Cellular genomic reporter assays for screening and evaluation of inducers of fetal hemoglobin

Jim Vadolas¹, Hady Wardan¹, Michael Orford², Robert Williamson¹ and Panayiotis A. Ioannou¹,²,*

¹Cell and Gene Therapy Research Group, The Murdoch Childrens Research Institute, The University of Melbourne, Royal Children’s Hospital, Flemington Road, Parkville 3052, Melbourne, Australia and ²The Cyprus Institute of Neurology and Genetics, PO Box 3462, Nicosia, Cyprus

Received September 16, 2003; Revised and Accepted November 12, 2003

Reactivation of fetal hemoglobin (HbF) expression using pharmacological agents represents a potential strategy for the therapy of β-thalassemia, sickle cell disease, HbE and other β-hemoglobinopathies. However, the drugs currently available have low efficacy and specificity and are associated with high toxicity. We describe the development of stable cellular genomic reporter assays (GRAs) based on the green fluorescence protein (EGFP) gene under the Gγ-globin promoter in the intact human β-globin locus. We show that human erythroleukemic cell lines stably transfected with a Gγ-EGFP β-globin locus construct can maintain a uniform basal level of EGFP expression over long periods of continuous culture and that induction of EGFP expression parallels the induction of the endogenous globin genes. We compared the EGFP-induction potency of a number of chemotherapeutic agents, including histone deacetylase inhibitors and DNA-binding agents. We show that hydroxyurea and butyrate result in moderate levels of induction (70–80%) but with an additive inductive effect. Among the DNA-binding agents tested, cisplatin was the most potent inducer of HbF expression, (442 ± 32%), a level which is comparable to hemin (764 ± 145%). These results indicate that cellular GRAs containing Gγ-EGFP-modified β-globin locus constructs can be used to develop novel inducers of HbF synthesis for the therapy of β-hemoglobinopathies.

INTRODUCTION

Elevated levels of adult γ-globin chain synthesis have been shown to reduce disease severity in β-thalassemia and sickle cell disease through the formation of fetal hemoglobin (HbF, zδγ) or mixed hemoglobin tetramers (zβδγ). Pharmacological induction of HbF is therefore being investigated as a potential therapeutic procedure in patients with β-thalassemia and sickle cell anemia.

Since the first report describing the elevation of HbF by 5-azacytidine (1), several other compounds have been found to induce HbF. Recent clinical trials using hydroxyurea have shown that it can significantly reduce the need for blood transfusion in some β-thalassemia patients, while in sickle cell anemia patients it can ameliorate the clinical symptoms and dramatically reduce the frequency of pain crisis (2). Strong evidence in favor of a direct effect of hydroxyurea on HbF synthesis by the nitric oxide-dependent activation of soluble guanylyl cyclase has been recently reported (3). A separate group of compounds also under evaluation are the short chain fatty acids such as butyrate and its derivatives, which have been shown to up-regulate HbF in experimental in vitro models and in β-thalassemia and sickle cell anemia patients (4,5). As with 5-azacytidine and hydroxyurea, the level of HbF induction with butyrate has been extremely variable. Despite a number of clinical trials investigating the potential of HbF-inducing chemical agents, there are still continued concerns over the long-term use of such agents. Many of these drugs have low efficacy, specificity and cytotoxicity, while some are potentially carcinogenic. There is therefore an urgent need to identify new types of pharmacological agents that can induce HbF with greater efficacy and less toxicity (6–8).

A two-phase liquid culture assay using primary human erythroid cells has been commonly used to evaluate potential inducers of fetal hemoglobin. Measurement of γ-globin gene expression in such cultures by the RNase protection assay, globin chain biosynthesis, high-performance liquid chromatography (HPLC) and staining by γ-globin specific fluorescent antibodies have all been used to identify fetal globin gene inducers (9–13), but such assays are not suitable for screening

*To whom correspondence should be addressed. Tel: +61 383416232; Fax: +61 393481391; Email: panos.ioannou@mcri.edu.au

Human Molecular Genetics, Vol. 13, No. 2 © Oxford University Press 2004; all rights reserved
large numbers of agents. More recently, an assay based on a dual luciferase construct in murine erythroleukemia GM979 cells has been reported (14). Simultaneous monitoring of expression of renilla luciferase, under the β-globin promoter, and of firefly luciferase, under the α-globin promoter, driven by a LCR cassette in mixed stable cultures, was used to examine the efficiency of various agents to preferentially induce γ-globin gene expression. A marked variability in induction was observed on different days due to the instability and heterogeneity of the mixed culture system and the lack of many endogenous regulatory sequences from the reporter construct, leading the authors to suggest that a Yeast Artificial Chromosome (YAC) based assay system may be necessary to overcome such limitations. Instability of reporter gene expression with various reporter gene constructs has also been reported in gene transfer experiments, designed to study globin gene expression (15,16). Host cells transduced with GFP-retroviral vectors, or stably transfected with GFP-containing plasmids carrying few, if any, of the endogenous regulatory elements, have been shown to express GFP for up to 2–4 weeks. Moreover, continuous culture often results in interrupted or variegated levels of gene expression (17,18). The molecular basis by which transgene expression is decreased is not totally clear, although randomly integrated minigenes are certainly subject to regulatory constraints imposed by flanking genomic sequences. In contrast, the intact β-globin locus has been shown to be isolated from such position effects and to produce regulated levels of globin gene expression in transgenic animal models (19–21). However, difficulties in the isolation, manipulation and transfection of the intact β-globin locus have limited its use in cellular transfection studies.

We have previously generated and characterised pEBAC/148β, a eukaryotic bacterial artificial chromosome clone carrying the intact β-globin locus, and developed the GET Recombination technology for its precise experimental modification in bacterial cells (22–24). We have shown in transgenic studies that this genomic fragment can rescue the embryonic lethality of homozygous knockout embryos, demonstrating that it contains all the elements for regulated expression of the human γ- and β-globin genes in transgenic mice (Vadolas et al., in preparation). We have used EGFP-modified β-globin locus constructs with the same genomic fragment to develop episomal genomic reporter assays (GRAs) in human erythroleukemia cells (25). These episomal assays offer significant advantages for studying expression from the β-globin locus, but are not convenient for high throughput screening due to the continuous requirement for antibiotic selection, the variation in the proportion of expressing cells, the heterogeneity in the cell population with respect to episome copy number, and the likelihood of random integration of the β-globin locus construct after long periods of selection.

In this study, we describe the development of cellular GRAs that employ human erythroleukemic cells stably transfected with constructs that contain the EGFP reporter gene under the control of the Gγ-globin gene promoter in the human β-globin locus. We demonstrate with these GRAs that hemin is a potent inducer of EGFP expression driven by the Gγ promoter and that the induction parallels that of endogenous globin chain synthesis, resulting in the accumulation of EGFP to 5–10% of the total cellular protein. We compared the EGFP-induction potency of a number of chemotherapeutic agents, which include histone deacetylase (HDAC) inhibitors, and DNA-binding agents. We demonstrate a direct effect of hydroxyurea on γ-globin synthesis, which is comparable to butyrate, and an additive effect when both agents are used. Among the DNA-binding agents tested, cisplatin was found to be the most potent inducer of EGFP, approaching a level that is comparable to hemin induction. We propose that these GRAs represent sensitive and physiologically relevant assays for the screening and initial evaluation of γ-globin gene inducers that should greatly facilitate a rational approach to drug design and development for the treatment of β-hemoglobinopathies. The accumulation of EGFP to 5–10% of the total cellular protein also indicates that this approach may be very useful for the high level production of recombinant proteins in human erythroid cells.

RESULTS

Generation and characterization of stable KEB clones

Under normal growth conditions, K562 cells express low amounts of ε-globin and γ-globin. Hemin, as well as a variety of other compounds, induces erythroid differentiation, which results in a sharp increase in embryonic and fetal globin gene expression (26–28). Hence this cell line has been investigated as an in vitro model system to study globin gene regulation and to identify and evaluate γ-globin inducers (29–31). The advantages and limitations of this cell line for the screening and evaluation of HbF inducers have been previously reviewed (25,32).

We previously reported that transient transfection studies in K562 cells with 21 kb (pEBAC160G) and 205 kb (pEBAC/148β) vectors yielded low levels of transfection (4 and 0.5% respectively) (25), imposing serious limitations on functional studies with the intact β-globin locus. To overcome these limitations KEB cell lines were generated by transfecting an EBNA1/Neo expression cassette into K562 cells (33), since previous studies have reported up to 100-fold increase in the transfection efficiency of oriP-containing plasmids with EBNA1 expression (34). Twenty-two out of 30 KEB clones appeared to be homogeneous for EBNA1 expression by FACS analysis after limiting cell dilution. In agreement with previous studies, an increased transfection efficiency was noted with all KEB clones using the 21 kb pEBAC160G vector, but there was only a weak correlation between the level of EBNA1 expression and the percentage of EGFP expressing cells (data not shown), indicating that low levels of EBNA1 expression are sufficient to facilitate increased transfection efficiency. A single KEB clone, denoted as 8.13, which displayed moderate levels of EBNA1 expression and high transfectability with pEBAC160G, was chosen for further studies.

Generation of stable KEB cell lines expressing EGFP-modified β-globin locus constructs

We could not generate K562 cells stably transfected with the EGFP-modified β-globin locus constructs (Fig. 1), due to the very poor transfection efficiency of large genomic fragments in K562 cells. In contrast, transfection of KEB cells with
linearized EGFP-modified β-globin genomic fragments followed by hygromycin selection readily led to the isolation of stable EGFP expressing clones. Since the hygromycin resistance gene and the oriP element are located on the vector backbone that was physically separated from the genomic insert in these transfection studies, it is presumed that transfection with the genomic insert was facilitated by its compaction and co-transfection with the vector backbone. However, EBNA1 has also been reported to increase reporter gene expression by affecting other intranuclear mechanisms (34), indicating that the increased efficiency for generating stable clones with the β-globin locus fragment in KEB cells may result not only from enhanced nuclear uptake, but also from effects on other intranuclear processes.

The EGFP expressing clones were isolated by limiting cell dilution in 96-well plates following 2–3 weeks culture in the absence of drug selection. Several independent clones expressing EGFP were selected for both the pEBAC148β::Gγ-Aγ EGFP (clones 1–13) and pEBAC148β::Gγ-β EGFP (clones 14–15) genomic reporter constructs (Table 1). Independent clones exhibited a range of EGFP expression profiles, with differences both in the proportion of EGFP-expressing cells, as well as in the basal median peak fluorescence (MPF) of the expressing cells (Fig. 2, Table 1). About 50% of the clones had >95% expressing cells that persisted after re-isolation by limiting cell dilution (data not shown), while the remaining clones had a mixture of expressing and non-expressing cells. No significant variation in the MPF of individual clones was observed over a period of at least one month of continuous culture, indicating a much greater stability of expression than with small plasmid constructs. Variations in MPF between different clones may reflect the number of intact copies of the β-globin locus fragment that are integrated in each clone, while the variation in the percentage of EGFP expressing cells may primarily reflect difficulties in dispersion of cell clumps prior to limiting cell dilution. However, the reported chromosomal heterogeneity of K562 cells (35) and position-of-integration effects may also contribute to this heterogeneity, although transgenic studies with the same β-globin locus fragment (Vadolas et al., in preparation) do not support the existence of large position-of-integration effects with this fragment. Further studies are necessary to understand the relative contribution of the different mechanisms to the observed heterogeneity, but for the purposes of this study we have focused on clones showing uniform EGFP expression in practically all the cells in each culture.

A characteristic property of K562 cells is their inducibility with hemin to undergo hemoglobinization. The responsiveness of individual KEB clones to hemin induction was therefore evaluated by measuring the percentage increase in MPF above that of the uninduced cells. The percentage of EGFP-positive cells before and after induction was also measured (Table 1). In contrast to the episomal cultures, no significant increase was noted in the percentage of EGFP positive cells.
Table 1. EGFP expression from stable $\gamma\beta$-globin genomic reporter constructs, before (H0) and after induction with 100 $\mu$M hemin (H100). Clone 12 generated with pEBAC/148$\beta$-globin genomic reporter construct and clone 14 generated with pEBAC/148$\beta$-globin genomic reporter construct were selected for further studies. The induction levels of KEB cells containing episomal vectors pEBAC/148$\beta$-globin genomic reporter construct into KEB cells were isolated by culturing transfected cells for 48 h, followed by hygromycin (400 $\mu$g/ml) selection for an initial period of 1 week. Clonal cells were isolated using limiting cell dilution in the absence of hygromycin for a further 2-3 weeks. The percentage of EGFP positive cells and relative levels of EGFP expression (MPF) were measured by flow cytometry.

<table>
<thead>
<tr>
<th>Clone</th>
<th>EGFP (MPF)</th>
<th>MPE, percentage</th>
<th>Percentage EGFP-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H0</td>
<td>H100</td>
<td>Increase</td>
</tr>
<tr>
<td>Stable clones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>23</td>
<td>137</td>
<td>496</td>
</tr>
<tr>
<td>2</td>
<td>312</td>
<td>1080</td>
<td>246</td>
</tr>
<tr>
<td>3</td>
<td>88</td>
<td>245</td>
<td>178</td>
</tr>
<tr>
<td>4</td>
<td>154</td>
<td>610</td>
<td>296</td>
</tr>
<tr>
<td>5</td>
<td>49</td>
<td>250</td>
<td>410</td>
</tr>
<tr>
<td>6</td>
<td>151</td>
<td>333</td>
<td>120</td>
</tr>
<tr>
<td>7</td>
<td>57</td>
<td>213</td>
<td>274</td>
</tr>
<tr>
<td>8</td>
<td>232</td>
<td>730</td>
<td>215</td>
</tr>
<tr>
<td>9</td>
<td>27</td>
<td>186</td>
<td>589</td>
</tr>
<tr>
<td>10</td>
<td>28</td>
<td>404</td>
<td>1343</td>
</tr>
<tr>
<td>11</td>
<td>49</td>
<td>234</td>
<td>378</td>
</tr>
<tr>
<td>12</td>
<td>242</td>
<td>2054</td>
<td>749</td>
</tr>
<tr>
<td>13</td>
<td>76</td>
<td>275</td>
<td>262</td>
</tr>
<tr>
<td>14</td>
<td>126</td>
<td>922</td>
<td>632</td>
</tr>
<tr>
<td>15</td>
<td>92</td>
<td>431</td>
<td>369</td>
</tr>
<tr>
<td>Episomal cultures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>147</td>
<td>741</td>
<td>404</td>
</tr>
<tr>
<td>B</td>
<td>212</td>
<td>1260</td>
<td>494</td>
</tr>
</tbody>
</table>

Stability of EGFP expression in $\beta$-globin genomic reporter assays

Initial studies indicated stability and uniformity in MPF of all clone isolates for at least one month of continuous culture (data not shown). To further extend these studies the two stable cell lines established with the pEBAC/148$\beta$-globin genomic reporter construct were monitored by flow cytometry during exponential growth in the absence of antibiotic selection (Fig. 3). It should be noted that the basal MPF for both clones under these conditions was at least 2-fold higher than under our standard hemin induction conditions (Table 1). This may reflect the turnover of EGFP while slowing down protein synthesis by the fifth day of the hemin induction protocol, in comparison to high levels of fresh protein synthesis in exponentially growing cells. Initially both cell lines had a very high percentage of EGFP expressing cells (>98%) up to 110 days of continuous culture. On further culture, expression remained constant for the $\gamma\beta$-EGFP cell line for at least 230 days, while the percentage of EGFP-expressing cells for the $\gamma\beta$-EGFP clone started to decline, falling to only 10% of the cells by day 170 (Fig. 3) and to undetectable levels by day 190 (data not shown). This decline was not associated with a reduction in viability or in the MPF of the expressing cells and was also accompanied by loss of hygromycin resistance, indicating non-specific chromosomal loss as the most likely mechanism for the ‘silencing’ of EGFP expression in this culture. Stocks of both cell lines from earlier passages can thus be grown and expanded for long periods with a high level of stability and uniformity in reporter gene expression, a feature that should greatly facilitate their use in high throughput screening for novel HbF inducers.

Induction of EGFP expression in the $\gamma\beta$-EGFP GRAs parallels the induction of the endogenous globin genes

Unlike small plasmid reporter constructs used by other workers to study globin gene expression and to develop screening assays (14), the $\gamma\beta$-EGFP genomic reporter constructs used in this study were designed to maintain the integrity of the LCR and
all other regulatory elements in the \( \beta \)-globin locus upstream of the \( \gamma \)-globin start codon (Fig. 1). EGFP expression from these constructs in stable cell lines should therefore reflect the endogenous activity of the \( \gamma \)-globin genes. In order to examine this possibility, total cellular proteins from K562, KEB and KEB-\( \gamma \)-\( \lambda \)-\( \gamma \) EGFP cells, before and after induction with hemin, were analyzed by polyacrylamide gel electrophoresis (Fig. 4). A 16 kDa band corresponding to all the globin chains was clearly seen to increase after hemin induction in the K562 and KEB cells, with a reduction in other higher molecular weight proteins, as expected during erythroid differentiation of K562 cells. Similar analysis of KEB-\( \gamma \)-\( \lambda \)-\( \gamma \) EGFP cells before and after hemin induction showed a higher basal level of globin gene expression and a sharp increase in the globin band on hemin induction, as well as a similar increase in a band corresponding in size to the recombinant EGFP protein. It is estimated that the EGFP band after hemin induction corresponds to about 5–10% of the total cellular protein and amounts to about half the intensity of the globin band. These results therefore demonstrate that EGFP expression from our genomic reporter constructs mirrors the high level of \( \gamma \)-globin gene expression after hemin induction. In addition, these studies also show that it is possible with this approach to harness the unique capacity of the intact \( \beta \)-globin locus to drive the production of high levels of recombinant proteins in human erythroleukemia cells.

**Evaluation of known fetal hemoglobin gene inducers with the \( \gamma \)-\( \lambda \)-EGFP GRA**

Since the \( \gamma \)-\( \lambda \)-EGFP clonal cell line maintained uniform basal levels of EGFP expression and a very high percentage of EGFP expressing cells for over 230 days, it was used to examine \( \gamma \)-globin gene induction by various agents previously reported to induce HbF expression in cell cultures or in vivo. A range of concentrations were tested for each agent overlapping with previously reported effective concentrations. Induction with hemin was included in each separate experiment as a positive control, to assess the uniformity and reproducibility of our results. Treatment with hemin (0–100 \( \mu \)M) for up to 5 days followed by flow cytometry caused a large dose-dependent shift in the MPF of EGFP-expressing cells, reaching a maximum 764 ± 145% increase with hemin at 100 \( \mu \)M after 5 days (Fig. 5). This increase is generally in line with the increase in EGFP expression seen directly by protein analysis (Fig. 4) and is quantitatively and qualitatively in general accordance with the reported increase in the expression of the \( \gamma \)-globin genes that occurs in K562 cells following hemin induction (26–28).

We further investigated the responsiveness of the \( \gamma \)-\( \lambda \)-EGFP cell line to hydroxyurea (0–100 \( \mu \)M) or butyrate (0–1000 \( \mu \)M) for up to 5 days. As with hemin, we noted a significant dose-dependent shift in the MPF of EGFP-expressing cells following induction (Fig. 5). Maximum induction (75 ± 10%) with hydroxyurea was observed at the highest dose tested (100 \( \mu \)M) after 5 days of induction. Similarly, maximal induction with butyrate (82 ± 30%) was observed after 5 days with the highest dose tested (1000 \( \mu \)M). Interestingly, combination of hydroxyurea and butyrate at the highest doses tested (100/1000 \( \mu \)M) for 5 days resulted in a 159 ± 72% increase in MPF, indicating an additive effect between these agents. Very similar results were also obtained using an early passage stock of the \( \gamma \)-\( \lambda \)-EGFP GRA after induction with hemin, hydroxyurea and butyrate (data not shown).
We compared the EGFP-induction potency of a number of other HDAC inhibitors with the G\(\gamma-A\)G EGFP modified β-globin locus. Total protein lysates from K562 cells, KEK cells, and K562 cells stably transfected with the pEBAC/148β::G\(\gamma-A\)G EGFP construct were separated by SDS-PAGE analysis and electrophoresed on a 15% polyacrylamide gel. Separated proteins were directly visualized by staining with Coomassie Blue. Lane 3, Biorad Low Range Prestained molecular weight markers; lanes 2 and 9, 0.5 μg EGFP marker (Clontech); lanes 3 and 4, non-induced and hemin-induced K562 cells; lanes 5 and 6, non-induced and hemin-induced KEK cells; lanes 7 and 8, non-induced and hemin-induced K562 cells stably transfected with the G\(\gamma-A\)G-EGFP construct.

We also compared the EGFP-induction potency of three commonly used chemotherapeutic agents carboxatin, carboxatin, and mithramycin, which have previously been reported to induce γ-globin (29,39). We noted that carboxatin and mithramycin displayed similar levels of induction (42±2% and 38±7%, respectively) when used at concentrations with maximum activity (Fig. 6, Table 2). In contrast, carboxatin induced a dramatic increase in EGFP expression (442±32%), which is comparable to hemin induction (764±32%).

**DISCUSSION**

Many studies focusing on the development of assays to evaluate HbF inducers have utilized small plasmid constructs with minimal regulatory elements from the β-globin locus. However, removal of the globin genes from their natural control elements, followed by random chromosomal integration, usually leads to poor and variegated transgene expression, often followed by transcriptional silencing. These events are usually the result of positional effects influenced by the site of chromosomal integration (40–42). Attempts to overcome positional effects have focused on the inclusion of LCR elements since a number of studies with the intact genomic β-globin locus indicate that it is less susceptible to positional effects and also more likely to give physiologically relevant responses. However, limitations in the genetic engineering and low transfection efficiencies have limited until now the development of reporter assays with the intact β-globin locus. To the best of our knowledge, this is the first report utilizing a fully functional β-globin locus for the development of stable genomic reporter assays in a erythroleukemia cell line.

We have observed a remarkable stability and uniformity in EGFP expression with our cellular GRAs (Fig. 3), extending to over 230 days of continuous culture with the G\(\gamma-A\)G EGFP clone and 110 days with the G\(\gamma-B\)G EGFP clone. In the latter case, ‘silencing’ of EGFP expression in the absence of selection was accompanied by loss of hygromycin resistance from the vector cassette, indicating non-specific chromosomal instability as the most likely explanation of silencing EGFP expression. Our results contrast with the high instability and variability of mixed stable cell cultures that have been previously reported with a dual luciferase assay for HbF expression (14).

We have demonstrated that the induction in EGFP synthesis with the G\(\gamma-A\)G EGFP GRA parallels the induction of the endogenous globin genes, reaching 5–10% of the total cellular proteins after 5 days of induction with hemin (Fig. 4). It is therefore possible with this approach to drive the expression of very high levels of recombinant proteins from the human β-globin locus in human erythroleukemia cells. Placing the therapeutic gene at the start codon of the e-globin gene with deletion of all downstream sequences should drive even higher levels of expression due to removal of competition of downstream promoters for binding to the LCR, as suggested by our studies with various globin-EGFP constructs in episomal format (25). Alternatively, the incorporation of a globin locus construct with a therapeutic gene placed at the start codon of the β-globin gene into a small proportion of primary hemopoietic stem cells may still enable the in vivo synthesis and release of sufficient levels of therapeutic proteins into the circulation of patients as a form of enzyme replacement therapy for a number of genetic conditions.

This study examines for the first time, in a side-by-side comparison, the relative capacity of several previously described HbF inducers to specifically activate γ-globin gene expression in a human erythroid cell line. Hemin reproducibly induced a strong, dose-dependent increase in MPF (764±145%; Table 2), and was used as an internal control in different experiments. In contrast, butyrate and pyruvate each induced only a 70–80% increase at the highest concentrations used, but had an additive effect (159±72%). This additive effect is in line with previous clinical trials in primates and in a few patients (43,44), confirming that they act through different mechanisms. Mechanistically, butyrate has been shown to inhibit histone deacetylase (45), thus affecting nucleosome stability, chromatin structure and histone binding to DNA (46,47). Such pleiotropic effects appear to be important in the transcriptional regulation of genes in general (31,46). As far as pyruvate is concerned, it irreversibly inhibits DNA synthesis in rapidly dividing cells by inhibiting ribonucleotide reductase, an enzyme required for DNA synthesis (48,49). However, the induction of γ-globin synthesis by pyruvate appears to be the direct effect of the nitric oxide-dependent activation of soluble guanylyl cyclase (3).

Several research efforts have been directed towards the development and identification of new γ-globin inducers, in particular towards butyrate derivatives and novel HDAC inhibitors. We compared the EGFP-induction potency of a number of HDAC inhibitors (Table 2). At concentrations with

---

**Figure 4.** EGFP and globin synthesis in KEK cells stably transfected with the G\(\gamma-A\)G EGFP modified β-globin locus. Total protein lysates from K562 cells, KEK cells, and KEK cells stably transfected with the pEBAC/148β::G\(\gamma-A\)G EGFP construct before and after induction with hemin (75 μM) for 5 days were isolated for SDS–PAGE analysis by sonication. Cell lysate was resuspended in Laemmli buffer and boiled for 5 min prior to loading 5 μg of protein per lane on a 12% polyacrylamide gel. Separated proteins were directly visualized by staining with Coomassie Blue. Lane 1, Biorad Low Range Prestained molecular weight markers; lanes 2 and 9, 0.5 μg EGFP marker (Clontech); lanes 3 and 4, non-induced and hemin-induced K562 cells; lanes 5 and 6, non-induced and hemin-induced KEK cells; lanes 7 and 8, non-induced and hemin-induced KEK cells stably transfected with the G\(\gamma-A\)G-EGFP construct.
maximum activity, HDAC inhibitors such as \( \alpha \) methylhydrocinnamic acid, sodium-2,2-dimethylbutyrate and trichostatin A were found to be relatively weak inducers. Thus the reported increase in HbF by these agents may not be primarily at the level of \( \gamma \)-globin gene transcription, a result which is consistent with the more generalized effects of \( \alpha \) methylhydrocinnamic acid and sodium-2,2-dimethylbutyrate (36). Apicidin has also been reported to be a more potent inducer of HbF expression than butyrate (38). However, in our assay apicidin increased EGFP expression to a level comparable with butyrate and hydroxyurea, suggesting that it may also act through other mechanism(s).

One of the most interesting classes of chemotherapeutic agents which have recently been the subject of a number of in vitro studies, are the DNA-binding agents cisplatin, and carboplatin. The platinum atom of these compounds forms intrastrand covalent linkages at neighbouring guanine bases through the N7 position of guanine. Such DNA-binding drugs display sequence specificity and are able to interfere with the DNA-binding activity of transcription factors. Enhanced reactivity of cisplatin at a CACC site in the \( \gamma \)-globin promoter in K562 cells was recently demonstrated (50), a site where FKLF , a member of the Sp1 transcription factor family of proteins, is thought to bind (51). In this study, we found that carboplatin induced low levels of EGFP expression (43/2/56 ± 7%), while cisplatin resulted in a level of induction (442/32/32%), which is comparable to hemin (764/145%; Fig. 6, Table 2). Our observations are in line with a report that carboplatin and cisplatin induce the expression of \( \gamma \)-globin and the erythroid differentiation of K562 cells, although no significant difference between the two agents was observed by measuring the number of benzidine positive cells (29). In this context it is important to note that cisplatin and carboplatin form identical bifunctional adducts, but the relative frequency of individual platinum-DNA cross-links and pharmacokinetic properties are quite different (52–54). This difference, together with the enhanced sensitivity and specificity of our assay compared to benzidine staining (29), may explain the large

Table 2. Comparison of \( \gamma \)-globin inducers with the \( \gamma \)-A\( \gamma \) EGFP genomic reporter assay. The table summarizes the EGFP-induction potential of chemotherapeutic agents examined in this study with the \( \gamma \)-A\( \gamma \) EGFP genomic reporter assay. The induction values correspond to maximum activity at the specified concentration for each agent. Each compound was tested three to five times, and the results were statistically compared by Student’s t-test between appropriate groups.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Percentage increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemin (100 μM)</td>
<td>764 ± 145</td>
</tr>
<tr>
<td>Hydroxyurea (100 μM)</td>
<td>75 ± 10</td>
</tr>
<tr>
<td>HDAC inhibitors</td>
<td></td>
</tr>
<tr>
<td>Butyrate (1000 μM)</td>
<td>82 ± 29</td>
</tr>
<tr>
<td>Butyrate (1000 μM) + hydroxyurea (100 μM)</td>
<td>159 ± 72*</td>
</tr>
<tr>
<td>ST7 (500 μM) (( \alpha ) methylhydrocinnamic acid)</td>
<td>24 ± 7</td>
</tr>
<tr>
<td>ST20 (2000 μM) (sodium-2,2-dimethylbutyrate)</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Trichostatin A (0.3 μM)</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Apicidin (1 μM)</td>
<td>66 ± 11</td>
</tr>
<tr>
<td>DNA-binding agents</td>
<td></td>
</tr>
<tr>
<td>Carboplatin (25 μM)</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>Cisplatin (25 μM)</td>
<td>442 ± 32*</td>
</tr>
<tr>
<td>Mithramycin (0.05 μM)</td>
<td>38 ± 7</td>
</tr>
</tbody>
</table>

*Mean value is significantly different from other HDAC inhibitors.

**Mean value is significantly different from other DNA-binding agents.
difference between the EGFP-inducing potential of cisplatin versus carboplatin. These results indicate that our assay may be used to study disruption of specific interactions between transcription factors and sequences on the β-globin locus with DNA-binding agents, as the first step to a rational drug design approach for the targeted disruption of such interactions by agents that modify the proteins involved rather than the DNA, thus improving the efficacy and reducing the toxicity of HbF inducers.

In conclusion, we report for the first time the development of a cellular genomic reporter assay based on a human erythroleukemic cell line stably expressing EGFP under the control of the Gγ-β-globin promoter in the context of the human β-globin locus, and compare the effects of a number of HbF inducers. The specificity and sensitivity of our assay enables the identification of agents that directly induce γ-globin expression from those agents that induce HbF expression indirectly. We anticipate that this in vitro model system may facilitate high-throughput screening of FDA-approved drugs, chemical libraries and traditional medicines for the identification of more effective and safer inducers of HbF. The development of a dual reporter GRA with different reporters will allow the preclinical evaluation of HbF inducers in erythropoietic cell cultures and in vivo in an adult erythropoietic environment. Lastly, given the high level of heterologous protein expressed from the β-globin locus in erythroid cells, it may be possible to produce high levels of therapeutic proteins in vitro and perhaps in vivo for diseases amenable to protein replacement therapy.

MATERIALS AND METHODS

Construction of genomic reporter constructs

The P1-derived artificial chromosome (PAC) clone (148O22) was identified from the RPCI I PAC library (www.chori.org/bacpac) to contain the β-globin locus (22). Sequencing of the T7 and Sp6 ends of the genomic insert and alignment with the human genome sequence (GenBank Accession number NT_028310.10) revealed that the genomic insert is 183 039 bp long, with 122 076 bp upstream of the start codon of the ε-globin gene and 17 672 bp downstream of the stop codon of the β-globin gene. However, these sequence assignments are approximate, since this clone could have large numbers of polymorphisms and other sequence variations from the published genome sequence. The 183 kb genomic fragment was retrofitted into the pEBAC140 cloning vector as a single NotI fragment to generate pEBAC/148β (Fig. 1, also referred to as pBH148β) (55,56). The construction of the pEBAC/148β::Gγ·β EGFP and pEBAC/148β::Gγ-β EGFP β-globin genomic reporter constructs (Fig. 1) has been previously described (24,25). The start codon of the EGFP gene was targeted in frame with the start codon of the Gγ·β-globin gene in both constructs, while the stop codon of the Neo/Kan gene in the EGFP-Neo/Kan cassette was placed at the termination codons of the Gγ- or β-globin genes respectively.

Preparation of pEBAC vectors

The pEBAC vectors were propagated in the E. coli strain DH10B (Invitrogen, Carlsbad, CA, USA). Bacterial cultures were routinely grown in LB liquid culture, or on LB agar plates (15 g/l) containing the following antibiotics as indicated in the text: 12.5 µg/ml chloramphenicol (Cm) or 25 µg/ml kanamycin (Km). Large-scale preparation of pEBAC constructs for transfection studies was performed using CsCl–ethidium bromide density gradient centrifugation.

Generation and characterization of KEB cell lines

The generation of stable K562 cells that constitutively express EBNA1 (KEB cells) has previously been reported (25). All KEB clones tested positive for the presence of the EBNA1 gene by PCR (data not shown). The EBNA1-specific rat MAb IH4 (57) was used to quantitate the level of EBNA1 protein expression in each clone, using a phycoerythrin polyclonal anti-rat Ig (BD PharMingen, Los Angeles, CA, USA) by measuring the median peak fluorescence (MPF) by flow cytometry using a FACSscan flow cytometer (BD PharMingen, Los Angeles, CA, USA). KEB cells and derivative cell lines were maintained in continuous culture in Dulbecco’s modified Eagles medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

Transfection of EGFP-modified pEBAC vectors into KEB cells

The pEBAC/148β::Gγ·β EGFP and pEBAC/148β::Gγ-β EGFP genomic reporter constructs were digested with NotI before
transfection, to liberate the intact β-globin locus from the pEBAC vector as a linear genomic fragment. Four micrograms of each digested construct, containing both the pEBAC vector domain and the β-globin locus genomic fragment, were transfected into KEB cells using DMRIE-C (Invitrogen, Carlsbad, CA, USA) at a 2:1 lipid to DNA ratio, as previously reported (25). The transfected cells were cultured for 48 h, followed by hygromycin (400 μg/ml) selection for an initial period of one week. Clonal cells were isolated in 96-well plates using limiting cell dilution in the absence of hygromycin for a further 2–3 weeks. Individual EGFP positive clones were picked using fluorescence microscopy and clonally expanded in media without hygromycin selection. KEB clones stably transfected with the pEBAC/148β::γ-Aγ EGFP and pEBAC/148β::γ-β EGFP constructs were grown without antibiotic selection in media containing 20% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and supplemented with an antioxidant mix (1 mM sodium pyruvate, 50 μM z-thioglycolerol and 20 μM bathocuprinedisulfonate) (58). The cell density was maintained at 1–8 × 10⁵ cells/ml and cultures were incubated at 37°C. Cells in continuous culture without antibiotic selection for up to 230 days were used to examine the stability and uniformity of EGFP expression with over time.

Analysis of EGFP expression

The percentage of EGFP positive cells and relative levels of EGFP expression (MPF) were measured by flow cytometry. The argon laser was set at 488 nm, and fluorescent cells were analysed with a 525 nm band-pass filter. Data acquisition and analysis was performed using WinMDI software (http://pingu.salk.edu/software.html). The percentage increase in MPF between induced and non-induced cells and the ratio of MPF between induced and non-induced cells was calculated by subtracting the value corresponding to non-induced cells from the value obtained in induced cells. Quantitative and qualitative differences in the induction of γ-globin gene expression were examined by measuring the percentage of EGFP-expressing cells and MPF by flow cytometry. Each compound was tested three to five times, and the results were statistically compared with appropriate groups.

Acknowledgements

We would like to thank Dr Susan Perrine at the Hemo-globinopathy-Thalassaemia Research Unit, Boston University School of Medicine, Boston, for the supply of α-methylhydroxycinnamic acid and sodium-2,2-dimethylbutyrate. This work was supported by grants from the National Health and Medical Research Council (NHMRC) of Australia, the Brockhoff Foundation, the Ronald Geoffrey Arnott Foundation and the Thalassaemia Society of New South Wales. M.O. was supported by a grant from the Thalassaemia International Federation and the Cyprus Thalassaemia Association. J.V. is currently a recipient of a research fellowship from the Cooley’s Anemia Foundation.

References


