Regulated gene expression in transfected primary chicken erythrocytes
(globin genes/transcription/enhancers/osmotic shock)

JOANNE E. HESSE, JOANNE M. NICKOL, MICHAEL R. LIEBER, AND GARY FELSENFELD

Laboratory of Molecular Biology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

Contributed by Gary Felsenfeld, February 10, 1986

ABSTRACT We describe a method for studying transient gene expression in primary avian erythroid cells that involves controlled osmotic shock, followed by DNA transfection using DEAE-dextran. Cells treated in this way reproducibly express high levels of chloramphenicol acetyltransferase (CAT) when transfected with a plasmid having the cat gene coupled to an appropriate viral promoter. An observed correlation between levels of CAT expression and extent of hemoglobin release during controlled shock makes it possible to choose optimal conditions for expression in erythroid cells at various stages of embryonic development. Using these techniques, we have investigated the effect on CAT expression of fusing to the cat gene various portions of the chicken adult β-globin (βA) gene. We show that in 9-day or 12-day embryonic erythrocytes, the promoter activity of the 5′ flanking region of the βA gene (in the absence of any viral promoters) is strongly stimulated by a downstream sequence, located in the region 110-588 base pairs on the 3′ side of the poly(A) signal, that acts as an enhancer. Its activity is reduced in 5-day embryonic cells and absent in primary chicken fibroblasts and mouse L cells, suggesting that this transient expression system will be useful in studying developmentally regulated globin gene expression.

The globin gene family has provided a prototype for the study of developmentally regulated cellular gene expression in eukaryotes. Avian erythroid development in particular offers well-defined embryonic stages; furthermore, erythrocytes at all of these stages are relatively easily isolated (1). For these reasons it has been possible to obtain considerable information from avian erythrocytes about chromatin structural features in the neighborhood of globin genes and their correlation with varying levels of transcriptional activity (2-5).

In our laboratory, work has focused on chromatin structure in the neighborhood of the chicken adult β-globin (βA) gene and especially on the nature of the nuclease-hypersensitive domain located in its 5′ flanking region (4). We have identified and partially purified protein factors that bind specifically within this domain and determined the DNA sequences to which they bind (5-7).

Despite its usefulness, such a structural analysis of the promoter region does not provide detailed information about the functional role of the proteins or their DNA binding sites. However, the availability of methods for introducing exogenous gene copies into eukaryotic cells offers the possibility of obtaining this information, provided appropriate cell types, consistent in species, tissue, and developmental stage, can be used for transfection. To date, functional studies of globin genes have employed neoplastically transformed cell lines. In the case of human genes, for example, mouse erythroleukemia cell lines (8, 9) have been used.

Conclusions from such studies must take into consideration uncertainties about the precise relationship of a transformed cell to its cell of origin.

We describe a system for studying transient expression in primary avian erythroid cells; this system allows us to examine the behavior of globin genes in a biologically relevant environment and as a function of developmental stage. We use the method to study the regulation of the βA-globin gene and confirm the presence of an enhancer element, previously observed in another laboratory (W. Schaffner, personal communication), in the 5′ flanking region of the gene. The enhancer is active in 9- and 12-day embryonic erythrocytes but has reduced or negligible activity when transfected into other kinds of cells, suggesting that developmental fidelity is preserved in the assay.

METHODS

Erythrocyte Isolation. Erythrocytes from chicken eggs 5, 9, or 12 days after laying were collected into 25 ml of phosphate-buffered saline (PBS) (0.726 g of anhydrous Na2HPO4 per liter, 0.21 g of anhydrous KH2PO4 per liter, 9 g of NaCl per liter) at 22°C. Erythrocytes were pelleted at 800 × g for 5 min at 22°C, washed once in 45 ml of PBS, and resuspended in PBS at about 10⁸ cells per ml. One-milliliter aliquots were distributed into 1.5-ml Eppendorf Microfuge tubes and used within 3 hr.

DEAE-Dextran Transfection of Erythrocytes. Samples were centrifuged in a Beckman Microfuge 12 for 1 min. Supernates were aspirated and pellets were softened and then resuspended by brief, gentle mixing in a Vortex. Reagents were sterile and used at 22°C unless otherwise specified. Cells (10⁶) were pelleted at 6300 × g and resuspended in 0.5 ml of DEAE-dextran-DNA (10, 11) [67% vol/vol L15 (Liebovitz) medium (Flow Laboratories), 0.05 M Tris-HCl, pH 7.45, 300 μg of DEAE-dextran (Pharmacia P-L Biochemicals, ~5 × 10⁵ daltons) per ml, and 1-3 μg of DNA per ml], which was prepared 5-120 min before use. The reaction mixture was incubated 10 min at 37°C, after which the cells were pelleted at 10,500 × g, washed once in 100% L15 medium, repelleted at 10,500 × g, resuspended in 0.25 ml of culture medium [92% (vol/vol) L15 medium, 5% fetal calf serum (Flow Laboratories), 2% chicken serum (GIBCO), and 100 μg of kanamycin sulfate (GIBCO) per ml], and incubated, with tubes tightly capped and lying on their sides, at 37°C for 48 hr.

Transfection of L Cells and Primary Chicken Fibroblasts. Mouse cell line DAP-3 (Ltk* apr) (kindly provided by R. D. Camerini-Otero) was grown under published conditions (12) with addition of 200 units of penicillin per ml and 200 μg of streptomycin per ml (final concentration). Primary chicken fibroblasts (American Type Culture Collection) isolated from 11-day embryos were grown under identical conditions, with the further additions of 1× nonessential

Abbreviation: CAT, chloramphenicol acetyltransferase.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
amino acids (Flow Laboratories) and 5% (vol/vol) tryptose phosphate broth (Flow Laboratories). Cells grown to 50% confluence in 150-cm² flasks were washed twice with 10 ml of 100% modified Eagle’s medium (ME medium) and incubated 2 hr at 37°C in 5 ml of DEAE-dextran-DNA, prepared as for erythroblast except for the use of ME medium and resuspended directly to 0.5 ml of DEAE-dextran-DNA. Preparation of this complex and the subsequent steps of the procedure were identical to those described above in the section on DEAE-dextran transfection of nonosmotically stressed erythrocytes. The supernate from the NH₄Cl incubation was saved and the A₄₁₂ of an appropriate dilution was measured to determine the amount of Hb released. The value corresponding to 100% release was determined from the A₄₁₂ of an appropriate dilution of the supernate from a cell sample lysed to completion by incubation in 1 ml of 150 mM NH₄Cl at 37°C for 40 min.

**Assay for Chloramphenicol Acetyltransferase (CAT).** Cells were harvested for assay 48 hr after transfection. Erythrocytes were pelleted at 10,500 × g for 1 min, washed once in 1 ml of PBS at 22°C, pelleted, resuspended in 0.16 ml of 0.25 M Tris-HCl (pH 7.55), dispersed by vigorous mixing in a vortex, and lysed by three cycles of freezing and thawing (dry ice/ethanol bath, 37°C bath), and the debris was pelleted at 10,500 × g for 5 min. L cells and primary chicken fibroblasts were washed three times with 10 ml of Dulbecco’s PBS (GIBCO) at 22°C, loosened from the culture flask by incubating 10–35 min at 37°C in 0.25% trypsin (GIBCO) in PBS, pelleted at 800 × g for 7 min, washed once in 10 ml of PBS, pelleted at 10,500 × g for 1.5 min, resuspended, and lysed as above. No reduction in CAT activity was observed in transfaction samples harvested with trypsin as compared to those harvested by scraping.

To measure CAT activity within the linear range of the assay, a suitable fraction of each cell extract was used. The assay procedure of Gorman et al. (13) as described by Lopata et al. (11) was followed except that each assay contained 20 μl of 50 mM acetyl-coenzyme A and 0.25 μCi of [³⁴Cl]-chloramphenicol (1 Ci = 37 GBq).

**RESULTS**

**Transient Expression.** We have found that erythrocytes isolated from chicken embryos transiently express genes in exogenously added DNA. The CAT expression vector pRSVcat, in which the Rous sarcoma virus long terminal repeat is fused to the cat gene (14), was introduced into erythrocytes from 9-day embryos using the DEAE-dextran method. As shown in Fig. 1 and Table I, an extract prepared from the transfected cells 48 hr after DEAE treatment (lane B) contained CAT, revealed by its ability to convert [³⁴Cl]-chloramphenicol to its acetylated derivatives. Extracts from mock-transfected cells contained no CAT (lane A).

We took advantage of the unique permeability properties of erythrocyte membranes to induce osmotic swelling and shock of the cells in solutions of NH₄Cl. Such treatment does not cause complete disruption of the cells but results in transient formation of holes in the membrane through which some cytoplasmic components can escape (see Discussion).

---

**Table 1. Effects of NH₄Cl wash on transient expression**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Plasmid</th>
<th>% Hb release*</th>
<th>Limited wash, Pre-DEAE</th>
<th>Extensive wash, Post-DEAE</th>
<th>CAT+</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>No DNA</td>
<td>0.4</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>pRSVcat</td>
<td>0.3</td>
<td>1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>pRSVcat</td>
<td>98.7</td>
<td>+</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>pRSVcat</td>
<td>0.4</td>
<td>+</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>pRSVcat</td>
<td>12.5</td>
<td>+</td>
<td>61.4</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>pRSVcat</td>
<td>11.8</td>
<td>+</td>
<td>+</td>
<td>2.0</td>
</tr>
<tr>
<td>G</td>
<td>No DNA</td>
<td>11.0</td>
<td>+</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>48-hr supernate of sample F</td>
<td>28.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*During NH₄Cl wash (limited or extensive) administered prior to DEAE treatment. Where no pre-DEAE NH₄Cl wash was given (samples A, B, and D), PBS prewash (1 ml, 22°C, 1 min) was used.

**Fig. 1. Autoradiogram (3-day exposure) of the TLC plate on which CAT assay components were separated, showing results obtained with samples described in Table 1. The presence of CAT activity is revealed by conversion of [³⁴Cl]-chloramphenicol (chl) to its three acetylated derivatives. Samples in lanes A–H correspond to samples in Table 1.**

Extensive exposure to NH₄Cl (Table 1 legend) affects all cells and results in total Hb release. An extract prepared from erythrocytes washed extensively with NH₄Cl either before or after DEAE transfection (Table 1 and Fig. 1, lane C) or 48 hr after DEAE treatment (lane D) contains markedly reduced levels of CAT when compared to an identical sample that received no wash (lane B). The responses to extensive NH₄Cl treatment either before or after transfection reflect the expected behavior of erythrocytes (see Discussion). However, when DEAE transfection was preceded by an NH₄Cl wash of limited extent (Table 1 legend), affecting only a fraction of the cells, there was a 38-fold increase in CAT activity (lane E) compared to samples that had received no wash but were otherwise treated identically (lane B). If the cells were subsequently given an extensive NH₄Cl wash 48 hr after transfection (lane F), the CAT activity was recovered quantitatively in the supernate (lane H).

The striking increase in transient expression that follows limited shock suggests that, under some conditions, cells can recover with increased capability to take up or express...
exogenous DNA. To examine further the relationship between erythrocyte shock and the uptake and expression of DNA, a series of identical 5-day or 12-day embryonic erythrocyte samples was given NH4Cl washes of increasing duration, followed by DEAE-dextran treatment with pRSVcat. Release of Hb as a function of time was monitored by measuring A\text{412} of each NH4Cl wash supernatant (Fig. 2). There was an initial lag in Hb release as cells swelled and approached the critical hemolytic volume, followed by a burst of Hb release. Cell extracts prepared 48 hr after transfection were assayed for CAT activity. CAT expression was increased by the exposure to NH4Cl, with a broad peak of activity correlated with \(-12\%\) Hb release in 5-day and 12-day cells. Activity fell off as Hb release approached 100%. Results similar to those for 12-day cells were obtained with 9-day cells (data not shown). Release of Hb and peak CAT expression from 5-day cells are delayed and protracted relative to 12-day cells (Fig. 2); this is related to the larger size of the primitive erythrocytes predominant in 5-day cells.

The correlation between time of Hb release and time of maximum CAT activity can be used as an empirical guide in establishing optimum conditions for shock-enhanced transient expression. However, additional and substantial amounts of Hb are released during DEAE-dextran treatment immediately following the NH4Cl wash (data not shown). Peak CAT activity occurs at 85% total Hb release. Furthermore, in a 12-day erythrocyte sample washed in NH4Cl sufficiently to release 90% of its Hb, and subsequently exposed to DEAE-dextran-DNA with release of additional Hb, the shocked, Hb-free cells that remain are able to synthesize 15 times more CAT than an identical sample that received no NH4Cl wash (Fig. 2). These results strongly suggest that the shocked, Hb-releasing cells, rather than those merely osmotically swollen, are responsible for the enhanced transient expression.

The relationship between DNA concentration and osmotic shock-enhanced transient expression was examined. A set of 9-day samples uniformly washed under conditions for peak shock enhancement was transfected with increasing amounts of pRSVcat per cell. A second set of samples was similarly washed and transfected with increasing amounts of the globin-con cat pACatBC (Fig. 3). CAT activity after a 48-hr incubation of the cells was directly proportional to DNA concentration up to 3 \(\mu\)g/ml. At a DNA concentration of 6 \(\mu\)g/ml, transient expression was no longer proportional for either vector (data not shown).

Regulated Transient Expression. To explore the usefulness of these methods for studying globin gene expression, CAT expression vectors were constructed (Fig. 3) in which the 5' flanking region of the chicken \(\beta\)-globin gene was placed upstream of the \(\alpha\)-globin structural gene, and various segments of the \(\beta\)-globin structural gene and associated 3' sequences were placed downstream of the \(\alpha\)-globin gene. Each of these plasmids was transfected into 9-day erythrocytes, and after 48 hr extracts were assayed for CAT (Table 2). Expression of pRSVcat serves as a control. When transfected into 9-day cells, the \(\alpha\)-globin gene was solely under the control of the vector.

Table 2. Transient expression of the \(\alpha\)-globin gene directed by pRSVcat or globin-cat constructs

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mouse L cells</th>
<th>Primary chicken embryonic fibroblasts</th>
<th>Primary chicken embryonic erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRSVcat*</td>
<td>2.0 ± 0.4</td>
<td>11 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>pRSVcat</td>
<td>13</td>
<td>76 ± 30</td>
<td>81 ± 16</td>
</tr>
<tr>
<td>pUC18cat</td>
<td>2.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>pACat</td>
<td>0.2 ± 0.1</td>
<td>0.5 ± 0.2</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>pACatBC</td>
<td>0.6 ± 0.1</td>
<td>3.3 ± 0.2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>pACatC</td>
<td>1.1 ± 0.1</td>
<td>7.4 ± 0.1</td>
<td>3.8 ± 0.9</td>
</tr>
<tr>
<td>pACatC(inv)</td>
<td>11 ± 4</td>
<td>4.5 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>pACatD</td>
<td>27 ± 10</td>
<td>9.9 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>pACatD(inv)</td>
<td>16 ± 2.0</td>
<td>11 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>pACatE</td>
<td>0.1 ± 0.1</td>
<td>40 ± 24</td>
<td>24 ± 2.3</td>
</tr>
</tbody>
</table>

Cells were transfected with DNA (1 \(\mu\)g/ml: primary chicken fibroblasts and mouse L cells; 3 \(\mu\)g/ml: primary chicken erythrocytes) using DEAE-dextran. Limited NH4Cl wash (9-day: 1 ml, 250 mM, 40 min, 22°C; 5-day: 1 ml, 250 mM, 67 or 90 min, 22°C) was administered to erythrocytes prior to DEAE treatment. Cell extracts were prepared 48 hr after transfection and assayed for CAT. Data are expressed as \(\%\) [\(^3\)H]chloramphenicol acetylated in 60 min. Where mean deviations are given, results are an average of three experiments in most cases and two or four experiments in others.

*Of the erythrocyte samples, only these were not treated with NH4Cl.
β-globin 5' region extending to approximately $-1000$ (pAcat) is transiently expressed at a low but detectable level, slightly higher than that of an identical construction devoid of globin sequences (pUC18cat). Addition to pAcat of the globin structural region extending from +43 to +1060 (pAcatB) yields similarly low levels of activity. Addition to pAcatB of a progressively more 3' globin region, +1060 to +3400 (pAcatBC), which includes sequences 3' of the poly(A) signal at +1507, yields ≈7 times higher activity than pAcat. This result suggests that sequences are contained within this region that increase CAT expression. This is confirmed by the 18-fold average increase over pAcat activity yielded by constructs in which this region, +1060 to +3400, in either orientation is added alone to pAcat (pAcatC, C(inv)). Subclones of this region appended to pAcat, +1480 to approximately +2600 [pAcatD, D(inv)] and +1617 to +2095 (pAcatE), yield still greater CAT activity, the latter 80 times higher than that of pAcat. High CAT activities were also observed when region E in either orientation was placed directly 5' of region A (data not shown). The sequence of globin region E is presented in Fig. 4. Other subclones of the region +1060 to +3400 appended to pAcat (dashed lines, Fig. 3) were tested and each yielded low levels of activity, within 0.5% of that of pAcat (data not shown).

To determine the extent to which transient expression is developmentally regulated, CAT vectors were introduced into a variety of cell types. In mouse L cells or chicken fibroblasts, pRSVcat directed CAT expression (Table 2). In fibroblasts, the globin constructions pAcat and pAcatE directed low levels of expression; in L cells, although there was some CAT expression with pAcat and pAcatC, there was no evidence for a stimulatory effect of sequences 3' of the globin promoter (Table 2). Transient expression in 5-day embryonic erythrocytes showed stimulation in the presence of these sequences (Table 2), but diminished compared to that observed in 9-day cells. In 17-day cells, expression of pRSVcat and pAcatE was very low but slightly greater than that of pAcat. None of these plasmids gave a signal in adult cells (data not shown).

**DISCUSSION**

Transfection of DNA into avian primary embryonic erythrocytes provides a valuable tool for the study of globin gene expression in vivo. The method makes it possible to examine the activity of modified globin gene sequences as a function of developmental stage in cells in which the globin gene is normally expressed. It avoids the use of neoplastically transformed cell lines and provides an environment in which the native transcriptional and developmental control elements remain in effect after transfection. The assay is extremely sensitive, reflecting a level of transient gene expression equalling that observed in comparable experiments with neoplastic cells.

The method involves induction of limited osmotic stress by washing with NH₄Cl, which results in shock and release of Hb. The shocked cells are subsequently exposed to a DEAE-dextran-DNA complex; during the 48-hr incubation that follows, the cells recover sufficiently to support transcription of plasmids and translation of the message.

The mechanism of action of NH₄Cl, widely used to rupture erythrocytes, is well understood (21). Free NH₄⁺ diffuses into cells, where it reequilibrates to form NH₃⁻. In nonerythroid cells this process is arrested at low concentrations of NH₃⁻ because neutralizing ions cannot enter the cell. Erythroid cells possess Band 3, a membrane protein that renders them permeable to Cl⁻ and other anions. The equilibration of NH₄Cl that follows ultimately leads to osmotic pressure differences that rupture the cell. In the case of human erythrocytes, it is known (22) that this does not cause total disruption of the cell structure but results in formation of a single hole in the membrane, which, under the ionic conditions used here, would be reduced within seconds to a radius $<20$ Å. Though our results make it clear that such cell shock can result in elevated levels of transient expression, it is not clear whether this effect reflects increased levels of DNA uptake as compared to an unshocked cell or a general stimulation of protein or mRNA synthesis that might follow partial loss of the cellular contents. A more complete examination of the role of shock and the mechanism of DNA uptake in this system will be presented elsewhere.

The active species in these studies is clearly the erythrocyte. First, transient expression is eliminated by extensive treatment before transfection with NH₄Cl (which specifically affects erythrocytes), whereas, if cells are shocked with NH₄Cl 48 hr after transfection, CAT activity is released quantitatively into the supernate. Second, peak transient expression is correlated with extent of Hb release under a variety of conditions (Fig. 2). Finally, microscopic examination of 9-day cell preparations reveals $<1%$ nonerythroid cells, unlikely to be capable of supporting the amount of CAT synthesis observed.

Our purpose in developing these methods was to provide an assay for the function of chicken globin genes in biologically relevant cells. We therefore tested the effect of introducing into erythrocytes various DNA constructions containing portions of the β-globin gene (15) fused to the structural gene for CAT. The first of these, pAcat (Fig. 3), in which the β-globin 5' region is attached upstream of cat, produces levels of CAT expression consistently above control levels, but low. There is evidence (8, 9) from work with human β-globin genes transfected into erythroleukemia cell lines that downstream DNA sequences are important in regulation of expression. Furthermore, W. Schaffner and his co-workers (personal communication) have detected an en
hancer element in the 3' flanking region of the chicken \( \beta^A \) gene based on transfection studies in virally transformed cell lines. To determine the possible effect of downstream sequences in our assay, we constructed pAcatBC (Fig. 3) in which the coding and 3' flanking regions of the \( \beta^A \)-globin gene are fused to the 3' end of pAcat. Transfection with this plasmid resulted in heightened levels of CAT expression relative to pAcat (Table 2).

We have carried out a number of experiments with related plasmids (Fig. 3, Table 2) in which various portions of the coding and 3' flanking regions are deleted or rearranged and transfected into 9-day embryonic erythrocytes. The results show that the stimulatory sequence is in the region 110–588 base pairs 3' of the \( \beta^A \)-globin gene poly(A) signal and that it functions when inserted in either orientation. [We note that a site hypersensitive to nuclease in erythrocyte chromatin is also located in this region (4).] The stimulatory effect of the sequence varies with its distance from the promoter; at distances of 1.6 kilobases (pAcatE), comparable to its separation from the promoter in the intact \( \beta^A \)-globin gene, \( \approx \)80-fold increases in expression are observed. We have not demonstrated directly that this increase results from an effect on transcription rate (as opposed to effects on translation or mRNA stability). The fact that the sequence also stimulates transcription when placed 5' of the promoter argues against such mechanisms. The results shown in Table 2 thus strongly suggest that the 3' segment acts as an enhancer, although we have not yet determined its action on the intact \( \beta^A \)-globin gene. Furthermore, it is inactive in transient expression when introduced into either mouse L cells or chicken fibroblasts and displays reduced activity in 5-day embryonic chicken erythrocytes, suggesting that it is specific with respect to cell type and perhaps developmental stage. It is not yet clear whether this specificity is conferred by the 3' region alone or whether contributions by promoter and 3' region are involved.

The method we have described here for studying transient expression in primary avian erythroid cells has the advantages of simplicity, high efficiency of transfection resulting in large signals, and reproducibility. It has the disadvantage that, at least in the cells we have studied so far, there is some question as to whether exogenous DNA will be packaged in chromatin and whether absence of chromatin structure might alter transcriptional behavior. Nonetheless, we think that it will be possible to use this method to obtain considerable information about the regulatory effects of DNA sequences and their corresponding protein factors during the development of avian erythroid cells.

We thank Dr. Walter Schaffner for permitting us to cite his unpublished data and Miss Betty Canning for preparing this manuscript.