Increased erythroid-specific expression of a mutated HPFH γ-globin promoter requires the erythroid factor NFE-1

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ABSTRACT

The −175 T ►C mutation in the promoter of the $^{A}\gamma$- or $^{G}\gamma$-globin gene causes a 50−100 fold increase of the expression of the respective gene in adult erythroid cells (Hereditary Persistence of Fetal Hemoglobin). We show here that this mutation increases 3−9 fold the expression of a γ-CAT reporter plasmid transfected into the erythroid cells K562, but not that of the same plasmid in non erythroid cells. The overexpression of the mutant is abolished by the mutation of the binding site for the erythroid specific factor NFE1; inactivation of the adjacent binding site for the ubiquitous factor OTF1 does not cause overexpression of the normal γ-globin promoter. Previous results demonstrated that the −175 mutation slightly increases the *in vitro* binding of NFE1 and almost abolishes that of OTF1; the present functional data indicate that altered binding of NFE1, but not of OTF1, is responsible for the observed overexpression of the mutated promoter.

INTRODUCTION

Hereditary persistence of Fetal Hemoglobin (HPFH) is a heterogeneous group of genetic disorders characterized by elevated expression in adult life of fetal (γ) globin genes [1,2]. In a subclass of HPFH, point mutations in the promoter of the overexpressed $^{A}\gamma$- or $^{G}\gamma$-gene are found; strong genetic evidence suggests that these mutations are the cause of the abnormal expression of these genes [3,4]. We have previously reported (5) that a T ►C mutation at position −175 of the γ-globin promoter (either $^{G}\gamma$- or $^{A}\gamma$-) greatly decreases the ability of the ubiquitous octamer binding factor (OTF-1) [6] to bind to its recognition sequence (octamer) in the γ-globin promoter, while increasing the ability of the erythroid-specific protein NFE1 to bind to its bipartite site flanking the octamer. In this work, we evaluate the functional roles of NFE1 and OTF1 binding sites by transfection experiments using normal and mutated γ-globin promoters.

METHODS

Plasmids for CAT assays

An AluI fragment (−299 to +35) of the human γ-globin promoter (normal or −175 HPFH) was joined by linkers to the HindIII site of the plasmid pSVo-CAT [7]. Other mutants were derived from these plasmids by site directed mutagenesis; 5′ oligonucleotides carrying the mutations indicated in Figure 1B, were used in conjunction with a 3′ oligonucleotide carrying a Hind III linker, to amplify *in vitro* (using the normal pSVo- γ-globin-CAT plasmid as template) with Taq polymerase the γ-globin promoter fragment extending from −210 to +35 (plus HindIII linker); after double-digestion with Apa I and Hind III, the amplified (and mutated) fragment was inserted into the normal pSVo γ-CAT, replacing

the corresponding normal Apa I-Hind III fragment. The doubly mutant fragment (NFE1-/HPFH) was obtained using the HPFH plasmid as template. Oligonucleotides used were:
5′ oligonucleotide (from position −210, top strand): 5′TTGGGGGCC-CCTTCCCCACACTATCCATATGCAAATATCT (the normal sequence is given; mutations as in Figure 1B)
3′ oligonucleotide (from position +35, bottom strand) preceded by Hind III linker: 5′GCGAAGCTT−CTTATTGATAATCTCAGA

Transfection

2×10^7 cells in 0.8 ml of phosphate buffered saline were transfected by electroporation in the presence of 10−70 μgs of the relevant plasmids. Carrier DNA (pGEM plasmid) was added (when necessary) to bring the total amount of DNA to 70 μgs/transfection; this resulted in optimal activity. Electroporation was at 400 Volts (K562 cells) and at 350 Volts (BJA/B cells) with a capacitance of 400 μF. HeLa cells were transfected by calcium phosphate precipitation. Two days after transfection, cells were recovered, washed with phosphate buffered saline and lysed by repeated cycles of freeze-thawing in 60 μl of 0.25 M Tris HCl pH 7.8.

CAT assay

20 μls of cell lysate were incubated for 4−6 hours at 37°C in 75 μls of a standard CAT assay reaction (7), including 0.2 μCi of ^{14}C-labelled chloramphenicol (Amersham). Addition of 3 μls of 10 mM acetyl-CoA every 2 hours maintained linearity of the reaction. Following steps were exactly according to ref. 7.

Alternatively, chloramphenicol was butyrylated and determined by the sensitive phase-extraction assay (xylene terminated) of ref. 8: briefly, 5−20 μls of cell lysate were incubated for 3−5 hours at 37°C in a total volume of 100 μls in the presence of 0.2−0.5 μCi ^{14}C-Chloramphenicol, 0.25 mM butyryl-Co A (Sigma), 100 mM Tris HCl, pH 7.8. The reaction was then terminated by extraction with two volumes of xylenes; after centrifugation for 1 minute, the organic phase was removed and extracted twice with 1 volume of 10 mM Tris HCl, pH 7.5, 1 mM EDTA. The remaining organic phase (containing the butyrylated chloramphenicol) and the acqueous phases (containing the non-butyrylated chloramphenicol) were counted.

RNAase mapping

A NdeI-EcoRI fragment of the pSV γ-CAT plasmid was cloned into the SmaI-EcoRI sites of pGEM4; this fragment has its 5′ end in the pSV sequence, and includes the whole γ-globin promoter linked to part of the CAT gene. The plasmid was linearized at the BamHI site (immediately 5′ to the NdeI site) and transcribed with SP6 polymerase (9) in the presence of ^{32}P-CTP (18 μM final concentration, 500 Ci/m mole). After RQ1 DNAase digestion and phenol-chlorophorm extraction, the labelled probe was ethanol precipitated and used without further purification.

1×10^8 K562 cells were transfected with normal or HPFH plasmids (see above), and total RNA was recovered after two days. Poly A$^+$-RNA was prepared from 100 μgs of total RNA; half of it was hybridized to the RNA probe ($0.5-1\times10^6$ cpm) for 14 hrs. at 45° in 30 μls of 80% formamide, 0.4 NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, containing 10 μgs of carrier tRNA and then digested for 90 min at 30°C in 300 μls with RNAases A and T1 (80 μgs/ml and 2μgs/ml, final concentration, respectively) followed by proteinase K digestion and phenol-chlorophorm extraction; the remainder was added to the hybridization mixture at the end of the hybridization, and processed in parallel with

the other samples. Gel electrophoresis was in a standard 6% acrylamide, 8 M urea sequencing gel.

RESULTS

To assay for γ-globin promoter activity, a fragment of this promoter was linked to a chloramphenicolacetyltransferase-based plasmid (pSVo-CAT [7] and used for transfection experiments. In addition to normal and HPFH plasmids, three mutants were tested (Fig. 1): NFE1- , a mutant unable to bind NFE1, but retaining OTF1 binding activity [5]; OTF1-, a mutant unable to bind OTF1, but retaining NFE1 binding activity [5]; NFE1-, HPFH, a double mutant carrying the HPFH mutation in association with the NFE1-mutation. These mutants were transiently transfected into human erythroid cells (K562), expressing ϵ and γ, but not β-globin chains. Figure 2A shows that the HPFH mutant reproducibly expresses greater (>3-fold, see below) CAT activity than the normal plasmid; however, the addition of the NFE1-mutation to the −175 HPFH plasmid abolishes this increased expression (Fig. 2B). Single NFE1- and OTF1- mutations have little or no effect (Fig. 2C).

The increased expression of the HPFH relative to the normal plasmid was consistently observed, using 7 different plasmid preparations in more than 50 transfections. Transfections carried out together with a tymidine kinase-driven reporter plasmid expressing growth hormone (to normalize for transfection efficiency) also gave identical differences between HPFH and normal plasmids (not shown); however, transfections carried out in parallel yield highly reproducible results (Fig. 2A) and we therefore preferred not to use cotransfection of growth hormone plasmids, in order to avoid possible competition between promoters.

To obtain a quantitative estimate of the efficiency of the various plasmids, densitometry and counting of radioactive spots were employed; however, for experiments yielding relatively low CAT expression, we found that a more precise quantitation could be readily obtained by the recently described chloramphenicol-butyrylation test [8]. Representative results obtained with the same cell extracts as in the experiments shown in Figure 2 are given in Table I; the activity of the −175 HPFH promoter exceeds that of the normal γ-globin promoter by three-four fold in most experiments, with an upper limit of 7−9 fold (average increase: ~4 fold).

To test for the cell specificity of the increased expression of the −175 HPFH mutant, the same plasmids as above were also transfected into non erythroid cell lines, like HeLa (epithelial cells) and BJA-B (B-lymphocytes). The activity of the plasmids was extremely

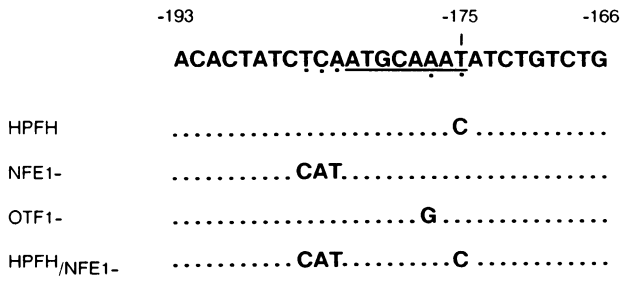

Figure 1. Mutations in the NFE1 and OTF1 recognition sites of the human γ-globin promoter. The effect of these mutations on binding is shown in ref. 5.

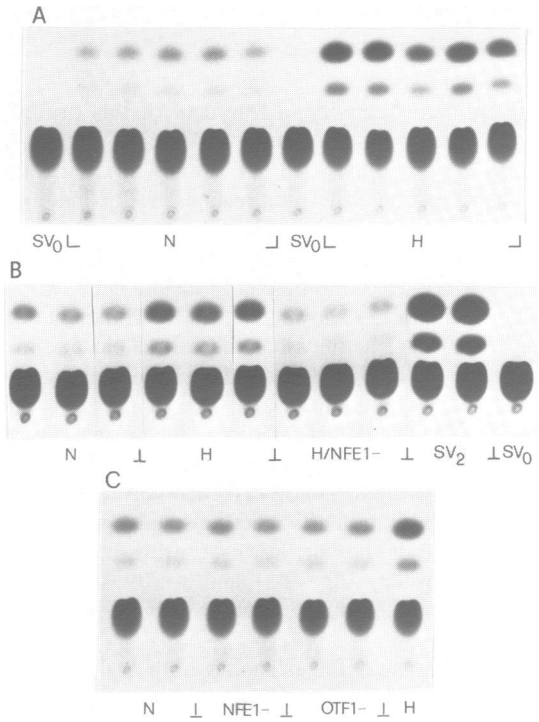

Figure 2. γ-globin promoter activity in K562 cells; CAT assay. N: normal promoter; H: −175 T ► C; NFE1-, OTF1- and H/NFE1-, see Figure 1; SVo and SV2 : pSVo and pSV2 (7). Each lane represents the activity of the cell extract from a single transfection; all transfections shown were carried out in parallel and the results can therefore be directly compared.

low, as compared with that of a reference SV40 enhancer containing vector (pSV2) [7], and no difference could be detected between the various γ-globin promoters (Fig. 3).

The increased expression in K562 cells of the −175 mutant could also be demonstrated by RNAase mapping experiments (Fig. 4)). Using poly A^+-RNA from cells transfected with the −175 mutant a band (B) corresponding in size to that expected for the normal CAP site is seen; a similar, but fainter, band, is also generated with RNA from cells transfected with the normal gene. Similarly, band A is increased using RNA from cells transfected with the mutant; the size of this fragment corresponds to that expected for RNA hybridizing to a shorter fragment of the probe, due to premature stop of *in vitro* transcription. None of these bands is visible when RNA is added to the hybridization mixture at the end of the hybridization, just before RNAase digestion, or when RNA from mock-transfected cells is used; these data indicate that the observed bands are not due to incomplete digestion of the RNA probe and are related to the transfected gene. The increased intensities of bands A and B obtained with RNA from cells transfected with the HPFH plasmid cannot be due to differences in the amount of poly A^+ RNA hybridized; in fact (Figure 4B) the 35 nucleotide-long RNA band generated by protection of the probe by endogenous γ-globin RNA, is identical using RNA from cells transfected with normal and HPFH plasmids. In addition to bands A and B, a further band (C) is seen with RNA generated from the

Table I. Chloramphenicol butyrylation by extracts from transfected cells.

	Conversion $\times 10^{-3}$							
Experiment 1	Normal		HPFH					
	4.2		62					
	4.4	Av.	59	Av.				
	5.5	6.3	26	51				
	11.9		65					
	5.6		45					
Experiments 2,3	Normal		HPFH		HPFH/NFE-		pSV2	
	1.2		6		3.1			
	1.6	Av.	7.7	Av.	2.1	Av.		
	2.2	1.7	6.9	6.9	2.3	2.5		
	1.2		7.8		0.3		45.2	Av.
	1.7	1.5	7.8	7.8	0.8	0.7	65.1	55.1
	1.7		7.8		1			
Experiment 4	Normal		OTF1-		NFE1-			
	10.7		12.2		6.7			
	7.5	Av.	7.8	Av.	11.1	Av.		
	6.8	8.9	6.9	9.9	9.4	9		
	10.7		12.8		8.6			

Values are given as proportion of ^{14}C-butyryl-chloramphenicol out of total chloramphenicol (0.2–0.5 μCi) after subtracting the corresponding values obtained with pSVo (typical pSVo values between $2.9-3.9\times10^{-3}$; background values with extracts from mock-transfected cells $1.7-2.8\times10^{-3}$).

−175 HPFH mutant; its 5′ end maps between positions −190 to −170. The nature of this band is unclear and its elucidation requires further studies; it might derive from upstream start sites, which have been previously reported [10,11], or represent an artefact.

DISCUSSION

We have shown in this paper both by CAT assays and by RNA determination that the −175 T ► C mutation in the γ-globin promoter known to cause HPFH is able to increase the activity of this promoter in transfection experiments in human erythroid cells; no similar effect is observed in transfections carried out with non-erythroid cells. The mechanism of this effect can be investigated by relating functional data to changes, induced by the mutation, in *in vitro* binding of nuclear proteins to γ-globin promoter fragments. We previously reported [5] that the −175 mutation greatly decreases the *in vitro* binding of the ubiquitous octamer binding factor (OTF-1) and slightly, but significantly, increases the binding of the erythroid-specific factor NFE1 (in a large series of binding and competition experiments carried out under different binding conditions the increase is approximately two-fold (A.R. and S.N., unpublished results); others [12,13] recently confirmed the decrease of OTF1 binding. By using a promoter with a mutation in the octamer sequence greatly decreasing the binding of OTF1, but leaving unaffected that of NFE1 [5], we rule out the hypothesis that the loss of OTF1 binding is, by itself, responsible for increased expression of the −175 HPFH promoter in transfection experiments. On the other hand, the erythroid-specific overexpression of the HPFH promoter suggests that NFE1 binding may be related to this effect; indeed, a mutation greatly decreasing NFE1, but not OTF1, binding [5], completely suppresses the overexpression of the HPFH mutant when introduced into the −175 HPFH promoter. This result clearly indicates that NFE1

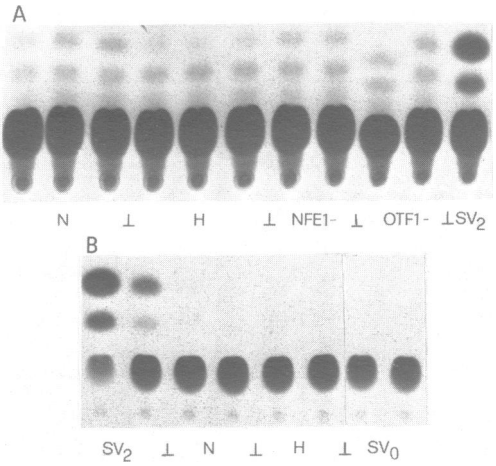

Figure 3. γ-globin promoter activity in BJA/B (A) and HeLa cells (B). In A the autoradiograph was greatly overexposed to allow detection of acetylated chloramphenicol; this underestimates relative pSV2 efficiency. In A 15 μgs of γ-globin plasmid were used for each transfection; pSV2 was at 15 and 3 μgs, in the two transfections in B.

partecipates in the activity of the HPFH promoter and is necessary for it.

It is likely, although not yet formally proven, that the −175 T ► C mutation increases the activity of the γ-globin promoter in transfection experiments by directly modifying NFE1 binding; this is suggested by the observations discussed below. First, no other K562 proteins (besides OTF-1 and NFE-1) appear to bind in the vicinity of position −175 [5]. Second, the −175 HPFH mutation increases NFE-1 binding (5; our unpublished data); although the effect on *in vitro* binding is smaller than the effect on efficiency of transcription, it is possible that increased activity results in part from subtle changes in binding induced by the mutation. For example, Martin *et al.* [13] detected decreased DNAaseI protection (in footprint experiments) of the 3′-end of the NFE-1 binding region of the HPFH mutant (although DMS interference experiments—ref. 5 and unpublished data quoted in ref. 13—do indicate that binding occurs in that region) suggesting that binding may become predominant (and increased, according to our data) at the 5′-end of the NFE1 recognition site.

Finally, the question arises whether the increased expression of the HPFH promoter detected in transfection experiments is related to the *in vivo* HPFH phenotype. While the data suggest this possibility, there is no real proof that this is the case. *In vivo*, HPFH γ-globin genes are overexpressed during the adult period, and, at least in the single case examined (British HPFH, −198 T ► C, refs. 14,15) there is no evidence that overexpression of the HPFH relative to the normal gene occurs before completion of the switch from γ to β-globin synthesis. The K562 cells used in this study show a fetal-embryonic hemoglobin pattern, and are thus not expected to (fully) reproduce the HPFH phenotype ('adult' type mouse erythroleukemic cells express transfected γ-globin plasmids at relatively high basal levels, and are equally not suitable for these experiments, see refs. 16,17); indeed, the increased expression (3 to 9-fold) of the HPFH gene in our transfection experiments is at least an order of magnitude less than the level of overexpression (50−100 fold) observed *in vivo* for $^{A}\gamma$- and $^{G}\gamma$-globin genes carrying the −175 mutation.

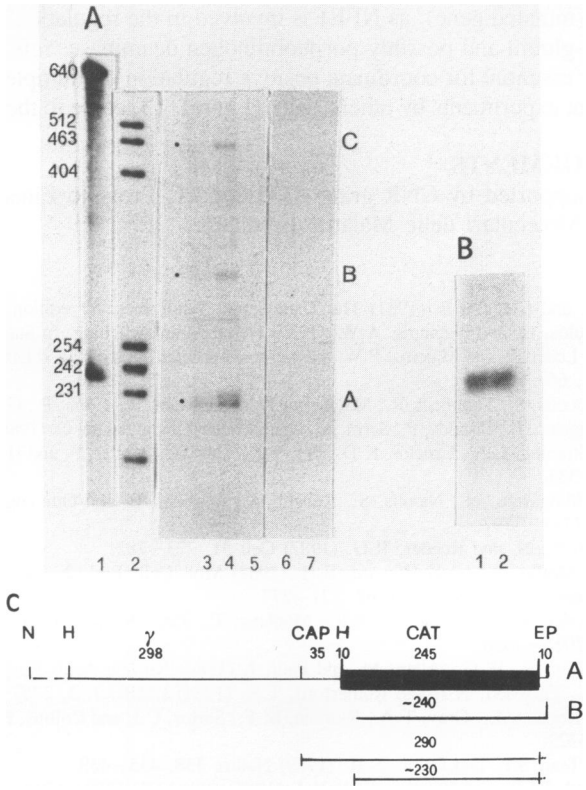

Figure 4. RNAase mapping of γ-globin CAT mRNA from K562 cells transfected with normal and HPFH plasmids. A: lane 1: labelled probe; lane 2: molecular weight markers (HpaII digested pGEM4); lanes 3,4: hybridization of poly A^+ RNA from cells transfected with the normal and HPFH plasmids; lanes 5,6: same RNA, added at the end of hybridization; lane 7: hybridization of RNA from mock transfected cells. B: lanes 1,2: protection of a 35 nucleotide fragment of the probe (from CAP to Hind III linker) by endogenous γ-globin mRNA from cells transfected with normal and HPFH plasmids, respectively: note that this fragment is from the very bottom, of the gel shown in A (lanes 3–4) and can therefore be used as an internal control for the procedure and to check that similar amounts of poly A^+-RNA are loaded onto the gel. C: NdeI-EcoRI fragment of the γ-globin CAT plasmid used as a probe. Transcription by SP6 polymerase starts 10 nucleotides upstream of the EcoRI site and extends 640 nucleotides to the Bam HI site of pGEM4 (probe A); premature termination also generates a short fragment (probe B). The expected protected fragments (290 and ~230 nucleotides) are shown below. pGEM fragments or DNA sequence of the probe (not shown) were used for size determination. B: Bam HI; N: NdeI; H: Hind III linker; E: EcoRI; P: SP6 promoter. RNA sizes are given in nucleotides.

It remains entirely possible that in adult cells additional regulatory proteins, not detectable in K562 cells, bind in the vicinity of position −175 (directly or via protein-protein interaction with either OTF1 or NFE1) and are affected by the −175 mutation. For these reasons at the present time we can neither conclusively prove the postulated role of NFE1 as an activator causing HPFH, nor entirely rule out a role of OTF1 in mediating repression in adult cells [5]. Our data, however, strongly indicate that the binding of NFE1 to the γ-globin promoter in fetal embryonic cells has a positive effect on transcription (at least

in the case of the mutated gene); as NFE1 is involved in the regulation of other erythroid specific genes (β-globin and possibly porphobilinogen deaminase, refs. 18–20) it might represent a factor essential for coordinate positive regulation of multiple cell-type specific genes. Very recent experiments by others, quoted in ref. 13, come to the same conclusion.

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REFERENCES

1. Weatherall, D.J. and Clegg, J.B. (1981) The Thalassemia Syndromes. 3rd edition, Blackwell, Oxford.
2. Stamatoyannopoulos, G. and Nienhuis, A.W. (1987) Hemoglobin switching. In Stamatoyannopoulos, G., Nienhuis, A.W., Leder, P. and Majerus, P.W. (eds), The Molecular Basis of Blood Diseases. W.B. Saunders, Philadelphia, pp. 66–105.
3. Ottolenghi, S., Nicolis, S., Taramelli, R., Malgaretti, N., Mantovani, R., Comi, P., Giglioni, B., Longinotti, M., Dore, F., Oggiano, L., Pistidda, P., Serra, A., Camaschella, C. and Saglio, G. (1988) Blood **71**, 815–817.
4. Stoming, T.A., Stoming, G.S., Lanclos, K.D., Fei, Y.J., Altay, C., Kutlar, F. and Huisman, T.H.J. (1989) Blood **73**, 329–333.
5. Mantovani, R., Malgaretti, N., Nicolis, S., Ronchi, A., Giglioni, B. and Ottolenghi, S. (1988) Nucleic Acids Res. **16**, 7783–7797.
6. Fletcher, C., Heintz, N. and Roeder, R.G. (1987) Cell **51**, 773–782.
7. Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Mol. Cell. Biol. **2**, 1044–1051.
8. Seed, B. and Sheen, J.Y. (1988) Gene **67**, 271–277.
9. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Nucleic Acids Res. **12**, 7035–7056.
10. Grindlay, G.J., Lanyon, W.G., Allan, M. and Paul, J. (1984) Nucleic Acids Res. **12**, 1811–1820.
11. Partington, G.A., Yarwood, N.J. and Rutherford, T.R. (1984) EMBO J. **3**, 2787–2792.
12. Gumucio, D.L., Rood, K.L., Gray, T.A., Riordan, M.F., Sartor, C.I. and Collins, F.S. (1988) Mol. Cell. Biol. **8**, 5310–5322.
13. Martin, D.I.K., Tsai, S.F. and Orkin, S.H. (1989) Nature **338**, 435–438.
14. Tate, V.E., Wood, W.G. and Weatherall, D.H.J. (1986) Blood **68**, 1389–1393.
15. Wood, W.G., Macrae, I.A., Darbre, P.D., Clegg, J.B. and Weatherall, D.J. (1982) Br. J. Haematol. **50**, 401–414.
16. Charnay, P. and Henry, L. (1986) Eur. J., Biochem. **159**, 475–478.
17. Ottolenghi, S., Giglioni, B., Comi, P., Mantovani, R., Malgaretti, N., Nicolis, S., Longinotti, M., Sciarratta, V., Pirastu, M., Camaschella, C. and Saglio, G. (1987) In 'Developmental Control of Globin Gene Expression', Stamatoyannopoulos, G. and Nienhuis, A.W. (eds.) Alan R. Liss, Inc., New York, pp. 373–382.
18. Evans, T., Reitman, M. and Felsenfeld, G. (1988) Proc. Natl. Acad. Sci. USA **85**, 5976–5980.
19. de Boer, E., Antoniou, M., Mignotte, V., Wall, L. and Grosveld, F. (1988) EMBO J. **7**, 4203–4212.
20. Mignotte, V., Wall. L., de Boer, E., Grosveld F. and Romeo, P.H. (1989) Nucleic Acids Res. **17**, 37–54.