Transcription Factor OTF-1 Interacts with Two Distinct DNA Elements in the $^{A}\gamma$-Globin Gene Promoter†

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ABSTRACT: A DNA region (site II) in the promoter of the human $^{A}\gamma$-globin gene (−182 to −168) is involved in transcriptional regulation. At least two nuclear proteins bind to this region: the erythroid-specific factor NF-E1/GF-1 and another factor present in many cell lines. In the present study, we demonstrate that the ubiquitous factor binding to site II has immunological identity with the octamer transcription factor OTF-1, which has been implicated in the regulation of expression of genes such as histone H2b and small nuclear RNA. In addition, we show that OTF-1 binds to site I (−291 to −267), a purine-rich region upstream of site II. Interestingly, OTF-1 binds to sites I and II with equal affinity. This was unexpected since the 14 bp site I binding site AAGAATAAATTAGA (−291 to −278), determined by methylation interference, does not show obvious similarities to the canonical octamer binding site for OTF-1 in site II (ATGCAAAT). Interaction of OTF-1 with functionally active binding sites in the γ-globin promoter suggests that this factor has a role in γ-globin transcription.

Developmental regulation of the human β-globin gene locus involves a switch from fetal (γ) to adult (β) globin which initiates at approximately 32 weeks of gestation. Six months after birth, the switch is complete, and fetal hemoglobin (HbF), containing γ-globin chains, decreases and remains less than 1% of all the β-like globins through adult life (Collins & Weissman, 1984). The mechanism by which this switch occurs is not understood. In the asymptomatic condition known as hereditary persistence of fetal hemoglobin (HPFH), the switch from γ- to β-globin is incomplete, and significant levels of γ-globin continue to be expressed during adult life [reviewed in Poncz et al., (1988)]. The coincidence of the HPFH phenotype with spontaneous point mutations within the immediate 200 bp 5′ of the γ-globin transcription start site at positions −117, −158, −175, −196, −198, and −202 (Weatherall et al., 1975; Friedman & Schwartz, 1976; Collins et al., 1984, 1985; Giglioni et al., 1984; Gilman & Huisman, 1985; Gelinas et al., 1985, 1986; Waber et al., 1986) indicates that this DNA region is involved in γ-globin gene expression. In this region of the $^{A}\gamma$-globin promoter, two of the sites that bind nuclear factors and appear to have functional significance have been identified by using gel mobility shift and functional assays (Lingrel et al., 1987; Mantovani et al., 1987, 1988; Gumucio et al., 1988, 1990; Lloyd et al., 1989; Martin et al., 1989; O'Neill et al., 1990). One of these sequences, site II, spans the position −182 to −168 bp and contains the conserved octamer motif ATGCAAAT. This octamer sequence is an important functional element which has been implicated in promoter and enhancer function of the immunoglobulin genes (Bergman et al., 1984; Falkner & Zachau, 1984; Mason et al., 1985; Ballard & Bothwell, 1986; Mizushima & Roeder, 1986) and other non-tissue-specific promoters such as those of the histone H2b (La Bella et al., 1988) and small nuclear RNA [reviewed in Carbon et al. (1987)]. Tissue-specific (OTF-2) and/or ubiquitous (OTF-1) nuclear factors interact with octamer elements in these genes. In the γ-globin promoter, a nuclear protein present in erythroid and nonerythroid cell lines binds to the ATGCAAAT octamer motif which is flanked by a bipartite binding site for the erythroid-specific factor NF-E1/GF-1. An HPFH (T → C) substitution at position −175 within the octamer sequence decreases binding of the ubiquitous octamer binding factor (Mantovani et al., 1987; Gumucio et al., 1988; Lloyd et al., 1989) and increases expression of $^{A}\gamma$-globin–CAT constructs in erythroid cells (Lloyd et al., 1989; Martin et al., 1989; Gumucio et al., 1990), thereby indicating the importance of this DNA region as a regulatory element. The other γ-globin promoter sequence (site I) is located at position −291 to −267. A clustered mutation of this region (−281 to −272) prevents protein binding of a widely distributed nuclear protein and has been proposed as a positive regulator (Lloyd et al., 1989). At present, not all the nuclear factors binding to these two regions have been well characterized. The presence of an octamer sequence in site II suggests that the octamer binding protein OTF-1 (OCT-1 or OBP 100) or possibly other octamer binding proteins are likely candidates for binding to this DNA region. In this study, we demonstrate using a gel mobility shift/immunoassay that the octamer transcription factor OTF-1 (OCT-1, OBP 100), present in K562 and HeLa nuclear extracts, indeed interacts with both site I and site II DNA elements in the human $^{A}\gamma$-globin gene.

Materials and Methods

Cells. Cell lines used were the following: erythroid cell lines, K562 and HEL cells (human myeloid leukemia cells synthesizing embryonic and fetal hemoglobins) and MEL (murine erythroleukemia cells producing mainly adult globin chains); nonerythroid cell lines, HeLa (human uterine carcinoma cells) and FAZA (rat hepatoma cells).

Nuclear Extracts. Nuclear extracts were prepared from the various cell lines following the method of Dignam et al. (1983). The final extracts, subsequently dialyzed for 4 h against 100 volumes of a buffer containing 20% glycerol, 20

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mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethanesulfonyl fluoride (PMSF), were stored in small aliquots at −80 °C. Protein concentrations were measured according to Bradford (1976).

Highly Purified OTF-1. The OTF-1 protein from HeLa cells was obtained according to Scheidereit et al. (1987). Briefly, the nuclear extract was chromatographed sequentially on DEAE-cellulose, BIO-REX 70, and single-stranded DNA–agarose columns. The fractions testing positive for OTF-1 binding were pooled and dialyzed against buffer BC 100 (50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 20% glycerol). The highly purified OTF-1 obtained by this procedure [for purity, see Murphy et al. (1989)] was diluted 1:4 in buffer BC 100 containing 100 μg/mL BSA instead of glycerol when used in binding assays.

Probes and Competitor DNAs. Different DNA samples were radiolabeled for use as probes or added unlabeled as competitors. Fragments, site I −300/−202 (*Bam*HI/*Apa*I) and site II −202/−140 (*Apa*I/*Nco*I), were obtained from the $^{A}\gamma$-globin gene subclone pIBI30-γRL (*Alu*I −300/+36) and subsequently purified by 0.9% agarose gel electrophoresis in 1× TAE. Oligonucleotides (OCTA, 5'-GGATCCATGCAAATAAGCTT-3'; SI, 5'-GATC^{-293}GGAAGAATAAATTAGAGAAAAACTGGA^{-267}-3'; SII, 5'-$^{-190}$CTATCTCAATGCAAATATCTGTCTG^{-166}-3'; H2b, 5'-TGCAG^{-31}TAGAATCGCTTATGCAAATAAGGTGAAGAGTTG^{-63}C-3') and their complements were synthesized, annealed, and purified by 15% PAGE (1× TBE). OCTA represents the canonical histone H2b octamer motif ATGCAAAT flanked by random sequences. SI and SII oligonucleotides contain a partial sequence of their parent fragments site I or site II (γ-globin gene promoter). H2b corresponds to a partial sequence of the histone H2b promoter. Probes were gel-purified on 8% polyacrylamide gels (1× TBE), after 5' end-labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP. Alternatively, overhangs were filled in with the Klenow fragment of DNA polymerase I, [α-^{32}P]dNTPs and excess unlabeled dNTPS. The nonspecific competitors used were either a 171 bp fragment from the human β-globin first intron or a 28 bp fragment from the IBI-30 polylinker region. The concentration of fragments and oligonucleotides was determined by the optical density at 260 nm.

Gel Mobility Shift/Binding Assay. Binding reaction mixtures (20 μL) contained 20 mM HEPES, 60 mM NaCl, 0.5 mM EDTA, 5 mM βME, 2 mM DTT, and 5% glycerol. DNA-radiolabeled probes (0.3–1.5 ng) were incubated with 0–10 μg of nuclear extract total protein in the presence of 1–4 μg of double-stranded poly(dI-dC) heteropolymer (Pharmacia, Inc.) used as nonspecific competitor. Specific DNA fragments or oligonucleotides were included as competitors to test the specificity of the binding or affinity of the protein factor for the DNA probe. Complete mixtures were incubated 30–60 min at 25 °C, followed by electrophoresis in 4% polyacrylamide gels [acrylamide to bis(acrylamide) ratio 20:1] in 0.25× TBE (22.5 mM Tris-HCl/22.5 mM boric acid/0.5 mM EDTA). Gels were run for 2–3 h (15 V/cm, 4 °C), dried, and exposed to XAR-5 film (Kodak). In the binding assays performed with partially purified OTF-1, the conditions of Poellinger and Roeder (1989) were followed, except that the reducing agents were omitted.

Gel Mobility Shift/Immunoassay. In this modification of a gel mobility shift/binding assay, a rabbit immune serum which reacts with the C-terminus of OTF-1 (A. Pierani, unpublished results) was incubated with OTF-1-containing samples for 20 min prior to the addition of the radiolabeled probe. Alternatively, the samples were incubated with preimmune serum used as a control. Subsequently, a gel mobility shift/binding assay was performed as described above. The complete incubation mixture (10 μL) consisted of 1 μL of diluted, highly purified OTF-1 or 5 μg of K562 nuclear extract total protein, 1 μL of serum, 1 μg of poly(dI-dC) double-stranded heteropolymer, and 0.3 ng (50 cps/μL) of radiolabeled probe in 10 mM HEPES, pH 7.9, 60 mM KCl, 4% ficoll, and 100 μg/mL BSA.

Methylation Interference Assay. The *Bam*HI/*Apa*I (−300/−202) fragment was 5' end-labeled at the *Bam*HI site using T4 polynucleotide kinase and [γ-^{32}P]ATP. The adenine and guanine residues of the end-labeled DNA were partially methylated with dimethyl sulfate (Maxam & Gilbert, 1980). The fragment was next used in gel mobility shift/binding assays with either K562 or HeLa nuclear extracts as described above. The free and protein-bound DNAs were electroeluted from a preparative binding assay gel and subsequently treated with 10% piperidine to cleave at the modified bases. Samples were resolved on a 10% sequencing gel and later autoradiographed.

Results

Characterization of Nuclear DNA Binding Proteins. Two DNA regions 5' of the canonical $^{A}\gamma$-globin promoter, site I (−291 to −267) and site II (−182 to −168), may be involved in transcriptional regulation of this gene (Lloyd et al., 1989; Nicolis et al., 1989; Martin et al., 1989; Gumucio et al., 1990). In vitro binding studies have shown that site I and site II restriction fragments bind protein factors present in cell nuclear extracts in a sequence-specific manner (Lingrel et al., 1987; Mantovani et al., 1987, 1988; Gumucio et al., 1988; Lloyd et al., 1989; O'Neill et al., 1990). Site I, enriched in purine residues, has binding activity for a factor found in HeLa and K562 cells which constitutes the most prominent binding activity in this region. A different pattern is found in site II, which has binding activity for at least two nuclear factors: a ubiquitous protein which binds the octamer motif ATGCAAAT, and the erythroid-specific factor NF-E1/GF-1 which interacts with a bipartite binding site adjacent to the octamer sequence (Martin et al., 1989). In the gel mobility shift assay depicted in Figure 1, two radiolabeled restriction fragments of similar size, containing either the site I or the site II sequences, were incubated with HeLa (a nonerythroid) or K562 (erythroid) cell nuclear extracts in order to compare their binding activities. Only the site II probe incubated with the K562 nuclear extract showed the erythroid-specific factor binding activities C-2 and C-3 (lane 4). This assay also revealed an apparent comigration of the non-erythroid-specific complexes C-1 (lanes 1–4), evidenced with both the site I and the site II probes. This result suggested that the same protein factor could be binding to both sites, or alternatively different protein(s) could be generating complexes with the same electrophoretic mobility. (Other minor complexes were also observed which can be attributed to nonspecific binding.)

Site I and II DNA Probes Compete for an Octamer Binding Factor. In order to distinguish if the same or different factors are responsible for the C-1 comigrating complexes, competition binding experiments were conducted. Site I and site II radiolabeled fragments were incubated with a K562 nuclear extract and electrophoresed. As shown in Figure 2A, the formation of the complex C-1 with each probe was specifically competed by homologous sequences (lanes 2 and 7) but not by an equivalent amount of a nonspecific competitor (lanes

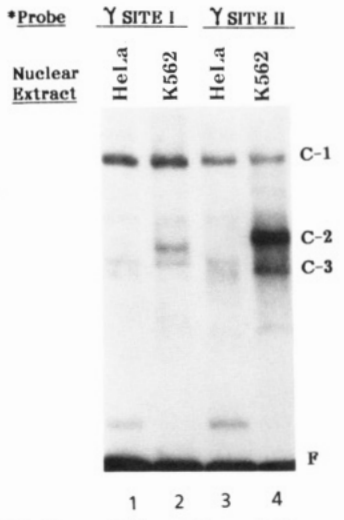

FIGURE 1: DNA binding activities of the γ-globin promoter fragments γ-site I (−300/−260) and γ-site II (−202/−140) incubated with HeLa or K562 nuclear extracts. (γ-site I had 5′-heterologous sequences from the polylinker region of IBI-30, included to make the length of γ-site I and γ-site II comparable). C-1 and C-2 and C-3 denote protein–DNA complexes identified according to their mobility relative to the origin. F indicates free or unbound radiolabeled probe.

1 and 5), demonstrating that the binding is specific. Efficient competition of the site I probe by a site II sequence was also achieved in this assay (lane 3). Similarly, site I effectively competed for the protein(s) binding to site II (lane 6). Taken together, these results suggest that the same factor binds in both sites. Our earlier studies (Lloyd et al., 1989) demonstrated that the ubiquitous factor binding in site II interacts with the octamer motif ATGCAAAT; therefore, it seems plausible that the factor interacting with site I may also be an octamer binding protein. This hypothesis is supported by the results obtained in the same experiment with OCTA, an oligonucleotide containing a core octamer motif flanked by random sequences, which efficiently competed for the C-1 binding with both the site I and the site II probes (lanes 4 and 8).

An Octamer Binding Factor Interacts with Site I, Site II, and a Histone H2b Promoter Sequence. Several octamer binding proteins have been recently characterized (Fletcher et al., 1987; Scheidereit et al., 1987; Staudt et al., 1988; Müller et al., 1988). It was of interest to determine if a sequence from the histone H2b promoter, known to interact with the ubiquitous octamer transcription factor OTF-1, could also exhibit a similar binding activity to that found for sites I and II with K562 nuclear extracts. To test this possibility, three different radiolabeled oligonucleotides, SI, SII, and H2b with analogous electrophoretic mobility, were incubated with a K562 nuclear extract and analyzed in a gel mobility shift assay (see Figure 2B). The protein–DNA complexes obtained with the H2b, SI, and SII probes in the presence of a nonspecific competitor comigrated in the assay (lanes 2, 4, and 6). The formation of the comigrating complexes with the three probes was inhibited by the addition of a 10× molar excess of SI oligonucleotide (Figure 2B, lanes 1, 3, and 5), indicating that the same factor binds to all three sites. This result suggested to us that the K562 nuclear factor binding to sites I and II in the γ-globin promoter could be OTF-1.

Cell-Type Specificity of the Nuclear Protein Binding to Site I. OTF-1 is a ubiquitously distributed protein. To study the tissue distribution of the nuclear protein interacting with site I, the site I binding activities of erythroid (K562, HEL, and MEL) and nonerythroid (HeLa and FAZA) cell nuclear extracts were compared in a gel mobility shift assay. Equivalent protein amounts of each of the nuclear extracts were incubated with the ^{32}P-end-labeled SI oligonucleotide. The K562 sample shown in Figure 3 (lane 3) gave rise to a major retarded protein–DNA complex (C-1) with the same electrophoretic mobility as the complexes formed with each of the other cell nuclear extracts (lanes 1, 5, 7, and 9). The specificity of the

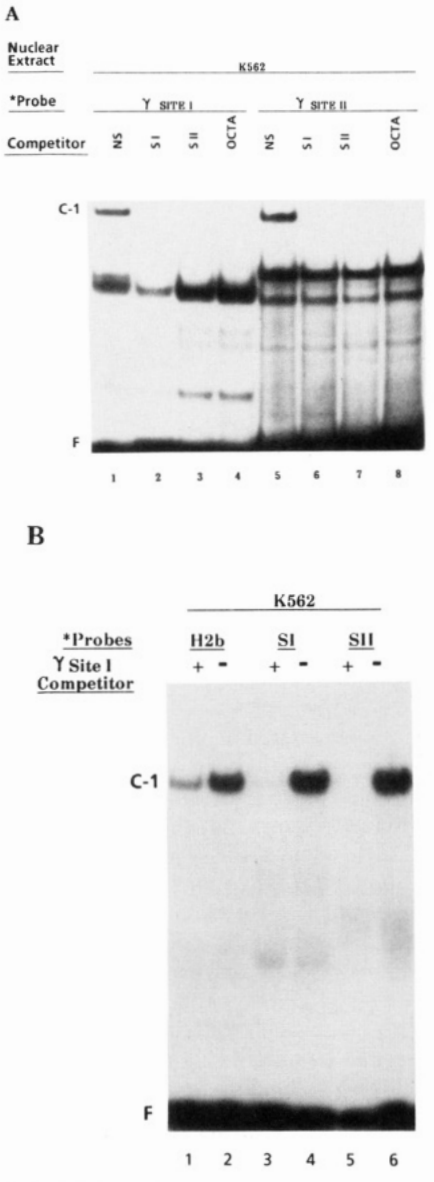

FIGURE 2: (A) Mobility shift assay to demonstrate the specificity of the binding activities in sites I and II. In lanes 1 and 5, γ-site I (−300/−260) or γ-site II (−202/−140) radiolabeled probes were incubated with a K562 nuclear extract to demonstrate in each case the formation of specific complexes. With each probe, the complex C-1 was specifically competed by a 20× molar excess of site I (SI), site II (SII), or OCTA unlabeled oligonucleotides as indicated in the autoradiograph, but not by an equimolar amount of a 28 bp nonspecific competitor (NS) obtained from the polylinker region of IBI-30. SI and SII represented footprinted sequences of the C-1 complexes obtained with site I or site II fragments, respectively. OCTA contains a core octamer motif flanked by unrelated sequences. (B) Mobility shift assay with a K562 nuclear extract to demonstrate comigration of the protein factor C-1 binding to H2b, SI, and SII oligonucleotides and specific competition with the SI sequence. H2b represents the octamer region of the histone H2b promoter, the binding site for the transcriptional regulator OTF-1 in this gene. Probes used in each case are indicated. Specific competitors were added (+ lanes) in 10× molar excess amounts relative to the probes. The control lanes (− lanes) had equimolar amounts of the above-mentioned nonspecific competitor.

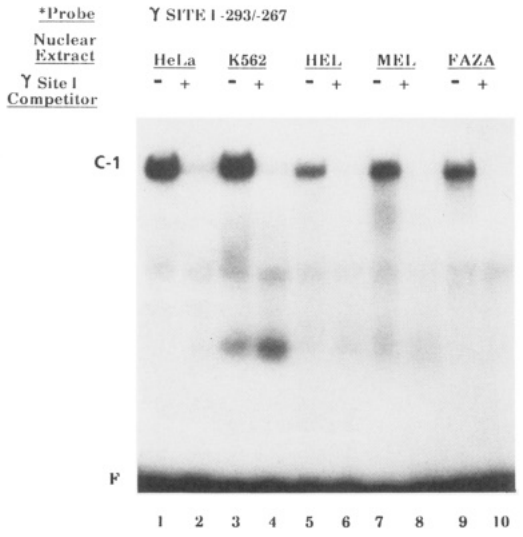

FIGURE 3: Distribution of the site I binding factor C-1 among different cell lines. Nuclear extracts were obtained according to Dignam et al (1983). The gel mobility shift assay was performed as previously described under Materials and Methods. Site I oligonucleotide competitor was added at a 10× molar excess amount relative to the probe. F indicates free probe.

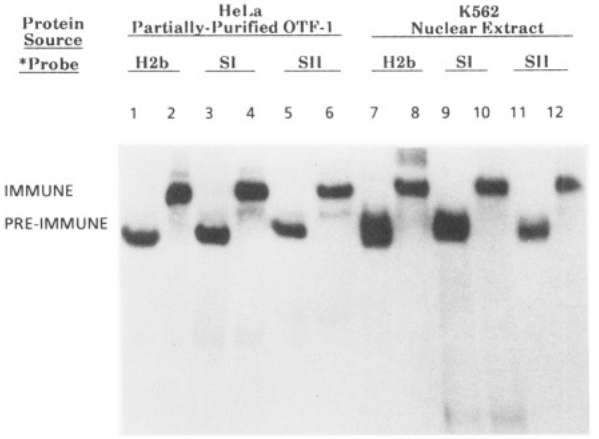

FIGURE 4: Gel mobility shift/immunoassay. Effect of an OTF-1-directed antiserum on the migration of proteins complexed to H2b; SI-, or SII-radiolabeled oligonucleotides. Protein source and probes are indicated. Appropriate amounts of immune (lanes 2, 4, 6, 8, 10, and 12) or control (preimmune) serum (lanes 1, 3, 5, 7, 9, and 11) were included in the incubation mixtures 20 min before addition of the probe. The position of the protein–DNA (preimmune) or protein–DNA–antibody (immune) complexes is indicated. The free probe was run off the gel to obtain better resolution of the complexes.

binding was demonstrated in all cases by a marked reduction of the C-1 binding activity by a 10× molar excess of the unlabeled SI oligonucleotide (lanes 2, 4, 6, 8, and 10). This experiment clearly shows that the nuclear factor binding to site I, like OTF-1, does not have a tissue-specific distribution.

Identification of OTF-1 as the Ubiquitous Octamer Binding Protein Interacting with both Site I and Site II. To determine if the C-1 binding activity is indeed OTF-1, highly purified OTF-1 and an antiserum that reacts with both recombinant and natural OTF-1 (Pierani and Roeder, unpublished data) were used in a gel mobility shift/immunoassay. In this assay, incubation of the samples with a specific antiserum precedes a gel mobility shift/binding assay. Simultaneous specific interaction of the protein with the antibody and the DNA probe results in ternary complexes of antibody–protein–DNA with slower electrophoretic mobility compared to protein–DNA complexes not bound by the antibody. Detection of immunoreactive complexes indicates which samples contain the protein bound by the antibody. The advantage of this technique over immunoprecipitation is that it permits simultaneous electrophoretic analysis of immunoreactive and nonimmunoreactive complexes. The experiment shown in Figure 4 depicts a gel mobility shift/immunoassay. In lanes 1, 3, and 5, three different probes, OCTA, SI, or SII were added to protein-containing samples which have been preincubated with a control serum. The resulting complexes had similar electrophoretic mobility to those C-1 complexes obtained with each of the probes and a K562 nuclear extract in the absence of serum (not shown). In contrast, preincubation of highly purified OTF-1 with the immune serum produced a further shift of the major retarded bands relative to the controls due to the formation of antibody–protein–DNA complexes (lanes 2, 4, and 6). The significant shift of the protein–DNA complexes caused by the antiserum indicates that OTF-1 or an antigenically closely related protein is part of these complexes. Similar results were obtained when highly purified OTF-1 was substituted by K562 nuclear extract in the assays (lanes 7–12). Comigration of the complexes obtained with either the purified protein or the nuclear extract suggests that no additional factors in the extract contribute to the C-1 complexes. Other minor bands were not immunoreactive, indicating that they are not related to OTF-1. These results prove that OTF-1 or an antigenically closely related protein in K562 nuclear extracts is the main factor of the C-1 complexes interacting with sites I and II of the γ-globin promoter in gel mobility shift assays.

Site I Binding Sequence. Having demonstrated that OTF-1 binds to both site I and site II, the differences in the nucleotide sequences of these two sites raise the question of how this interaction occurs. Our studies have clearly shown (Lloyd et al., 1989) that the nonerythroid factor binding to site II, here identified as OTF-1, binds specifically to the ATGCAAAT octamer motif. The nucleotides in site I which interact with OTF-1 were elucidated by methylation interference analysis. Binding assays were performed by incubating K562 or HeLa nuclear extracts with a ^{32}P-end-labeled site I (−300/−202) DNA fragment which had been partially methylated at the A and G residues using dimethyl sulfate. Free (F) and bound (B) DNAs, resolved in a gel retardation assay, were subsequently treated with piperidine to cleave at the modified bases. The electrophoretic analysis of the piperidine-treated samples on a sequencing gel is shown in Figure 5. It revealed the A and G residues essential for the binding which can be detected by decreased cleavage in B relative to F in the autoradiograph. Residues in site I, which undergo contact with the protein, encompass positions −291 to −278 on the coding strand with both the HeLa and the K562 samples. The adenine residues at positions −286, −282, and −281 on the noncoding strand also appear to interact with the protein, thus complementing the results obtained with the coding strand (unpublished data). The site I binding sequence for the ubiquitous factor, as summarized in Figure 5, spans 14 bp, AAGAATAAAT-TAGA, and shares only the underlined four nucleotides with the canonical octamer binding site in site II. However, it exhibits greater similarity to some other octamer-derived OTF-1 binding sites such as the TAATGARAT (R = purine) sequence found in the herpes simplex virus 1 immediate early genes (Baumruker et al., 1988).

Relative Affinity of γ-Globin OTF-1 Binding Sites for the Purified Factor. Because of the demonstrated dissimilarities in the site I and site II OTF-1 binding sequences, the relative affinity of OTF-1 for either site was compared. The binding of a limited amount of partially purified OTF-1 to a ^{32}P-labeled

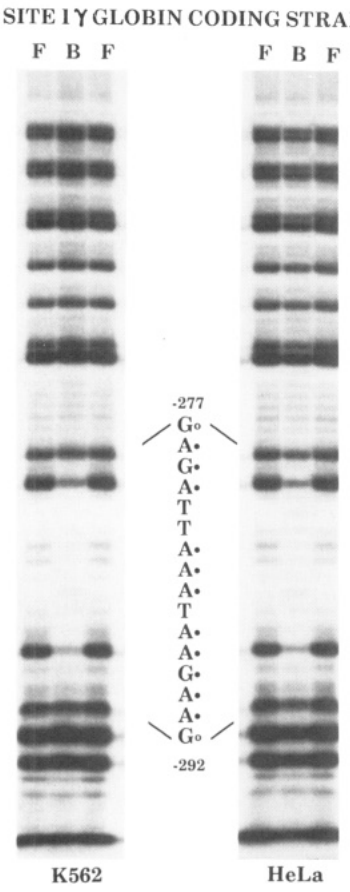

FIGURE 5: Methylation interference analysis of proteins binding to the (−300/−202) site I fragment using K562 or HeLa nuclear extracts. The assay was performed as described under Materials and Methods. Methylated G and A residues that interfere with the protein binding in the coding strand are indicated by closed circles. The open circles denote partially protected residues. B (bound) lanes represent assays with the C-1 complexes and F lanes assays with free DNA.

site I probe, was competed with excess equimolar amounts of unlabeled nonspecific competitor (NS), site I (SI), or site II (SII) oligonucleotides in a gel mobility shift assay (Figure 6). Quantitation of the C-1 protein–DNA complex formed in each case was obtained by scanning the resultant autoradiograph by laser densitometry (unpublished data). Equally effective competition was acquired with either site I or site II unlabeled oligonucleotides. For example, competition of the OTF-1 binding to radiolabeled site I, with a 7× molar excess of either site I or site II competitor, abolished about 85% of the binding relative to the control (NS). These results indicate that purified OTF-1 binds to site I and II DNA elements with approximately the same affinity. In the same experiment, the OTF-1 binding properties of a site II unlabeled oligonucleotide with an HPFH-like T → C substitution of the octamer motif (ATGCAAAT) at position −175 were investigated. The affinity of OTF-1 for the −175 HPFH oligonucleotide was markedly reduced as compared to the wild-type site I or site II sequences as demonstrated by a decreased ability of this oligonucleotide to compete with site I for OTF-1 binding. These results suggest that under circumstances in which OTF-1 is limiting, site I and site II compete equally for OTF-1 binding. In contrast, in the −175 HPFH mutants, the binding of OTF-1 to site II could be decreased, and the factor would preferentially bind to site I.

Discussion

It has been hypothesized that proteins shown to bind DNA elements within the 300 pb upstream of the $^{A}\gamma$-globin promoter

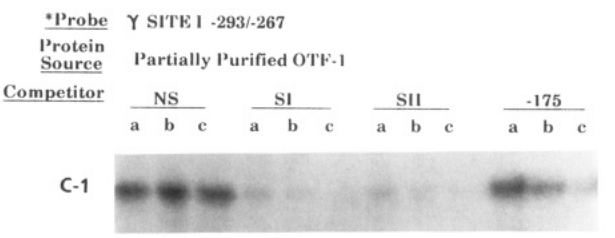

FIGURE 6: Gel mobility shift/competition assay. A detail of the assay shows only the C-1 complexes in order to compare the affinities of SI, SII, and −175 oligonucleotides for OTF-1. The sequence of the −175 oligonucleotide is the same as the SII oligonucleotide but with a T → C substitution at position −175. Each incubation mixture (20 μL) consisted of 1 μL of highly purified OTF-1, 0.3 ng of γ-^{32}P-labeled site I oligonucleotide, 1 μg of poly(dI-dC), and 7× (a), 14× (b), or 21× (c) molar excess of the respective competitor (SI, SII, −175 or NS). The 28 bp fragment (NS) obtained from the IBI-30 polylinker region was used as nonspecific competitor. Incubation, electrophoresis, and autoradiography were performed as stated under Materials and Methods. Gels had an acrylamide to bis(acrylamide) ratio of 30:0.8 and 0.01% (v/v) NP40.

cap site could be involved in transcriptional regulation of this gene. Therefore, we have undertaken the study of these factors. Here we demonstrate using electrophoretic mobility shift and competition assays that a common factor, designated C-1, specifically binds to site I (−291 to −267) and site II (−182 to −168), two distinct DNA elements within this upstream region. The tissue distribution of C-1 as evidenced with a site I probe and HeLa, FAZA, K562, HEL, and MEL cell nuclear extracts was confirmed to be non-erythroid-specific. Previous reports have shown a similar tissue distribution for the homologous binding activity at site II, which is known to be an octamer binding protein (Gumucio et al., 1988). The octamer binding nature of the C-1 factor binding to sites I and II was demonstrated by competition with an oligonucleotide which contains a core octamer motif. Interestingly, the comparison of the site I and the site II sequences showed some dissimilarities which suggested that the proteins binding to either site could be different. Nevertheless, the mobility of the complexes obtained with the site I and the site II probes in electrophoretic DNA binding assays is the same. These apparently identical complexes could also be cross-competed, indicating that most likely the same factor was binding to both sites. Also a similar binding activity was found with a nonerythroid histone H2b sequence, which is a binding site for the well-characterized octamer transcription factor OTF-1. Furthermore, we demonstrated that these three DNA elements bind a factor with identical mobility when incubated with highly purified OTF-1 or a crude nuclear extract. To confirm the identity of the factor, we used an antiserum which specifically reacts with OTF-1 in a gel mobility shift/immunoassay. This antiserum interacts with an epitope present in the C-terminus of OTF-1, does not react with OTF-2, and is not likely to cross-react with the other OTFs since there is no sequence similarity among them within this region (A. Pierani, unpublished results). We proved that the ubiquitous protein which generates comigrating complexes with site I, site II, and the H2b DNA element in K562 nuclear extracts is immunologically indistinguishable from the octamer transcription factor OTF-1 (Fletcher et al., 1987; Sturm et al., 1988).

We and others have previously reported that the ubiquitous protein–DNA complex obtained in the γ-globin −182 region footprints the octamer motif ATGCAAAT (Mantovani et al., 1987; Gumucio et al., 1988; Lloyd et al., 1989; O'Neill et al., 1990). Here we show that the nucleotide sequence that establishes contact with OTF-1 at the distal binding site, AAGAATAAATTAGA, bears little similarity to the canonical

octamer. Degeneracy in the DNA sequences recognized by transacting factors interacting with promoter regions has been previously demonstrated (Davidson et al., 1986; Pfeifer et al., 1987; Johnson et al., 1987; Costa et al., 1988). In fact, octamer binding factors, members of the POU domain family (POU: Pit-1, OTF-1, OTF-2, Unc-86) of transcriptional activators, have been shown to interact with degenerate octamer sequences present in the promoters of homeotic genes of *Drosophila melanogaster* (Thali et al., 1988; Ko et al., 1988), in the promoter of the 7SKRNA gene (Murphy et al., 1989), and in the SV40 enhancer (Sturm et al., 1988). Another recent study has also shown that OTF-1 binds not only to an octamer (ATGCAAAT) but also to a heptamer (CTCATGA), adjacent motifs in the immunoglobulin VH promoters (Poellinger & Roeder, 1989; Kemler et al., 1989). As expected, OTF-1 binds to these two unrelated sequences with different affinities. This does not seem to be the case for the site I and site II OTF-1 binding sites in the $^{A}\gamma$-globin promoter. Surprisingly, we found that despite the fact that their footprints differ in length (site I, 14 bp; site II, 8 bp) and that the sequence of site I is not homologous to the canonical octamer, OTF-1 (within the limits of the methodology used) interacts with both sequences with similar affinities. This was not expected, since transversional mutations of the octamer demonstrate that nucleotides 1–7 are essential for OTF-1 (NF-A1) binding (Staudt et al., 1986).

Two possible explanations for this finding can be proposed. First, we speculate that the affinity of OTF-1 for the degenerate octamer sequence at site I could be the result of its immediate context. Evidence supporting this hypothesis has been obtained by Baumruker et al. (1988), who demonstrated by mutational analysis that sequences flanking degenerate octamer motifs which are not essential for binding can determine the affinity of OTF-1 (OBP 100) for the site. The sequences adjacent to the degenerate octamer site I binding site may be strengthening the affinity of OTF-1 for the sequence, making it closer to the affinity of the factor for the consensus octamer in site II.

As a second possibility, one could invoke protein–protein interactions as an explanation for the similar affinities observed with the site I and site II probes. The POU domain of OTF-1 is known to specifically interact with both AT-rich promoter sequences similar to site I and viral or cellular proteins involved in transactivation of the respective gene. One of these sequences is the TAATGARAT (R = purine) motif, which is implicated in transcriptional activation of the HSV-1 immediate early gene promoters by the viral protein VP16 (Vmw65) in conjunction with OTF-1 (Gerster & Roeder, 1988; Triezenberg et al., 1988; apRhys et al., 1989; Kristie et al., 1989). Since this sequence bears some similarity to the OTF-1 site I binding site GAATAAATT (underlined), it would be interesting to know if there is a cell-specific homologue of VP16 that could transactivate the γ-globin gene via protein–protein interaction with OTF-1 at this site.

The role of OTF-1 in γ-globin transcriptional regulation is not clear. In humans, a point mutation at position −175 within the site II octamer binding site in the $^{A}\gamma$-globin gene promoter correlates with a 50–80-fold increase in expression of the fetal γ-globin gene in adult life (Schwartz et al., 1987). As in the spontaneous mutants, the −175 T → C HPFH promoter tested in transient assays is associated with up-regulation of expression in erythroid cells and decreased binding of OTF-1 when tested in binding assays (see the introduction). On the basis of this evidence, it has been proposed that OTF-1 could be a direct repressor of transcription or an indirect negative regulator by preventing the binding of a positive regulatory factor. The mechanism of control of transcription exerted at this DNA locus does not seem to be as simple as proposed. Neither the −175 HPFH γ-globin promoter transfected in nonerythroid cells nor mutations of the octamer that prevent OTF-1 binding other than the −175 affect transcription in transient assays. This suggests that OTF-1 is not a repressor. Since the bipartite erythroid factor NF-E1/GF-1 binding site flanks and partially overlaps the OTF-1 proximal binding site, it has been proposed that OTF-1 could prevent the binding of this putative positive regulatory factor (Martin et al., 1989; O'Neill et al., 1990). Although some studies report increased binding of NF-E1/GF-1 relative to wild type with the −175 mutants, this effect is not consistent and has not been seen with other octamer mutants. Furthermore, γ-globin site II mutations that prevent binding of NF-E1/GF-1 or both NF-E1/GF-1 and OTF-1 result in wild-type expression of CAT constructs transfected into K562-transfected cells. In conclusion, in the up-regulation of transcription observed in the context of the −175 mutation, which prevents OTF-1 binding, there appear to be other undefined factors influencing transcription. Also site I, encompassing the distal OTF-1 binding site, could have a positive role in transcriptional regulation of the $^{A}\gamma$-globin gene (Lloyd et al., 1989). In addition, Perez-Stable and Constantini (1990) have shown, by expressing 5′ γ/β-globin gene hybrids in transgenic mice, that a −383 to −201 γ-promoter fragment containing site I but no other known transcription factor binding sites is independently capable of activating the β-globin gene in embryonic cells. Contrary to site II, no spontaneous human mutations at this site correlate with altered transcription. However, since OTF-1 has a positive regulatory role in the transcription of other genes with similar TAATGARAT-like sequences, this factor is attractive as a positive modulator of γ-globin expression in conjunction with other cellular transactivating factors. The fact that OTF-1 binds with relatively high affinity to the distal and the proximal sites suggests that in the wild-type promoter both sites are occupied while in the −175 HPFH mutants most of the OTF-1 binding would occur predominantly at the distal site. The protein–DNA interactions within the 300 bp region upstream of the $^{A}\gamma$-globin gene are multiple and complex. Additional studies will be required to elucidate these interactions.

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