Protein – DNA interactions upstream from the Human A gamma globin gene

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ABSTRACT

We have used DNAase I footprinting and the gel mobility shift assay to study proteins which bind to promoter elements located between – 140 and – 382 upstream of the human A gamma globin gene. Footprints are found with both erythroid and non-erythroid nuclear extracts at three sites: from – 294 to – 264, – 242 to – 227, and – 189 to – 172 from the transcription initiation site. An erythroid-specific footprint is identified from – 194 to – 189. We demonstrate that two known transcription factors, the ubiquitous octamer-binding protein OTF-1 and the erythroid regulatory factor NFE-1, bind to the – 194 to – 172 region and that their footprints overlap. Binding of OTF-1 to this region is reduced by a mutation at – 175 associated with a form of non-deletion hereditary persistence of fetal hemoglobin. We conclude that OTF-1 may compete with NFE-1 for the – 175 binding site, possibly functioning as a repressor of gamma globin transcription.

INTRODUCTION

The human beta-like globin genes are a model system for the study of the regulation of eukaryotic transcription, since they represent a family of genes which are expressed in a tissue-specific manner and are switched on and off in succession at specific developmental stages. There are two switches during development, the first occurring in early fetal life, when the transcription of the epsilon globin gene is switched off and replaced by that of gamma globin; the second occurs shortly following birth, when gamma globin synthesis is replaced by that of beta globin. In cultured cell lines, globin transcription is also inducible with a variety of chemical compounds (e.g. hemin or DMSO) (for review, see 1).

The regulation of transcription has been shown in other systems to be controlled by the sequence-specific binding of regulatory proteins to promoter and enhancer elements (2–4). The level of transcription is thought to be controlled by the interaction of the promoter and enhancer-binding factors with other regulatory proteins (4,5). Almost all of the proteins identified to date which bind to globin regulatory elements are ubiquitous factors present

in a variety of tissues and throughout development (6–10). An erythroid specific DNA-binding protein, variously termed NFE-1 or GF-1 in mammals or Eryf1 in chickens, has been recently identified (9,11), and since this paper was originally submitted, its cDNA has been cloned from both mouse and chicken libraries (12,13). NFE-1 appears to be a transcriptional activator, but is present throughout development and seems to serve in the general activation of erythroid-specific genes, rather than in hemoglobin switching (13,14). In the chicken system a 'stage selector element' has been described in a globin promoter (15), and a stage-specific regulatory protein has been recently identified (16). The factors responsible for the switch from fetal to adult globin synthesis in mammals, however, remain uncharacterized.

In this report, we examine the role of proteins in human erythroleukemia (K562) cells which bind to DNA upstream from the human A Gamma globin promoter, from – 140 to – 382 from the transcription initiation site. This region is of interest since there are a number of naturally occurring point mutations in humans, at – 175 (17,18), – 158, – 196, – 198 and – 202 (1) which are associated with the abnormal persistence of gamma globin expression into adult life (non-deletion hereditary persistence of fetal hemoglobin, or HPFH). It would be expected that promoter mutations might affect the binding of regulatory proteins, and an understanding of these molecular events might help clarify the mechanism of the fetal to adult hemoglobin switch. We show that two transcription factors, NFE-1 and OTF-1, bind to overlapping sites in the – 175 region, and that the – 175 HPFH mutation results in decreased binding of OTF-1.

MATERIALS AND METHODS

Growth of Cell Lines and Preparation of Nuclear Extracts

The human erythroleukemia cell line K562 was grown in RPMI 1640 medium with 10% fetal bovine serum (FBS). For induction, log phase cells were cultured in the presence of 20 micromolar bovine hemin (Sigma) and the cells were harvested after 4 days. HeLa cells were grown in spinner culture in Joklik's minimum essential medium (Flow Labs) with 10% FBS. EL-4 cells were grown in RPMI 1640 medium with 5% FBS and 50 micromolar 2-mercaptoethanol.

Nuclear extracts were prepared as described (19) with no

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modifications except for the addition of leupeptin, pepstatin and aprotinin (1 microgram/ml each) to all solutions to inhibit proteolysis.

Fractionation of K562 Nuclear Extract on Heparin Sepharose

A column with a 65 ml bed volume (2.6×12 cm) of heparin sepharose (Pharmacia) was prepared according to the manufacturer's instructions. Approximately 60 mg of crude K562 nuclear extract was passed over the column in buffer A (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.2 mM phenylmethanesulfonyl fluoride, and 1 microgram/ml aprotinin, leupeptin and pepstatin)(20) containing 50 mM (NH₄)₂SO₄ at a flow rate of 10 ml/cm²-hr. The flow-through peak was collected, and protein bound to the column was eluted in steps with buffer A containing 200 mM, 300 mM and 400 mM (NH₄)₂SO₄. Protein from each elution step was precipitated by the addition of 0.35 g/ml solid (NH₄)₂SO₄ and resuspended in a small volume of buffer D (20 mM HEPES-KOH pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, PMSF, aprotinin, leupeptin, pepstatin)(19). The samples were then dialysed exhaustively against buffer D, divided into small aliquots, frozen in liquid nitrogen and stored at -135 degrees C. OTF-1 binding activity was in the 0.2 M fraction, while most DNA-binding proteins were present in the 0.4 M fraction (data not shown).

Preparation of DNA probes and Competitor Oligonucleotides

The probe for DNAase I footprinting was an NcoI-StuI restriction fragment from -140 to -382 upstream from the A gamma globin cap site. The probe was 5' end-labeled on the non-coding strand with T4 polynucleotide kinase (21). A 5' end-labeled HaeIII-AvaII restriction fragment extending from -161 to -203 was used for the gel mobility shift assays. For the competition mobility shift assays, competitor oligonucleotides and their complementary strands were purchased from Genetic Designs, Houston, TX. Complementary strands were annealed in TE buffer by heating to 85 degrees C for 2 minutes, then incubating for 15 minutes each at 65, 37, 21 and 0 degrees.

Gel Mobility Shift Assay

The mobility shift assay was performed essentially as described (22,23). Ten microliters of crude nuclear extract containing 20 micrograms of protein (5 to 10 micrograms when using heparin sepharose-fractionated protein) was mixed with 10 microliters of binding buffer (10 mM Tris-HCl pH 8.0, 5 mM MgCl₂, 100 mM KCl), 1 to 4 micrograms double-stranded poly (dI-dC) competitor DNA (Pharmacia), and 10,000 CPM (approximately 0.1 ng) of 5' end-labeled DNA probe, and incubated on ice for 30 minutes. The reaction mixture was then loaded directly on a 5% polyacrylamide gel and electrophoresed with 0.5X TBE (21) at 200 V for 1 to 3 hours at room temperature. The gels were then dried and analyzed by autoradiography.

DNAase I Footprinting

DNAase I footprints were performed essentially as described (24), with modifications. One microliter of an end-labeled DNA fragment (approximately 0.5 ng) was added to a reaction mixture containing 13 microliters of nuclear extract in buffer D (see above), 10 microliters of footprint buffer (25 mM HEPES-NaOH pH 7.9, 80 mM NaCl, 12.5 mM MgCl₂, 1 mM DTT, 10% glycerol and 0.1 mg/ml BSA), and one microliter of double-stranded poly (dI-dC) competitor DNA. For crude nuclear

Figure 1. DNAase I footprint, -140 to -382 A gamma globin probe, 5' end-labeled on non-coding strand. Lanes g, ga, tc and c: chemical sequencing ladders. Lanes 1 and 2, no nuclear extract; lanes 3 and 4, K562 nuclear extract; lanes 5 and 6, EL-4 nuclear extract. All footprint reactions are performed in duplicate (lanes 1 and 2 are identical, as are lanes 3 and 4, as are lanes 5 and 6). Footprints with K562 nuclear extract are designated by brackets labeled 'K,' EL-4 footprints by brackets labeled 'E.' EL-4-specific footprint marked by open arrow, K562-specific footprint by filled arrow.

extracts, 40 micrograms of protein and 4 micrograms of poly (dI-dC) were used in the incubation mixture, which was treated with 50 ng of DNAase I (Worthington) for 5 min on ice. For heparin sepharose fractions, 20 micrograms of protein was incubated with 1 microgram of poly (dI-dC), and the reaction mixture was treated with 10 to 15 ng of DNAase I for 5 minutes on ice. Control reactions contained no poly (dI-dC) and were treated with 5 ng DNAase I for 5 min on ice. DNAase I was stored at -20 degrees C as a 2 mg/ml stock solution in 20 mM Tris-HCl pH 7.5, 1 mM MgCl₂ and 50% glycerol.

RESULTS

DNAase I Footprinting Using Erythroid and Non-Erythroid Nuclear Extracts

A DNAase I footprint of the 242 bp NcoI-StuI fragment (-140 to -382 with respect to the A Gamma globin cap site) is shown in figure 1. Nuclear extracts from two different cell lines were

Figure 2. Gel mobility shift assay, -161 to -203 A gamma globin (gamma-42) probe. Lane P, no nuclear extract; lane H, HeLa nuclear extract; lane K-, uninduced K562 nuclear extract; lane K+, induced K562 nuclear extract.

used: the human erythroleukemia cell line K562, which expresses the embryonic and fetal epsilon and gamma globin genes, but not the adult beta globin gene, and a human T lymphocyte cell line, EL-4, which does not express globin mRNA. Three footprints are readily seen with both extracts, from -294 to -264, from -242 to -227, and from -189 to -172 (fig. 1). A K562-specific footprint is seen from -194 to -189, and possibly from -172 to -167. K562-specific DNAase I hypersensitive cleavages are seen at about -200. An EL-4 specific footprint is seen from -264 to -252.

NFE-1 and OTF-1 Bind to the -194 to -172 Region

To further explore the protein-DNA interactions which occur in the -194 to -172 region, we performed gel mobility shift assays using a 42 base pair HaeIII-AvaII fragment (gamma-42), which includes DNA from -203 to -161. In figure 2 we compare the gel shift patterns seen with uninduced K562 nuclear extract, K562 nuclear extract prepared from cells given a 4 day induction with hemin, and extract from HeLa (human cervical carcinoma) cells. All extracts produced a band of low mobility (arrow A), while two higher mobility bands (arrows B and C) are seen only with K562 nuclear extracts. The intensity of band C appears to decrease with hemin induction, although this varies with individual preparations of nuclear extract. Bands of identical mobility to B and C are seen using murine erythroleukemia

Figure 3. Gel mobility shift assay, gamma-42 probe. Lane 1, no nuclear extract; lanes 2-6, K562 nuclear extract. Lane 2, no competitor DNA; lanes 3 and 4, 10 and 50 ng, respectively, of human 3' beta globin enhancer competitor DNA containing an NFE-1 recognition site (5'-GTCTGCTGGCTCCCTTATCATGTCCCT-3'); lanes 5 and 6, 10 and 50 ng of -187 to -170 A gamma globin competitor DNA containing the octamer sequence (5'-TCTCAATGCAATATCTG-3').

(MEL) nuclear extract, and are not seen in gel shifts using EL-4 nuclear extract (not shown). Band A was seen with all extracts tested. All bands could be competed away by unlabeled gamma-42 probe, but not by an unrelated 30 bp fragment of gamma globin promoter DNA (-161 to -131, data not shown).

In order to determine if band A represented OTF-1, and if either or both of the erythroid-specific bands, B and C, represented NFE-1, we performed mobility shift assays using two unlabeled competitor oligonucleotides: one a -187 to -170 gamma globin oligonucleotide with the octamer sequence, the other a human beta globin enhancer oligonucleotide (9) which binds only NFE-1 (fig. 3). Band A was completely competed by the octamer oligonucleotide, but not by the NFE-1 binding site, while both bands B and C were competed away by the NFE-1 binding site, but not by the octamer sequence. These results suggest that both bands B and C represent NFE-1 binding activity. We believe that bands B and C may represent either different forms of the same protein or different proteins that bind the same sequence, or that band C represents a proteolytic

Figure 4. Gel mobility shift assay, gamma-42 probe, K562 nuclear extract. Lane 1, crude K562 nuclear extract; lane 2, 0.2 M ammonium sulfate fraction eluted from heparin sepharose column; lane 3, 0.4 M ammonium sulfate fraction.

cleavage product of the protein represented by band B (as has been previously suggested (8)).

Footprinting of Partially Purified NFE-1 and OTF-1

In order to better characterize the binding sites of OTF-1 and NFE-1, we first separated them by chromatography of crude K562 nuclear extract on heparin sepharose. As seen in figure 4, OTF-1 binding activity elutes from the column with 0.2 M ammonium sulfate, while NFE-1 activity elutes with 0.4 M ammonium sulfate. We then compared the footprint of the 0.2 M fraction with the 0.4 M fraction using the same probe as in figure 1. Figure 5 shows that the NFE-1 and OTF-1 footprints partially overlap, the 0.4 M (NFE-1) footprint extending from -194 to -175, and the 0.2 M (OTF-1) footprint from -189 to -172. In addition, there is a faint suggestion that the 0.4 M footprint extends to -167, covering a degenerate (6 out of 8 match) downstream NFE-1 recognition sequence. The K562-specific DNAase I hypersensitive sites at -200 elute in the 0.4 M fraction. The results are summarized in figure 6.

It is interesting to note that the -242 to -227 footprint obtained with crude nuclear extract (fig. 1) is not present in either ammonium sulfate fraction. In addition, the protein (or proteins) responsible for the -294 to -264 footprint in figure 1 elute(s) from heparin sepharose with 0.2 M ammonium sulfate, although the footprint is now smaller (-294 to about -280; the protection could extend downstream of -280, but the lack of DNAase I cleavage of the control DNA prevents a clear interpretation). It

Figure 5. DNAase I footprint, fractionated K562 nuclear extract. -140 to -382 A gamma globin probe, 5' end-labeled on non-coding strand. Lane ag, A+G chemical sequencing ladder; lanes 1 and 2, no nuclear extract; lanes 3 and 4, 0.2 M ammonium sulfate heparin sepharose fraction; lanes 5 and 6, 0.4 M ammonium sulfate fraction. Footprint reactions are performed in duplicate, as in figure 1. Footprints from 0.2 M fraction are marked by brackets labeled '0.2,' footprint from 0.4 M fraction with bracket labeled '0.4.' In the -175 region (lower brackets), the 0.2 M footprint extends from nucleotides -189 (top of bracket) to -172 (bottom of bracket), while the bracket marking the 0.4 M footprint extends from -194 (top) to -175 (bottom). The arrow designates a faint extension of the 0.4 M footprint to -167.

is possible that proteins which interact with these upstream sites may have been removed during purification, although all of the known sequence-specific DNA-binding proteins that we have attempted to purify elute in either the 0.2 M or 0.4 M fractions (data not shown).

The -175 HPFH Mutation Results in Decreased Binding of OTF-1 to its Recognition Site

To determine the effect of the -175 HPFH mutation on the binding of NFE-1 and OTF-1, we employed the gel mobility shift

Figure 6. Summary of DNAase I footprinting data. NFE-1 and OTF-1 footprints are marked by the open and filled rectangles, respectively. The region faintly suggestive of a downstream continuation of the NFE-1 footprint is designated by the line extending to the right from the open rectangle. The octamer recognition sequence is underlined, and the -175 nucleotide is marked by an *. The two potential NFE-1 recognition sequences are marked by brackets (the consensus NFE-1 recognition sequence is A/C Py T/A ATC A/T Py (9)). The upstream sequence is an 8 for 8 match with the consensus, while the downstream site is a 6 for 8 match. The T to C HPFH substitution at -175 changes the downstream site to a 5 for 8 match, in addition to changing the octamer sequence.

assay using unlabeled competitor oligonucleotides. Figure 7 shows that band A (which represents OTF-1) is competed away by the -187 to -170 oligonucleotide containing the wild type octamer (ATGCAAAT) sequence (as in fig. 3), but not by the same oligonucleotide containing the -175 T to C mutation. In addition, direct binding studies using the -175 mutant oligonucleotide as a probe show a marked reduction in the intensity of band A relative to the wild-type probe (not shown).

DISCUSSION

By comparing the DNAase I footprints of partially purified NFE-1 and OTF-1 we have obtained evidence that NFE-1 binds to a site upstream from but overlapping with the binding site for OTF-1. The two binding sites have a 15 base pair region in common, from -189 to -175 . In addition, we show that NFE-1 binds only very weakly to a degenerate (6 out of 8 match) NFE-1 recognition sequence just downstream from the octamer sequence. This recognition site has the -175 nucleotide in common with the octamer sequence. These data are summarized in figure 6. Competition experiments show that a -187 to -170 oligonucleotide with the -175 HPFH mutation has decreased affinity for OTF-1 compared to an oligonucleotide with the wild-type sequence (figure 7).

Since the submission of this paper, a number of groups have published data on DNA-protein interactions in the -175 region, using the gel mobility shift assay, DNAase I footprinting, methylation interference and transfection of genes with normal and mutant gamma globin promoters into mammalian cells (14,25,26). The results of these and earlier reports (8,27) have been somewhat conflicting, particularly concerning the size and position of the respective footprints for OTF-1 and NFE-1. Previous studies have shown footprints of the two binding activities in the -175 region by eluting the different bands from a gel shift assay and cleaving the bound DNA using the copper-orthophenanthroline method (8). These studies show that gel shift bands in both erythroid and non-erythroid nuclear extracts correspond to a footprint over the octamer sequence, extending from -184 to -171 , while erythroid specific binding activity shows an upstream, overlapping footprint protecting nucleotides -191 to -180 .

Two other groups performing DNAase I footprints of this region have separated the octamer-binding protein (OTF-1) from

Figure 7. Gel mobility shift assay, gamma-42 probe, K562 nuclear extract. The lanes designated 'octa' represent the addition of 60 and 120 ng of -187 to -170 A gamma globin competitor oligonucleotide (5'-TCTCAATGCAAATATCTG-3'). The lanes designated ' -175 M' represent the addition of 60 and 120 ng of competitor DNA identical to the -187 to -170 oligonucleotide except for the -175 mutation (5'-TCTCAATGCAAAATCTG-3').

the erythroid-specific binding activity (NFE-1) by fractionation of K562 nuclear extracts on heparin sepharose columns (14,25). Both groups report similar findings: the OTF-1 footprint covers the octamer sequence, extending from -190 to either -174 (14) or -168 (25), while the NFE-1 footprint is much larger than previously reported (8), extending both 5' and 3' from the OTF-1 footprint and covering a potential downstream NFE-1 recognition site.

Our results differ from these latter two reports. We demonstrate a footprint for NFE-1 similar to that originally described (8), upstream from and overlapping with the OTF-1 footprint. Our studies show that the potential downstream NFE-1 recognition sequence is bound only very poorly, if at all, by NFE-1. This interpretation of the footprinting data is supported by the gel shift in figure 3, which demonstrates that an oligonucleotide containing the octamer sequence and the downstream NFE-1 recognition sequence does not compete the NFE-1 binding activity of the -203 to -161 probe, even in 100-fold molar excess. These results may be explained by the 8 of 8 nucleotide match of the upstream NFE-1 recognition sequence with the known consensus recognition sequence for NFE-1 (9), as compared to only a 6 of 8 match for the potential downstream NFE-1 sequence.

While there is general agreement that the -175 T to C mutation significantly reduces OTF-1 binding, the affinity of NFE-1 for the -175 region has been variously reported to be increased by the mutation (27), or to be unaffected by it (8,26). In addition, Martin *et al* (14) suggest that the mutation may alter the way

in which NFE-1 binds to the gamma globin promoter. By comparing footprints of affinity-purified NFE-1 on wild-type and -175 mutant probes, these studies show that with the -175 mutation the downstream NFE-1 recognition sequence is no longer protected. This report (14) also indicates that mutations in the octamer binding sequence other than at the -175 position abolish OTF-1 binding but do not alter the level of transcription of reporter genes linked to these mutant gamma globin promoters when transiently expressed in erythroid cell lines. These data suggest that OTF-1 does not mediate the increased expression of gamma globin in this form of HPFH, and that OTF-1 may not act as a repressor of gamma globin transcription by blocking NFE-1 binding, as has been proposed by other groups (8,26,27). Alternatively, it was suggested that the conformational change in NFE-1 binding following the mutation may mediate the elevated level of gamma globin transcription (14).

Our data conflict with this interpretation, since we demonstrate that the downstream NFE-1 recognition sequence is bound only very poorly by NFE-1, even without the -175 mutation. Thus, our data are consistent with a competition between octamer and NFE-1 proteins for binding in this region. We do not exclude the possibility, however, that the -175 mutation may directly affect NFE-1 binding. We are currently using purified octamer and NFE-1 proteins to further define these interactions.

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REFERENCES

1. Stamatoyannopoulos, G. and Nienhuis, A.W. (1987) 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.
2. Jones, N.C., Tigby, P.W.J. and Ziff, E.B. (1988) *Genes Dev.*, **3**, 1845-1859.
3. Maniatis, T., Goodbourn, S. and Fischer, J.A. (1987) *Science*, **236**, 1237-1244.
4. Ptashne, M. (1986) *Nature*, **322**, 697-701.
5. Ptashne, M. (1988) *Nature*, **335**, 683-689.
6. Emerson, B.M., Lewis, C.D. and Felsenfeld, G. (1985) *Cell*, **41**, 21-30.
7. Mantovani, R., Malgaretti, N., Giglioni, B., Comi, R., Capellini, N., Nicolis, S. and Ottolenghi, S. (1987) *Nucleic Acids Res.*, **15**, 9348-9363.
8. Gumucio, R., Rood, K., Gray, T., Riordan, M., Sarter, C. and Collins, F. (1988) *Mol. Cell. Biol.*, **8**, 5310-5322.
9. Wall, L., deBoer, E. and Grosveld, F. (1988) *Genes Dev.*, **2**, 1089-1100.
10. deBoer, E., Antoniou, M., Mignotte, V., Wall, L. and Grosveld, F. (1988) *EMBO J.*, **7**, 4203-4212.
11. Evans, T., Reitman, M. and Felsenfeld, G. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5976-5980.
12. Tsai, S., Martin, D.I.K., Zon, L.I., D'Andrea, A.D., Wong, G.G. and Orkin, S.H. (1989) *Nature*, **339**, 446-451.
13. Evans, T. and Felsenfeld, G. (1989) *Cell*, **58**, 877-885.
14. Martin, D.I., Tsai, S. and Orkin, S.I. (1989) *Nature*, **338**, 435-438.
15. Choi, O.B. and Engel, J.D. (1988) *Cell*, **55**, 17-26.
16. Gallarda, J.L., Foley, K.P., Yang, Z. and Engel, J.D. (1989) *Genes Dev.*, **3**, 1845-1859.
17. Surrey, S., Delgrosso, K., Malladi, P. and Schwartz, E. (1988) *Blood*, **71**, 807-810.
18. 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.
19. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) *Nucleic Acids Res.*, **11**, 1475-1489.
20. Sorger, P.K., Ammerer, G. and Shore, D. (1989) In Creighton, T.E. (ed.), *Protein Function, a Practical Approach*. IRL Press, Oxford, pp. 199-223.
21. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
22. Strauss, F. and Varshavsky, A. (1984) *Cell*, **37**, 889-901.
23. Sinh, H., Sen, R., Baltimore, D. and Sharp, P. (1986) *Nature*, **319**, 154-156.
24. Lichtsteiner, S., Wuarin, J. and Schibler, U. (1987) *Cell*, **51**, 963-973.
25. Catala, F., deBoer, E., Habets, G. and Grosveld, F. (1989) *Nucleic Acids Res.*, **17**, 3811-3827.
26. Lloyd, J.A., Lee, R.F. and Lingrel, J.B. (1989) *Nucleic Acids Res.*, **17**, 4339-4352.
27. Mantovani, R., Malgaretti, N., Nicolis, S., Ronchi, A., Giglioni, B. and Ottolenghi, S. (1988) *Nucleic Acids Res.*, **16**, 7783-7787.