irreversibly; RNA syn-

thesis diminishes and the nucleus becomes condensed and there is some other evidence that other specific molecules accumulate. The accumulation of haemoglobin is accompanied by an accumulation of globin messenger RNA (Ross, J., Ikawa, Y. and Leder, P. 1972; Harrison, P. R., Gilmour, R. S., Affara, N. A., Conkie, D. and Paul, J. 1974; Gilmour, R. S., Harrison, P. R., Windass, J. D., Affara, N. A. and Paul, J. 1974). This may occur slightly ahead of haemoglobin accumulation but for practical purposes, the two events occur almost simultaneously.

At what level does control of haemoglobin synthesis occur? – To determine whether globin messenger RNA synthesis is the controlling step for subsequent processing and translation, we undertook experiments in which cDNA was used to measure the concentration of globin messenger RNA sequences in nuclear, polysomal and cytosol RNA (the cytosol being the non-polysomal cytoplasmic compartment of the cell) (Harrison, P. R., Gilmour, R. S., Affara, N. A., Conkie, D. and Paul, J. 1974; Gilmour, R. S., Harrison, P. R., Windass, J. D., Affara, N. A. and Paul, J. 1974). To our surprise, in the two substrains we first studied, we found evidence for different mechanisms. In one, designated clone M2, we found low levels of messenger RNA in all three cell compartments in uninduced cells but, on induction with demethylsulphoxide, messenger RNA increased both in nucleus and cytoplasm, although the increase in the polysomes was considerably greater than that in the nucleus. This argues for transcriptional control (and possibly translational control) in this cell line. In the other substrain, designated line 707, however, we found that the increase in messenger RNA on induction was confined to the polysomes; there was no evidence of any increase following induction in nuclei of these cells. Hence, the evidence is strongly in favour of post-transcriptional controls. Further support for these conclusions came from experiments in which chromatin was isolated from the two different cells and transcribed with *E. coli* polymerase. It was found that the concentration of globin messenger RNA sequences in transcripts from chromatin from uninduced M2 cells was low and was much higher in transcripts from chromatin from induced M2 cells; in contrast, it was equally high in chromatin from both uninduced and induced 707 cells. Since clone M2 was originally isolated from the 707 cell population, we concluded that the original population had probably contained cells of M2 types in which both transcriptional and post-transcriptional controls and were rather tightly linked. We postulated that during the course of continuous culture, a cell had arisen in which transcriptional control had become relaxed. This cell had eventually extensively overgrown the original cell so that the culture had the characteristics of the variant, but sufficient of the original cells were present that we were able to pick one out by cloning. Whether this speculation is correct or not, these findings immediately suggested a much higher degree of variation among Friend cell clones than we might have expected.

This observation encouraged us to undertake somatic cell genetic studies with a view to elucidating the situation further (Paul, J. and Hickey, I. 1974). It was found that non-inducible variants could be isolated by culturing Friend cells continuously in DMSO. Those cells which differentiated failed to divide further and the population rapidly became overgrown by non-differentiating cells. We therefore attempted to isolate a number of lines of non-inducible cells, to characterise them and to fuse them with other cells. Inadvertently we had already obtained one non-

inducible cell line in the laboratory. This cell line, Fw, is therefore of different origin from the other non-inducible variants which will be referred to.

Using standard procedures, lines of cells were now developed which lacked certain of the salvage enzymes of nucleic acid synthesis, namely thymidine kinase (TK) and hypoxanthine-guanine phosphoribosyl transferse (HGPRT). TK^- cells and $HGPRT^-$ cells will not grow in HAT medium (containing hypoxanthine, aminopterin and thymidine) whereas wild-type cells will. However, if a TK^- $HGPRT^+$ cell is fused to a TK^+ $HGPRT^-$ cell, the hybrid will survive in HAT medium by complementation. Accordingly, we first prepared stocks of cells which were either TK^- or $HGPRT^-$ to facilitate the isolation of hybrids. In an early experiment, a hybrid cell was produced by fusing the non-inducible Fw cell line to an inducible Friend cell. The hybrid cell was found to be inducible although to a lesser degree than the parent. This experiment has now been repeated several times and the same result has always been obtained. It would seem, therefore, that in this fusion, inducibility is dominant and exhibits dosage effect.

Other non-inducible variants were produced by mutagenising the two different cell stocks and isolating variants in the presence of DMSO. Many resistant cell lines were isolated in this way but most of them reverted. The selection was, therefore, performed repeatedly until stable resistant cell lines were obtained. One of these was then fused to an inducible line. In this instance, the hybrid was non-inducible. This cross, therefore, provides evidence for transdominant repression.

The nature of the lesion in the Fw cell was further clarified by the finding that, while DMSO would not by itself induce haemoglobin synthesis, addition of haemin to the medium would. In this instance, it would seem that the defect may lie in the haem synthetic pathway.

Further evidence concerning the control steps was obtained by attempting to obtain hybrids between Friend cells and non-erythroid cell lines. In particular, a hybrid was obtained between an inducible Friend cell and a clone (Ly-T) of the L5178Y lymphoma cell. This cell never synthesises haemoglobin although, interestingly enough, very low concentrations of globin messenger RNA can be measured in nuclear RNA. The hybrid cell line proved incapable of synthesising haemoglobin, but it was found to contain quite significant amounts of globin messenger RNA in both nucleus and cytoplasm. Moreover, on treatment with DMSO, the concentration of globin messenger RNA molecules increased although there was little other evidence of erythroid differentiation. When this cell was treated with haemin as well as DMSO, it was then found that it could make haemoglobin. In other words, this hybrid behaved very much like the non-inducible Fw line which had arisen spontaneously from the original Friend cell line. The behaviour of this hybrid accentuates points which have been already made. Clearly, haemin releases a translational block. On the other hand, hybrid cells can make globin messenger RNA and the levels can be induced to higher levels with treatment with DMSO although this has little or no effect on haemoglobin synthesis in the absence of haemin.

Conclusions

Before attempting to draw conclusions from these results, it is necessary to consider the relationship between DMSO stimulation of the Friend cell and erythropoietin induction of mouse foetal liver cells. When mice are inoculated with Friend virus, there immediately ensues an acute condition called Friend disease which is a polycythemia. This occurs in hypertransfused mice which are producing no erythropoietin as well as in normal mice. In this disease, therefore, the normal erythropoietin machinery seems to be by-passed. Moreover, Friend cells are non-responsive to erythropoietin, confirming the suggestion that in these cells the normal process of erythropoietin regulation is in some way by-passed. It seems quite possible that the Friend cell represents a transformed cell closely related to the erythropoietin-sensitive cell.

It is very likely that in normal differentiation, erythropoietin is responsible for an early event, which results in the commitment of ESC to erythroid differentiation. Experiments with cultured foetal liver suggest that the continued presence of erythropoietin accelerates completion of maturation. In the course of normal erythropoiesis, it may be assumed that there is adequate production of haemin but this may be rate-limiting in many Friend cells and the DMSO effect may have to do with increasing haem availability.

The regulation of maturation of erythroid cells clearly involves a series of coordinated events, two of which have been considered here. One is the rate of transcription of the globin gene which appears to increase on induction of many inducible Friend cells. The other is the rate of translation for which there is evidence in all varieties of the Friend cell studied. That this may be an important mechanism *in vivo* is also suggested by the fact that globin messenger can be detected in large amounts in basophilic erythroblasts before haemoglobin synthesis is detectable.

Despite their sophistication, one is aware of the deficiencies of some of these methods. In particular, because the measurement of haemoglobin is insensitive, we have to be cautious about drawing hard and fast conclusions about some of these phenomena. Nevertheless, these studies have given us considerable insight into some of the probable mechanisms involved in differentiation and promise quite soon to yield a detailed understanding.

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