Induction by HMBA and DMSO of genes introduced into mouse erythroleukemia and other cell lines by transient transfection

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We have found rapid induction of various genes, including human globin genes, in response to hexamethylene bisacetamide (HMBA) and dimethyl sulfoxide (DMSO) in transiently transfected cells. In mouse erythroleukemia cells (MELCs), this effect is detected within 1 hr of exposure of the cells to inducer before the endogenous mouse globin genes are induced. It does not require protein synthesis and is reversed if the inducer is removed. This and other evidence suggest that the mechanism involves a change in activity of a factor intimately involved with transcription, probably as a result of post-translational modification. As such, it may represent an early triggering event in terminal differentiation, and its relevance to the expression of human globin genes in stable transfectants and to induction of the mouse globin genes is discussed. Other cell lines (K562 and NSO) also show this response, which may therefore involve a ubiquitous mechanism. We also found that HMBA depresses the expression of endogenous globin genes in K562, the opposite of this differentiation inducer's effect on MELC.

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Hexamethylene bisacetamide (HMBA) induces terminal differentiation in a variety of cell lines (Marks and Rifkind 1989). Of these, mouse erythroleukemia cells (MELCs), a virally transformed cell line that is blocked on the path to terminal erythroid differentiation, has attracted attention as a model for the study of both globin gene expression and terminal differentiation (Friend et al. 1971; Marks and Rifkind 1989). The potential for terminal differentiation is retained indefinitely by these cells and can be expressed by exposure to a variety of inducers. Among the changes in gene expression that accompany this process is the induced transcription of the mouse α - and β^{maj} -globin genes (Profous-Juchelka et al. 1983; Ganguly and Skoultchi 1985), seen only after a lag of between 12 and 24 hr (Salditt-Georgieff et al. 1984; Sheffery et al. 1984). The intervening time is occupied by a complex series of events that may include changes that are prerequisites for globin gene expression. For example, induction is followed rapidly by changes in inositol metabolites (Faleto et al. 1985) and in the expression of a number of proto-oncogenes (Ramsay et al. 1986). Constitutive expression of one of these proto-oncogenes, *c-myc*, can block terminal differentiation (Coppola and Cole 1986). In addition, cellular DNA undergoes a cyclical change in cytosine methylation (Razin et al. 1988). These early events occur at a time when the differentiation process requires the continued presence

of inducer, but after 8–12 hr, the cells reach a committed state, in which terminal differentiation continues, and the mouse globin genes are expressed in the absence of inducer (Gusella et al. 1976; Chen et al. 1982).

MELCs have provided a useful system for studies on the regulation of human globin genes. Introduction of part of human chromosome 11 or all of 16 into MELCs leads to induced expression of the β - or α -globin genes at levels comparable with those of the endogenous mouse genes (Deisseroth and Hendrich 1979; Nandi et al. 1988). Stable transfectants containing the human α - or β -genes give low levels of expression, and although the β -globin gene is inducible, the α -globin gene is constitutive (Chao et al. 1983; Wright et al. 1983, 1984; Charnay et al. 1984). The more recent identification of a dominant control region (DCR) sited at the 5' end of the β -globin gene cluster (Grosveld et al. 1987; Ryan et al. 1989b; Talbot et al. 1989; Tuan et al. 1989; van Assendelft et al. 1989) has allowed high levels of human β -globin expression, equal to those of the endogenous mouse genes, when it is linked to the human gene in MELC stable transfectants (Talbot et al. 1989). Although the DCR provides a tissue-specific level of regulation, it is developmental stage independent (Tuan et al. 1989), and regulatory sequences closer to the globin genes may be responsible for stage-specific expression (Kollias et al.

1986; Chada et al. 1987; Trudel et al. 1987). Regulatory sequences lying 5' and 3' close to the human β gene have been identified in stable MELC transfectants and in transgenic mice (Behringer et al. 1987; Kollias et al. 1987; Trudel et al. 1987; Antoniou et al. 1988). The involvement of both distant and local sequences in the regulation of the human α -globin genes remains more of an enigma. However, the ability of the β -globin cluster DCR to support high levels of tissue-specific expression of the α -globin gene in transgenic mice (Hanscombe et al. 1989; Ryan et al. 1989a) suggests the presence of an analogous sequence in the human α -globin gene cluster (Higgs et al. 1989). The question of involvement of sequences close to the α -globin genes remains more open.

We used electroporation to transfect MELCs transiently. Applied to the process of terminal differentiation, transient transfection may allow identification of individual events on the pathway to terminal differentiation and globin gene expression. We describe a rapid, labile stimulation of transcription of a variety of apparently unrelated genes and artificial promoters following exposure to inducer. This is independent of protein synthesis and is likely to involve post-translational modification.

Results

Dependence of expression on plasmid concentration

Preliminary experiments showed that the human α - and β -globin genes could be rapidly induced following transient transfection of MELCs. However, it was important to establish that their expression did not saturate and that induction did not vary over the range of plasmid concentrations used for electroporation.

Uninduced MELCs were transfected with the human α_1 -globin gene (pSPN α N) at a number of DNA concentrations and grown with or without inducer (5 mM HMBA) for 6 hr before harvesting (Fig. 1A). At input DNA concentrations where the human α -globin transcript was detected, induction was associated with a 10- to 20-fold increase in human transcript. As expected, there is no evidence for induction of the mouse α -globin genes by 6 hr after exposure to HMBA (Salditt-Georgieff et al. 1984; Sheffery et al. 1984). The accumulation of human α transcript was not related in a simple manner to the DNA concentration used for electroporation, and there was a threshold of 20–50 μ g/ml below which either transfection or expression appeared to be inhibited. A similar result was obtained with the human β -globin

Figure 1. Human globin gene expression following electroporation of MELCs at a number of plasmid concentrations. MELCs were electroporated in the presence of 5–200 μ g/ml pSPN α N (A) or 10–200 μ g/ml pSPN β N (B). Transfected cells were divided and grown with (+) or without (–) 5 mM HMBA for 6 hr (A) or 4 hr (B) before harvesting and RNA analysis. (A) Five micrograms of total RNA and samples of human (H) and mouse (Mu) bone marrow RNA hybridized with human [pSP6 α 132] and mouse [pSP64 Δ M α] α -globin probes gave the expected protected fragments, representing human (H α) and mouse (M α) α -globin mRNA. The molecular weight markers (M) in this and subsequent experiments are end-labeled *Hpa*II-digested pBR322. Below is shown the genomic DNA insert containing the human α_1 -globin gene in pSPN α N. (B) Twenty-microgram samples of total RNA and a sample of human bone marrow RNA (H) were hybridized with a human β -globin probe [pSP64H β 142] to give 142- and 210-bp fragments protected by human β mRNA (H β). Below is shown the genomic DNA insert containing the human β -globin gene in pSPN β N.

gene (pSPN β N; Fig. 1B). By 4 hr after exposure of transfected cells to inducer, the amount of human β transcript increased 10- to 20-fold over that in uninduced cells. As with the human α -globin gene, there was a threshold to expression between 20 and 50 μ g/ml of DNA.

Because this threshold could be explained by either specific or nonspecific binding of plasmid to sites that

inhibit transfection or expression, the effect of cotransfecting with carrier DNA was examined (Fig. 2). MELCs were transfected at concentrations of pSPN α N ranging from 2 to 100 μ g/ml, but with the total DNA concentration maintained at 100 μ g/ml by the inclusion of vector DNA (pSPNKN). Cells were transfected in duplicate at each pSPN α N concentration and grown in the presence (Fig. 2B) or absence (Fig. 2A) of inducer. Radioactivity in

Figure 2. Human α -globin expression following electroporation of MELCs at different concentrations of pSPN α N in the presence of carrier DNA. (A and B) MELCs were electroporated at a constant plasmid concentration of 100 μ g/ml of which 2–100 μ g/ml was made up of pSPN α N and the remainder of pSPNKN (plasmid vector). Duplicate transfections were performed for each pSPN α N concentration, and the transfected cells then divided and grown for 6 hr without (A) or with (B) 5 mM HMBA. Aliquots of 10 μ g (A) or 5 μ g (B) of RNA were analyzed with pSP6 α 132 and pSP64 Δ M α to give human α (Ha)- and mouse α (Ma)-protected bands. (C and D) The radioactivities in human α RNA-protected bands were divided by those in mouse α RNA (Ha/Ma), and these ratios were plotted against the concentration of pSPN α N used for electroporation. The results of this for uninduced (C) and induced (D) cells are shown. (E) The ratios (Ha/Ma) in induced cells were divided by the corresponding ratios in uninduced cells to give induction ratios for each pSPN α N concentration. (F) Total plasmid molarity (pSPN α N and pSPNKN) was calculated for each concentration of pSPN α N, and the ratio of human α RNA to mouse α RNA (Ha/Ma) was divided by this number to give a measure of pSPN α N expression per mole of plasmid, which was then plotted against pSPN α N concentration for induced transfectants. (G) MELCs were transfected as for A and B, but using pSPN α N Δ Eco (5.8 kb) and pJB8 (5.4 kb). Duplicate transfections were performed with 2 (lanes 1, 6), 8 (lanes 2, 7), 20 (lanes 3, 8), 50 (lanes 4, 9), and 100 (lanes 5, 10) μ g/ml pSPN α N Δ Eco, the total DNA concentration being maintained at 100 μ g/ml by the inclusion of pJB8. Shown is the analysis of 5 μ g of RNA for the presence of human (Ha) and mouse (Ma) α -globin transcripts in induced transfected cells. (H) The ratio Ha/Ma in G was plotted against pSPN α N Δ Eco concentration.

the protected human α RNA bands was expressed as a ratio to mouse α RNA for each DNA concentration (Fig. 2C,D). In both uninduced and induced cells, the amount of human α transcript increased linearly between 20 and 50 $\mu\text{g/ml}$, but the system appeared to saturate above these values. However, induction is consistent throughout the range of pSPN α N concentrations used (Fig. 2E). Thus, provided carrier DNA was present, induction was seen at pSPN α N concentrations as low as 2 $\mu\text{g/ml}$. At this concentration, the volume of an erythro-leukemia cell would be occupied by ~ 100 molecules of this plasmid. Because the DNA is in solution and complete equilibration of cell contents with the external medium is an unlikely consequence of electroporation, this figure is probably an upper limit for the copy number per cell.

If induction is an effect on transcription, then saturation for α -globin expression at similar concentrations of pSPN α N in both uninduced and induced MELCs could imply that induction does not affect binding of a rate-limiting factor but, rather, affects its ability to activate transcription once bound. However, pSPN α N is a 9-kb plasmid, whereas the vector with which it was cotransfected is 3 kb. Thus, although DNA concentration was maintained at 100 $\mu\text{g/ml}$, plasmid molarity declined as pSPN α N made up an increasing proportion of the DNA. If the amount of α -globin transcript in induced cells is corrected for total plasmid molarity and plotted against pSPN α N concentration (Fig. 2F), the relationship is linear. The same is true for uninduced cells (not shown). To test this relationship directly, cells were transfected with various concentrations of pSPN α N Δ Eco (5.8 kb, derived from pSPN α N and behaving indistinguishably from it; see Methods), together with pJB8 (5.4 kb) so that both the total DNA concentration and plasmid molarity were approximately constant (Fig. 2G). The amount of human α transcript was then linearly related to pSPN α N Δ Eco concentration (Fig. 2H), in which case expression was directly proportional to the number of copies of the transfecting gene and the total molar concentration of plasmid. Therefore, this system does not saturate for either expression or induction when up to 100 $\mu\text{g/ml}$ of plasmid containing the α -globin gene is used for electroporation, provided carrier DNA is present. The dependence on molarity, whether of pSPN α N or the related cosmid vector pJB8, would suggest that specific sequences in these plasmids affect either transfection or expression by binding with an inhibitor. We have excluded the possibility that plasmid sequences contribute to induction by inserting the human α -globin gene in the *NotI* site of the unrelated vector pCos6EMBL (Ehrlich et al. 1987; not shown). Linearization of plasmids before transfection also does not affect induction (not shown).

Time course of human α -globin expression following induction

The rapidity with which the human genes were induced following transfection and induction was surprising in

view of the lag of 12–24 hr characteristic of the response of the mouse globin genes. We therefore examined in more detail the accumulation of human transcript with time (Fig. 3). The mouse α -globin genes responded to induction with a small increase in transcript detected by 12 hr and an increase of 20-fold by 24 hr. The human gene, on the other hand, gave a detectable response by 1 hr after induction, and the amount of α -globin transcript increased rapidly to a maximum between 3 and 6 hr before declining. The same decline in expression is seen following transfection of MELCs with other genes (not shown).

The useful span of this transient assay is therefore limited to the first 3–6 hr following transfection. The subsequent decline in the amount of transcript may have any of a number of causes, including cell death, destruction of plasmid, or inhibition of transcription. The stability of globin mRNA in MELCs (Volloch and Housman 1981) makes RNA degradation a less likely cause. We analyzed isolated nuclei for the presence of plasmid following transfection and found no significant decrease in the amount of plasmid (not shown) and no evidence for the entry of plasmid DNA into nucleosomes (Enver et al. 1988). However, because we did not know what fraction of the DNA associated with the nuclei was transcriptionally active, we considered that these analyses would be difficult to interpret.

Dependence of expression on the presence of inducer and on protein synthesis

The rapid response to induction of the transfecting globin genes could be due to either a stable or unstable

Figure 3. Expression of the human α gene with time following transfection. MELCs were electroporated in the presence of 10 $\mu\text{g/ml}$ pSPN α N and 90 $\mu\text{g/ml}$ pSPNKN. The pool of transfected cells was divided and grown with (+) and without (-) 5 mM HMBA for the times shown. Five micrograms of RNA and samples of human (H) and mouse (Mu) bone marrow RNA were analyzed, by use of pSP6 α 132 and pSP64 Δ M α , to give protected fragments representing human (H α) and mouse (M α)-globin mRNA.

Rapid induction in transients

change in the cells. To distinguish between these alternatives, the effect on expression of removing inducer from induced transfected cells was examined (Fig. 4A, lanes 1–4). Two aliquots of cells transfected with pSPN α N were grown for 2 hr in the presence of HMBA. One was harvested (Fig. 4A, lane 2), and the other was grown for an additional 4 hr without inducer (Fig. 4A, lane 3). Both of these cultures contained the same amount of human α RNA and half as much as a culture grown with inducer for the full 6 hr (Fig. 4A, lane 4). Therefore, the induction response is reversed by removal of HMBA. The same conclusion was drawn from analogous experiments with cells transfected with the human β -globin gene. Thus, rapid induction of transient transfectants must be either a direct response to the inducer, which seems unlikely, or must involve an unstable intermediate.

When MELCs are exposed to inducer for 12 hr or longer, stable changes in the cells allow subsequent mouse globin expression even if the inducer is removed (Gusella et al. 1976; Chen et al. 1982). Longer exposures to inducer may therefore lead to commitment to expres-

sion of the transfecting genes, as well as the mouse genes. To test this, uninduced cells and cells grown with HMBA for 36 hr were cotransfected with SPN α N and pSPHAPRT and then grown with or without HMBA for an additional 6 hr (Fig. 4D). The plasmid pSPHAPRT contains the hamster APRT gene, which is expressed constitutively in both transiently transfected MELCs (shown here) and in stable MELC transfectants (Chao et al. 1983). The human α -globin gene was expressed at a similar level with respect to the APRT gene whether or not the cells had been exposed to HMBA before transfection. A similar experiment showed the same to be true of the human β -globin gene (Fig. 4D). Furthermore, irrespective of the time of exposure of the cells to inducer, sequences contained in the transfecting globin genes are not responsive to stable changes that allow the endogenous mouse genes to be expressed in the absence of inducer.

The rapid response of the human α -globin gene to induction and its lability suggested that de novo protein synthesis might not be required. Cells were transfected with pSPN α N, then divided and grown with or without

Figure 4. The dependence of induced expression of human globin genes on the presence of inducer, on protein synthesis, and on exposure of cells to HMBA prior to transfection. (A) MELCs were electroporated with 10 μ g/ml pSPN α N and 90 μ g/ml pSPNKN and grown without HMBA for 6 hr (lane 1), with 5 mM HMBA for 2 hr (lane 2), with 5 mM HMBA for 2 hr and, after harvesting, washing, and resuspension in fresh medium, for an additional 4 hr without HMBA (lane 3) or for 6 hr with 5 mM HMBA (lane 4). Transfected cells were also grown without (lanes 5 and 7) or with (lanes 6 and 8) 5 mM HMBA and without (lanes 5 and 6) or with (lanes 7 and 8) 1.5 μ g/ml cycloheximide for 6 hr. Five micrograms of RNA and samples of human (lane 9) and mouse (lane 10) marrow RNA were hybridized to give human (H α)- and mouse (M α)-protected fragments. (B) MELCs were transfected with 100 μ g/ml of pSPN β N and grown with (+) or without (-) 5 mM HMBA and with (Cyc) or without 1.5 μ g/ml cycloheximide for 6 hr. Twenty micrograms of RNA and an aliquot of human marrow RNA (H) were hybridized with a human β probe (pSP64H β 142), giving protected fragments representing the first and part of the second exon of human β mRNA (H β). (C) MELCs were transfected as in B, except that cells were electroporated with 100 μ g/ml pSPN γ N, and 20- μ g samples of RNA and an aliquot of human marrow RNA (H) were hybridized with the human γ probe (pSP65H γ 145) to give fragments protected by human γ RNA (H γ). (D) Cells grown without inducer (0 hr) or with 5 mM HMBA for 36 hr prior to transfection (36 hr) were electroporated with 75 μ g/ml pSPN β N and 75 μ g/ml pSPHAPRT (β) or with 10 μ g/ml pSPN α N, 75 μ g/ml pSPHAPRT, and 65 μ g/ml pSPNKN(α). The cells were then grown for an additional 6 hr with (+) or without (-) 5 mM HMBA. Ten-microgram samples of RNA were analyzed for the presence of human β mRNA (H β), and 5- μ g RNA samples were analyzed for human α RNA (H α) with pSP64H β 135, which protects most of the first exon of β mRNA, and pSP64 α 132, respectively. Aliquots of 0.25 μ g RNA were analyzed for the presence of mouse α -globin mRNA (M α) with pSP64 Δ M α , and 15- μ g aliquots were analyzed for hamster APRT transcripts (hAPRT). To correct for variation in transfection efficiency, radioactivities in human globin-protected fragments were divided by those in hAPRT. These ratios, for transfected cells grown with inducer, are shown below.

cycloheximide for 15 min before HMBA was added (Fig. 4A, lanes 5–8). Cycloheximide did not abolish induction, which therefore does not require protein synthesis. The human β - and γ -globin genes also induced in the presence of cycloheximide (Fig. 4B,C). If proteins mediate this induction response, they must do so by being reversibly modified.

Specificity of induction

To explore the responsiveness of genes other than the human globin genes, and to identify sequences that confer inducibility, we used constructs in which the chloramphenicol acetyltransferase (CAT) gene is driven by various viral promoters, or by the α - or β -globin promoters (Fig. 5). pMMTVcat contains the mouse mammary tumor virus long terminal repeat (LTR) (J. Devine, unpubl.), and pSrM2cat contains the Moloney murine sarcoma virus LTR (Laimins et al. 1982). pRSVcat contains the Rous sarcoma virus LTR (Gorman et al. 1982a), pSV2cat contains the early promoter from SV40 (Gorman et al. 1982b), and pAT153cat contains no eukaryotic promoter. MELCs were transfected with each of these constructs, and one of three such experiments, all giving similar results, is shown (Fig. 5A). None of these virally promoted CAT genes gave significant increases in expression on induction, though in subsequent experiments, we detected a small and reproducible response by pRSVcat. The largest of any increase was seen when cells transfected with pMMTVcat, which contains a glucocorticoid responsive element (Lee et al. 1981), were exposed to dexamethasone.

Constructs in which the α - and β -globin promoter regions replaced the early SV40 promoter in pSV2cat gave inducible but weak responses in similar experiments. Higher levels of expression were consistently obtained when the plasmid vector sequence was replaced by pUC13 (see Methods). pUC α cat, pUC β cat, and

pUCSV2cat contain the α - and β -globin, and early SV40 promoters. pUCcat contains no eukaryotic promoter. pIRV, which contains a β -galactosidase gene driven by the rat β -actin promoter (constructed by J. Morgenstern and H. Land, ICRF, London), was cotransfected with each construct (Fig. 5B). Both pUC α cat and pUC β cat were inducible, whereas pUCSV2cat was expressed constitutively. However, pIRV also responded to induction with a 5- to 10-fold increase in β -galactosidase activity. Thus, on the one hand, the APRT gene (Fig. 4D) and a number of viral promoters do not respond to induction, and on the other hand, α -, β -, and γ -(not shown) globin and rat β -actin promoters do. We show below that the herpes simplex virus-1 (HSV-1) thymidine kinase (TK) gene promoter is also inducible. These results demonstrate that the induction response is not globin specific.

Cis-acting sequences responsible for induction

To define further the *cis*-acting sequences responsible for induction, a series of deletions were made in pUC α cat by use of exonuclease III, and for deletions close to the CAAT box, a series of oligonucleotides were used to give deletions extending down to and through the CAAT box. The response of these constructs following transfection and induction was measured by both CAT and RNA analyses (not shown). Deletion of sequences from -1375 to -76 relative to the cap site gave a decrease in the level of expression similar to that described previously (Whitelaw et al. 1989), but induction remained unaffected. Removal of an additional 8 bp 3' to position -76 destroyed the CAAT box and caused the loss of measurable expression. Induction must therefore be mediated by sequences between an intact CAAT box and the translation initiation codon.

To eliminate the possibility that sequences between the α -globin cap site and the initiation codon are responsible for induction, a universal leader sequence (uls), de-

Figure 5. Expression of virally promoted and α - and β -globin-promoted CAT genes. (A) MELCs were transfected with 100 μ g/ml pMMTVcat (MMTV), pSrM2cat (SrM2), pAT153cat (AT153), pRSVcat (RSV), and pSV2cat (SV2). Transfected cells were grown for 12 hr, with (+) and without (-) 5 mM HMBA and, in one case, with 2 μ M dexamethasone (D). Shown below is the percent conversion of [14 C]chloramphenicol to its major acetylated derivative per milligram of protein (%). (B) MELCs were transfected with 75 μ g/ml of pIRV and 75 μ g/ml of each of the following: pUCcat (pUC); pUCSV2cat (SV2); pUC α cat (α); pUC β cat (β). Transfected cells were grown with (+) or without (-) 5 mM HMBA for 12 hr. Shown below is the percent conversion of [14 C]chloramphenicol per milligram of protein (%) and β -galactosidase activity in milliunits per milligram (mU/mg) of protein.

signed to be linked to a number of inducible promoters, was inserted into pUCSV2cat. Into this construct the minimal promoter elements of the α - and β -globin genes and the HSV-1 TK promoter were inserted (Fig. 6). These constructs preserve the normal spacing between the TATA box and a potential new cap site. Expression of these constructs was less than that of those containing the normal cap site and leader sequences, and this prevented the use of RNase protection analysis.

When transfections were performed in the absence of pIRV (Fig. 6A), the promoterless CAT control (pUCcat) gave low uninducible levels of CAT activity, whereas pUCSV2cat was expressed at a higher constitutive level (Fig. 6A). These relative levels of expression were seen consistently. pUC α ulscat Δ -76 lacks the normal cap site and leader sequence present in pUC α cat Δ -76 but still shows induction. However, its level of expression is reduced, probably as a result of changes to the normal

sequence around the cap site (Smale and Baltimore 1989). pUC β ulscat Δ -79 is also inducible, but again its expression is much reduced compared with pUC β cat Δ -371, which contains both the normal cap site and leader sequence and more sequence 5' to the CAAT box. pUCTKulscat was also induced. Thus, inducibility is conferred by sequences lying between or including the CAAT and TATA boxes of the α - and β -globin genes and upstream from the cap site of the HSV-1 TK gene.

Cotransfection of these constructs with pIRV affected induction (Fig. 6B). pIRV, itself, gave an induction ratio of ~ 10 -fold in all transfections. The more strongly expressed constructs, pUC α cat Δ -76 and pUC β cat Δ -371, remained inducible in the presence of pIRV, though less so than in its absence. The ulscat constructs, pUC α ulscat Δ -76, pUC β ulscat Δ -79, and pUCTKulscat, were then all constitutive, and the promoterless control construct, pUCcat, was inhibited on induction. This is pre-

human α -globin ULS CAT

Spe I actagGCCAGCCAATG AGCGCGCCCG GGCGGGCGT GCCCGCGCGC CCCAAGCATA AACCTGGCG CGCGggcgcg gcgtgactcc tagcttgagg ag

human β -globin ULS CAT

Spe I ccactaGTTG GCCAATCTAC TCCAGGAGC AGGGAGGGCA GGAGCCAGGG CTGGGCATAT AAGTCAGGGG cGCGcgccg acggtgact cctagcttgg cgag

HSV-1 TK ULS CAT

Xho I cctcgaggCTGCTTCATC CCCGTGGCCC GTTGCTCGCG TTGGCTGGC GGTGTCCCC GAAGAAATAT ATTGTCATGT CTTTAGTTCT ATGATGACAC AAACCCGCC

Eco RI CAGCGTCTTG TCATTGGCGA ATTGGAACAC GCAGATGCAG TCGGGCGGC GCGTCCAG GTCCACTTCG CATATTAAGG TGACGCGcg cgcgcgctg actcctagct

Figure 6. Expression of constructs containing minimal promoter regions and a common cap site and leader sequence. (A) MELCs were transfected with 100 μ g/ml pUCcat, pUC α ulscat Δ -76 (α uls), pUC β ulscat Δ -79 (β uls), pUCTKulscat (TKuls), pUCSV2cat (SV2), pUC α cat Δ -76 ($\alpha\Delta$ -76), or pUC β cat Δ -371 ($\beta\Delta$ -371), and the transfected cells were grown with (+) or without (-) 5 mM HMBA for 18 hr. The percent conversion of [14 C]chloramphenicol per milligram of protein was calculated for each sample, and the induction ratios for each transfection are shown below (ind. ratio). (B) MELCs were transfected as in A, except that 50 μ g/ml of pIRV was included in each transfection. Shown below are the induction ratios for CAT activity and β -galactosidase activity in milliunits per milligram (mU/mg) of protein. Also shown are the sequences of the promoter regions of the uls CAT constructs. CAAT and TATA boxes and some restriction sites are underlined. Nucleotides corresponding to those in the intact genes are in uppercase letters.

sumably due to competition imposed by the presence of pIRV, the effect being greatest on the most weakly expressed constructs.

Cell specificity of the induction response

This induction response is not specific for globin genes, and to determine its cellular specificity, MELCs, K562, a human erythroid cell line, and NSO, a mouse myeloma cell line, were transfected with pUCacat Δ -603 and grown with or without 5 mM HMBA or 1.4% dimethyl sulfoxide (DMSO). All three cell types showed a 10-fold induction of CAT with either inducer (not shown). Thus, the mechanism of induction is not cell-type specific.

Although the α - and β -globin genes were both induced in transiently transfected MELCs, expression of the α -globin gene was much greater than that of the β -globin gene, a situation similar to that in nonerythroid cells (Banerji et al. 1981; Mellon et al. 1981; Humphries et al. 1982; Treisman et al. 1983; Whitelaw et al. 1989). This suggested that the transiently transfecting globin genes gained no advantage from being expressed in an erythroid environment. Their expression was therefore compared with that of a construct containing a well-defined tissue-specific promoter and enhancer. Ig β consists of an immunoglobulin gene promoter fused to the body of the human β -globin gene and including the IgH enhancer downstream (see Methods; K. Weston, unpubl.). This construct would be expected to express well in a myeloma cell line and poorly in MELCs (Banerji et al. 1983; Grosschedl and Baltimore 1985; Picard and Schaffner 1985). MELCs and NSO cells were cotransfected with the α -globin gene and either the β -globin gene or Ig β (Fig. 7). To enable comparison of β -globin gene expression with that of the other constructs, five times as much pSPN β N was used for transfection and five times as much RNA was analyzed so that the β -globin signal was multiplied 25 times relative to those of the α -globin and Ig β genes. The α - and β -globin genes were induced in both cell lines and expressed at similar levels relative to each other, implying that the expression of the β -globin gene, like that of the α -globin gene, shows little regard for cell type in a transient expression system. This contrasts with the behavior of the Ig β gene in the same system. Its transcripts are undetectable in MELCs, compared with the α -globin gene, but it is expressed at a relatively high and nearly constitutive level in the myeloma cell line NSO, making its behavior comparable to that of strongly expressed virally promoted CAT constructs in MELCs (Fig. 5). The 5-kb BglII fragment containing the human β -globin gene that has been used in these experiments includes sequences implicated in tissue-specific expression in MELCs and transgenic mice (Charnay et al. 1984; Wright et al. 1984; Townes et al. 1985; Behringer et al. 1987; Kollias et al. 1987; Antoniou et al. 1988). These regulatory elements do not appear to confer on the β -globin gene the high-level tissue-specific expression conferred by immunoglobulin regulatory sequences.

The observation of induction in transiently trans-

Figure 7. Comparison of the induced expression of the α - and β -globin genes and of Ig β in MELC and a mouse myeloma cell line NSO. MELCs (MEL) or NSO cells were cotransfected with 10 μ g/ml pSPN α N and either 50 μ g/ml pSPN β N or 10 μ g/ml Ig β (see Methods), the total DNA concentration being made to 100 μ g/ml with pSPNKN. The transfected cells were then grown for 6 hr with (+) or without (-) 5 mM HMBA. 2.5 μ g (α , Ig β) or 12.5 μ g (β) of RNA were analyzed.

fected K562 cells suggested that the endogenous globin genes might also respond. K562 cells were therefore grown with hemin, an inducer of K562 (Rutherford et al. 1981), with HMBA, or with both inducers (Fig. 8). As expected, hemin induced both the α -globin and the embryonic ζ -globin genes. However, HMBA caused a dramatic reduction in the amount of both transcripts, irrespective of the presence or absence of hemin. Visual inspection of the cell pellets showed that the treated cells were virtually white compared with the yellow/orange color of untreated cells or the red of hemin-induced cells. Although growth was inhibited by HMBA, microscopy showed no evidence of extensive cell death. Therefore, as well as stimulating expression of constructs in transiently transfected K562, HMBA must also act at a regulatory step that is independent of that affected by hemin.

Discussion

We have shown that in transiently transfected MELCs, exposure to HMBA results in a rapid increase in expression of the human α -, β -, and γ -globin genes and HSV-1 TK and rat β -actin-promoted constructs. Although this response is not specific for globin genes, it does not extend to strong retroviral or viral promoters or to the hamster APRT gene and thus shows some template specificity. Replacement of the normal transcribed sequences in several inducible genes does not prevent induction, making changes in RNA processing, transport, or stability unlikely as an explanation and leading us to

Figure 8. Expression of the endogenous α - and ζ -globin genes in K562 cells following exposure to HMBA or hemin. K562 cells were grown without additions (lane 1) with 5 mM HMBA (lane 2) or 50 μ M hemin (lane 3), or with both inducers (lane 4) for 48 hr. Aliquots of 2.5 μ g of RNA were analyzed for the presence of α , ζ , and actin transcripts. The rat actin probe was also hybridized in the absence of K562 RNA (S).

conclude that induction acts at the level of transcription.

The response depends on the continued presence of inducer at both early and late times following induction. Furthermore, pre-exposure of transfected cells to cycloheximide shows that new protein synthesis is not required. These properties do not distinguish between a direct or indirect effect of inducer on transcription, but neither HMBA nor DMSO affect *in vitro* expression of the human α -globin gene in a MELC extract (P.L. Campbell and R.W. Jones, unpubl.); thus, we conclude that a direct effect is unlikely. We note that in the case of the human β - and γ -globin genes, but not the α -globin gene, cycloheximide, by itself, causes an increase in the accumulation of RNA and a superinduction when it is combined with HMBA. This superinduction phenomenon has been observed in a number of inducible systems (Greenberg et al. 1986; Sen and Baltimore 1986).

The regions of the human α - and β -globin promoters bounded by the CAAT and TATA boxes (pUC α ulscat Δ -76 and pUC β ulscat Δ -79; Fig. 6) are sufficient for induction. More recently (P.L. Campbell and R.W. Jones, unpubl.), we detected induction of correctly initiated transcript from constructs lacking functional promoter sequences other than the TATA box and cap site upstream from the rabbit β -globin structural gene and the SV40 enhancer (Westin et al. 1987; Muller et al. 1988). Thus, sequences upstream from the TATA box may not be required. This possibility is strengthened by a lack of correlation between upstream sequences and inducibility. For example, the CAAT boxes of the inducible HSV-1 TK promoter and the constitutive Moloney murine sarcoma virus LTR bind the same protein (Graves et al. 1986), and the HSV-1 TK promoter also shares functional SP1 sites (McKnight et al. 1984) with the noninducible SV40 early promoter (Dynan and Tjian 1983). This supports the view that induction acts at a

step intimately involved in transcription initiation and is compatible with the findings of Cowie and Myers (1988), who used DEAE-dextran to transfect MELCs transiently with constructs containing the mouse β -globin promoter. Mutations through this promoter affected expression and the extent of induction, but none abolished it.

The labile nature of the induction response and the absence of a requirement for protein synthesis suggest that it may involve secondary modification of a transcription factor, for example, by O-glycosylation (Jackson and Tjian 1988) or phosphorylation (Sen and Baltimore 1986; Sorger et al. 1987; Sorger and Pelham 1988; Yamamoto et al. 1988; Bagchi et al. 1989; Hiebert et al. 1989; Raychaudhuri et al. 1989). RNA polymerase, which is known to be phosphorylated (Cisek and Corden 1989; Lee and Greenleaf 1989), would be among the candidates for this role. Changes in protein kinase activity, observed as an early event in MELC differentiation (Marks and Rifkind 1989; Melloni et al. 1987), could provide the means of secondary modification, leading to increased binding of a factor intimately involved in transcript initiation. Constitutive promoters, such as those of retroviral LTRs, may differ from inducible promoters in not requiring this factor or they may not be rate-limited at the inducible step. Other early events in MELC induction, such as changes in the expression of a number of proto-oncogenes (Ramsay et al. 1986) and a cyclical change in DNA methylation (Razin et al. 1988), also do not require protein synthesis and may share a common transduction pathway with this induction response.

Although HMBA causes induction of transiently transfecting genes in MEL, K562 and NSO cells, the cellular consequences of induction differ according to the cell type. Although endogenous globin gene expression is switched on in MELCs, it is switched off in K562 cells. Loss of erythroid and monocytic markers, with a concomitant increase in expression of megakaryocytic markers, has been described following induction of K562 cells with phorbol 12-myristate 13-acetate (PMA) (Tabilio et al. 1983; Siebert and Fukuda 1985; Alitalo et al. 1988). The effect of HMBA on K562 cells may be similar to that of PMA, and we speculate that the apparatus of globin gene expression in K562 cells is unstable and that HMBA causes terminal differentiation at the expense of globin expression down a nonerythroid pathway dictated by the cell. Many transformed cell lines undergo terminal differentiation following exposure to HMBA (Marks and Rifkind 1989), and induction probably involves changes that are not specific to any one differentiation program, whereas the program itself is largely predetermined. The available evidence suggests that this may be so for globin expression in MELCs. Thus, in stably transfected cells, the DNase I-sensitive sites in the DCR show the same erythroid-specific pattern before and after induction (van Assendelft et al. 1989). Also, the erythroid-specific protein-binding patterns around the β -globin gene and the abundance of the erythroid transcription factor, GF-1, show little, if any,

change (de Boer et al. 1988; Wall et al. 1988; Tsai et al. 1989). Furthermore, regulatory events determining whether γ - or β -globin genes will express in MELC/human fetal erythroid cell hybrids occur before induction (Papayannopoulou et al. 1986), showing that mechanisms for regulating globin gene expression are already in place. Consequently, globin gene expression in MELCs may be blocked at steps that are not erythroid specific.

Changes detected in transient transfectants as a non-specific increase in the activity of the transcription apparatus could provide a positive component in the activation of cellular and stably integrated genes during MELC differentiation. If so, then one expectation would be of a correlation between the behavior of genes in transient and stable MELC transfectants. In stably transfected MELCs, the β - and γ -globin genes and the HSV-1 TK promoter have all been observed to induce, whereas the APRT gene is expressed constitutively (Chao et al. 1983; Wright et al. 1983; Charnay and Henry 1986). These observations correlate with the behavior of these genes in transiently transfected MELCs. On the other hand, constitutive expression of the human α -globin gene in stable transfectants (Charnay et al. 1984) appears to be at odds with its induction in transients. However, constitutive expression only implies that the rate-limiting step is constitutive and does not exclude the presence of an inducible step that may be rate-limiting in other circumstances, for example, in transient transfectants or when the gene is linked to a DCR. The variability in the induction response seen with stably integrated genes (Wright et al. 1983, 1984; Charnay et al. 1984) appears to be a function of the integration site, an effect that is abolished when the gene is linked to the β -globin DCR (van Assendelft et al. 1989). The effects on induction of both integration sites and globin local regulatory sequences (Antoniou et al. 1988) may be due to their binding of inducible factors, but these sequences may also determine whether or not a single inducible step acting elsewhere is rate-limiting and therefore measured.

Induction of stable transfecting genes and the mouse globin genes is delayed for 12–24 hr following exposure to inducer (Salditt-Georgieff et al. 1984; Sheffery et al. 1984). This delay indicates that the early stimulation of transcription seen in transient transfectants is not sufficient to cause increased expression of chromosomally integrated genes. This could be due to a requirement for de novo synthesis of transcription factors. Alternatively, the delay may be a function of chromosomal integration. For example, evidence exists that access of protein factors to DNA is inhibited by incorporation of DNA into chromatin (Emerson and Felsenfeld 1984; Workman and Roeder 1987; Workman et al. 1988; Cheng and Kelly 1989), and this would not apply to naked DNA introduced into the cell by transient transfection. Thus, proteins modified by induction may depend for access on cell-cycle events (Conkie et al. 1981), replication (Enver et al. 1988), or changes in DNA methylation (Razin et al. 1988).

A property of endogenous mouse globin gene induction is that it shows eventual commitment or inducer independence (Gusella et al. 1976; Chen et al. 1982). No data are available to determine whether the same is true of stably integrated inducible genes, but the transient response does not show this property. Thus, other events in the terminal differentiation process would be required to explain commitment.

The rapid induction response that we have described is of interest from several points of view. We provide evidence that the activity of a ubiquitous component of the cellular transcription apparatus is regulated by secondary modification. It is likely that its abnormal regulation by HMBA or DMSO has a normal counterpart that has a role in cellular regulation of transcription. Loss of this normal control may be related to the transformed phenotype and may contribute to the block on terminal differentiation. Thus, elucidation of this process may contribute to an understanding of the transformed cell phenotype, the requirements of the terminal differentiation process, and, in the case of MELCs, globin gene expression. To do this, we are attempting to identify the factor modified by induction by using an *in vitro* transcription system.

Methods

Cell culture

MELCs 707 B10/1 were grown in suspension in RPMI 1640 medium (GIBCO), supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 μ g/ml). To induce differentiation, HMBA (Sigma) was added to the medium at a final concentration of 5 mM. Alternatively, 1.4% DMSO was used. Cycloheximide was used at a concentration of 1.5 μ g/ml, sufficient to inhibit protein synthesis in MELCs by >95% (Kaneda et al. 1985), and dexamethasone at 2 μ M. A non-secretory mouse myeloma cell line (NSO) and the human erythroid cell line (K562) were grown under the same conditions as MELCs.

Transfection

MELCs were transfected by electroporation (Boggs et al. 1986; Chu et al. 1987). Cells were grown to a density of between 0.5×10^6 and 1.0×10^6 /ml. They were harvested, washed once with PBS, and resuspended in 1 ml of PBS containing, usually, 100 μ g of supercoiled plasmid DNA. The cell suspension was put into a 4-ml capacity plastic cuvette, into which were inserted two flat electrodes facing each other, 8 mm apart. A 1000 μ F capacitor array, charged at 425 volts from an LKB 2197 power supply, was discharged through the cells. After electroporation, the cells were resuspended in medium and aliquots were distributed between flasks to which any additions had already been made. The whole procedure was conducted at room temperature. NSO and K562 cells were electroporated in the same way, except that 400 volts was used.

Where necessary, a constitutively expressed plasmid (pSPHAPRT) was included as a control for transfection efficiency. Alternatively, variation in transfection efficiency was allowed for by duplication of the transfection. Transfection efficiency for one batch of cells generally varied within a two- to threefold range. Cells (4×10^7) transfected and grown for

various times provided between 50 and 100 μ g of total RNA, and multiples of this number of cells were electroporated to provide sufficient transfected cells for the number of experimental conditions.

Constructs used for transfection

The polylinker sequence of pSP65 was altered by use of standard methods to give the following sequence of restriction sites: *EcoRI*, *SacI*, *SpeI*, *SacII*, *SfiI*, *NotI*, *BamHI*, *KpnI*, *BamHI*, *XhoI*, *NotI*, *PstI*, and *HindIII*. Genomic DNA fragments containing globin genes were inserted into this vector (pSPNKN) in the same orientation, the 5' end of the gene being nearest the *EcoRI* site. A 6.0-kb *BglII*–*XhoI* fragment containing the human α_1 -globin gene from $\alpha 3'$ Bg [Nicholls et al. 1987] was inserted between the *BamHI* and *XhoI* sites of pSPNKN to give pSPN α N. Deletion between the *EcoRI* and *XhoI* sites 3' to the α gene in pSPN α N gave pSPN α N Δ Eco. Insertion of a 5-kb *BglII* fragment containing the human β -globin gene into the *BamHI* site of pSPNKN gave pSPN β N. pSPN γ N was made by insertion of the 3.3-kb *HindIII* fragment containing the human γ gene into the *BamHI* site of pSPNKN. pSPHAPRT was made by transfer of the hamster APRT gene in a 7.8-kb *HindIII* fragment [Lowy et al. 1980] into the *HindIII* site of pSPNKN.

Ig β is pV473 β GTIg3 [a gift from K. Weston (unpubl.)]. It consists of the mouse V_H promoter from pSV-V μ 1 [Neuberger 1983] fused to the human β -globin structural gene with the IgH enhancer inserted 3'.

Globin-promoted CAT constructs

pUC α cat and pUC β cat were made as follows. The *NcoI* sites in pSPN α N and pSPN β N were cut and made flush using mung bean nuclease, and a *HindIII* linker was inserted. *EcoRI*–*HindIII* fragments containing the respective promoter regions were inserted between the *AccI* and *HindIII* site of pSV2cat [Gorman et al. 1982a] after the *EcoRI* and *AccI* ends had been filled by use of Klenow DNA polymerase. The *SacI*–*BamHI* fragments containing a globin promoter contiguous with the CAT structural gene were transferred to the same sites in the pUC13 polylinker to give pUC α cat and pUC β cat. pUCSV2cat was made by cutting of pSV2cat [Gorman et al. 1982b] with *AccI*, filling of the ends with Klenow DNA polymerase, and transfer of an *AccI*–*HindIII* fragment between a filled *SpeI* and a *HindIII* site of pUC α cat, which thereby replaced the α -globin promoter with the SV40 early promoter. pUC β cat Δ –371 was made by deletion between the *NotI* site 5' to the promoter region in pUC β cat and an *AccI* site at position –371.

5' Deletion of the α -globin promoter region

pUC α cat Δ –603 was made by deletion between the *SpeI* site of the 5' polylinker and a *SpeI* site at –603 in pUC α cat. Exonuclease deletions were made by use of the *SpeI* and *SacI* sites 5' to the promoter of pUC α cat Δ –603 [Hoheisel and Pohl 1986].

Oligonucleotide-constructed α -globin promoter deletions were also made. Single-stranded oligonucleotides corresponding to the antisense strand of the α -globin promoter extended from the *EagI* site at –56 from the cap site to positions at and between –68 and –112 from the cap site. The 3' ends of these were abutted to a *SpeI* site and extended an additional 6 bp to form a terminal 18-bp palindrome. These oligonucleotides were made double-stranded by mutually primed synthesis [Hill et al. 1986], the products were digested with *SpeI* and *EagI*, and these fragments were inserted between the *SpeI* and *EagI* sites of pUC α cat Δ –603 to give pUC α cat Δ –68 to Δ –112 inclusive.

Constructs with a common cap site and leader sequence

A 31-mer oligonucleotide, 5'-CTAGAGGGCGCGCGGCC-GACGCGTGA Δ CTCCT-3', was annealed with a complementary 27-mer to give a double-stranded oligonucleotide with a blunt 3' end and a *SpeI* complementary 5' overhang. It also contained *BssHII*, *EagI*, and *MluI* sites to enable different promoters to be inserted, preserving the distance between the TATA box and the new potential cap site. The *HindIII* site in the pUC13 polylinker 3' to the CAT gene in pUC α cat Δ –76 was cut, filled in using Klenow polymerase, and religated. The resulting plasmid was cut at the *HindIII* site at the junction of the α promoter and the CAT gene, the ends were filled, and the plasmid was cut with *BssHII*. The double-stranded oligonucleotide (above) was cut with *BssHII*, and the resulting *BssHII*/blunt fragment was inserted between the *BssHII*/blunt *HindIII* sites of pUC α cat Δ –76 to give pUC α ulscat Δ –76.

A double-stranded oligonucleotide, spanning the CAAT and TATA boxes of the β -globin promoter, was made: 5'-CCACTAGTTGGCCAATCTACTCCCAGGAGCAGGG-AGGGCAGGAGCCAGGGCTGGGCATATAAGTCAGGGCGCGCCCA-3'. This was cut with *SpeI* and *BssHII*, and the resulting fragment was inserted between the equivalent sites in pUC α ulscat Δ –76 to give pUC β ulscat Δ –79.

pUCTKulscat was made by insertion of the promoter region of the HSV-1 TK gene into pUC α ulscat Δ –76. A 2-kb *XhoI*-linked *PvuII* fragment containing the HSV-1 TK gene [McKnight 1980] was made blunt-ended, by use of Klenow DNA polymerase, and cut with *MluI*. The 190-bp *MluI*/blunt fragment containing the HSV-1 TK promoter region was inserted between the *SpeI* site (filled) and the *BssHII* site of pUC α ulscat Δ –76 to give pUCTKulscat.

Plasmid growth and preparation

All plasmids were propagated by transformation of *Escherichia coli* RRI. Plasmid DNA was prepared by alkaline SDS lysis and purified by CsCl equilibrium centrifugation [Maniatis et al. 1982].

RNA preparation

A composite of existing methods was used [Auffray and Rougeon 1980; Maniatis et al. 1982]. MELCs were harvested, washed once with PBS, and homogenized in 10 ml of 6 M urea, 3 M LiCl for 1 min. After standing at 4°C overnight, the RNA was pelleted at 10,000 rpm in a Sorvall SS34 rotor. The pellet was dissolved in 0.5 ml of 6 M guanidinium thiocyanate, 10 mM Tris (pH 7.4), 0.5% Sarkosyl, 200 mg of CsCl dissolved in this solution, and 0.5 ml layered on 0.5 ml of CsCl, 1.7 g/ml, in 10 mM Tris (pH 7.4), and 10 mM EDTA. After centrifugation at 85,000 rpm in the Beckman TLA 100.2 rotor for 4.5 hr at 20°C, the supernatant was removed and the RNA was dissolved in 0.4 ml of 10 mM Tris (pH 7.4), 10 mM EDTA, and 0.1% SDS. The RNA was ethanol-precipitated and redissolved in 80% formamide hybridization buffer. The RNA was quantitated by measurement of the E_{260} of a diluted aliquot.

RNA analysis

RNA was analyzed by RNase protection with the SP6 system [Melton et al. 1984]. RNA recovery was monitored either by observation of cross-hybridization of the human probe to mouse sequences or by inclusion of a probe to cell RNA. Uniformly labeled RNA probes were made by use of [α - 32 P]GTP (>400 Ci/mmol, Amersham). RNA–RNA hybridization, with

5×10^5 cpm of radiolabeled probe and 0.25–20 μ g of total cellular RNA, was performed at 50°C for 18 hr. RNase digestion was carried out at 21°C for 30 min. The protected RNA fragments were run on a denaturing 8% or 12% polyacrylamide gel. End-labeled *HpaII*-cut pBR322 was used as molecular weight marker. Fuji RX medical X-ray film was exposed to the dried gels at –70°C with an intensifying screen. Quantitation was done by cutting out the radioactive bands, together with appropriate backgrounds, and the radioactivity was measured by scintillation counting with an LKB-Wallac Beta-plate counter.

SP6 probes

The 5' human α probe pSP6 α 132 (Charnay et al. 1984) and the 5' mouse α probe pSP64M α (Baron and Maniatis 1986) were gifts from Tom Maniatis. We used a derivative of pSP64M α (pSP64 Δ M α), made by deletion between a *BalI* site in the first exon and a mung bean nuclease-treated *EcoRI* site in the poly-linker sequence. This is protected by a 91-bp fragment from mouse α -globin mRNA. Two 5' human β probes were used. One a 555-bp *BamHI*–*BalI* fragment extending from +476 to –79 of the β -globin gene and cloned into pSP64 (pSP64H β 142; Kulozik et al. 1988), is protected by 142- and 210-bp fragments of the first and second exons of β mRNA. The other, a gift from Bernie Morley, is a 226-bp *BstNI* fragment protected by 135 bp of the first exon of the human β gene (pSP64H β 135). The 5' human γ probe is a 1502-bp *BstEII*–*HindIII* DNA fragment from +159 to –1343 of the human γ globin gene cloned in pSP65, giving a 145-bp protected fragment representing the first exon of the γ gene (pSP65H γ 145). The human ζ probe contains a 662-bp *EcoRI*–*BglII* fragment, overlapping the 5' end of the ζ -globin gene and inserted in pSP64, to give a 110-bp protected fragment. The 5' hamster APRT probe contains a 862-bp *HindII*–*BamHI* fragment overlapping the 5' end of the hamster APRT gene (Lowy et al. 1980) inserted between the *HindII* and *BamHI* sites of pSP64 (pSP64APRT). The actin probe contains a 276-bp *AvaI*–*HindIII* fragment inserted in pAM19, which protected the 76-nucleotide first exon of the rat β -actin transcript (Nudel et al. 1983). Cross-hybridization of this probe to human RNA was used as a control for RNA recovery. The 5' cat α probe was made by inserting the *SpeI*–*PvuII* fragment of pUCat Δ –112 between the *XbaI* and *HindII* sites of pSP64 (pSP64 α cat).

CAT assay

MELCs transfected with constructs containing the CAT gene were grown for 8–20 hr, harvested, and washed once with PBS. The cells were resuspended in 200 μ l of 0.25 M Tris (pH 7.4) and subjected to three cycles of freezing and thawing. Cell debris was removed by centrifugation, and aliquots of the supernatant, containing between 100 and 500 μ g of protein, were used for the assay of CAT activity (Gorman et al. 1982b). For each assay, 0.5 μ Ci of [¹⁴C]chloramphenicol (50 mCi/mmol, Amersham) was used. Following chromatography, the TLC plate was autoradiographed overnight, the positions of [¹⁴C]chloramphenicol and its acetylated derivatives identified, cut from the plate, and their radioactivity measured by scintillation counting. The protein content of cell extracts was measured by use of the Bradford colorimetric assay (Bradford 1976).

β -Galactosidase assay

β -Galactosidase was assayed by use of o-nitrophenyl- β -D galactopyranoside (Herbomel et al. 1984). β -Galactosidase (Sigma) was used as a standard, and activities were calculated as millounits per milligram of protein.

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