The β -globin stage selector element factor is erythroid-specific promoter/enhancer binding protein NF-E4

James L. Gallarda, Kevin P. Foley, Zhuoying Yang, and James Douglas Engel

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208 USA

The analysis of transcriptional regulatory proteins is often hampered because such factors are present in cells in only sparing abundance. Although direct biochemical purification has been successfully applied to the analysis of many of these factors, such methods are labor intensive and expensive. We have developed an alternative strategy to identify and characterize such *trans*-acting factors and have used it to analyze the proteins that interact with the chicken adult β -globin gene enhancer and promoter. The methodology involves (1) a sensitive 'reverse' radioimmunoassay used for the identification of antibodies to sequence-specific DNA-binding proteins, and (2) a monoclonal antibody-based DNase I footprint selection technique, which unambiguously identifies proteins responsible for particular footprints. Because this methodology relies on the isolation of antibodies to sequence-specific DNA-binding proteins, it should be of general utility in studying any *trans*-acting regulatory factor for which a specific DNA-binding sequence can be identified. In the present analysis, we report the identification of a 65-kD protein that is present only in mature definitive (adult) chicken erythroid cells. We show that this protein (termed NF-E4) binds to closely related sequences present in both the β -globin promoter and enhancer. Biochemical analysis of extracts prepared from both nonerythroid and a variety of erythroid cell types suggests that NF-E4 is the *trans*-acting factor that confers definitive erythrocyte stage-specific transcriptional activation to the adult β -globin gene.

[Key Words: β -Globin; promoter; enhancer; *trans*-acting factor]

Received May 18, 1989; revised version accepted September 28, 1989.

During ontogeny, chicken erythropoiesis is characterized by the sequential development of two populations of erythroid cells. In early embryogenesis, primitive erythroid cells selectively express the embryonic β -like globin genes ρ and ϵ (Bruns and Ingram 1973). Beginning ~5 days postincubation, these primitive erythroid cells are rapidly replaced by a population of definitive cells that selectively express the adult β -globin gene (Brown and Ingram 1974). We have shown recently that the tissue- and stage-specific regulation of the ϵ - and β -globin genes is elicited by at least two physically separate genetic elements. The β -globin enhancer confers overall erythroid tissue specificity for both genes (Choi and Engel 1988; Nickol and Felsenfeld 1988), and a second element, designated the β -globin developmental stage selector element (SSE), is not only required for definitive cell stage-specific expression of the adult β -globin gene but also for the concomitant suppression of embryonic ϵ -globin gene transcription in definitive cells (Choi and Engel 1988). The enhancer is located ~2000 bp, 3' to the β -globin mRNA cap site (Choi and Engel 1986; Hesse et al. 1986), whereas the SSE is an intrinsic part of the adult β -globin promoter.

Both of these *cis*-acting regulatory elements have been shown to be binding sites for a variety of *trans*-acting factors present in extracts from erythroid cells (Emerson et al. 1987; Lewis et al. 1988; Engel et al. 1989; Gallarda et al. 1989). It is thereby inferred that these sequence-specific *trans*-acting factors constitute the transcriptional apparatus that is responsible for the tissue- and stage-specific regulation of β -globin gene expression during erythroid cell development.

Although direct biochemical analysis has been successfully employed to elucidate the role of sequence-specific DNA-binding proteins in the process of selective gene expression, the extremely low concentration of many of these factors represents a significant impediment to their physical characterization. We have developed an alternative strategy to study such *trans*-acting factors and have applied it to the analysis of proteins that interact with the promoter and enhancer of the chicken adult β -globin gene.

Here, we report the immunochemical characterization of a definitive erythroid cell-specific DNA-binding protein that has high affinity for a specific β -globin promoter sequence within the genetically defined SSE, as

well as to a closely related sequence within the enhancer. The β -globin promoter binding site is shown to be unprotected [by DNase I footprinting] in extracts prepared from embryonic erythroid, immature definitive erythroid, and liver cells, none of which expresses the adult β -globin gene. The binding site for this protein, when mutated, leads to less efficient transcription of β -globin in vivo. In a variety of assays, the protein binding to this promoter sequence appears to be the unique factor required for activation of the β -globin SSE. A group of monoclonal antibodies that appear to recognize this specific factor has been isolated. One of these antibodies has been characterized in detail and is shown to specifically select a protein that corresponds to the *trans*-acting factor required for β -globin definitive erythroid cell-specific activation.

Results

In vitro analysis of proteins that bind to the β -globin promoter and enhancer

Previous observations have demonstrated that the DNA sequence constituting the β -globin SSE (functionally defined as nucleotides -112 to -20 , relative to the β -globin cap site) confers positive genetic regulation to β -globin transcriptional activation (Choi and Engel 1988). Thus, we might anticipate the appearance of a *trans*-acting factor (or factors) that associates with regulatory elements of the β -globin gene in definitive erythroid cells and is absent (or inactive) in cells in which this gene is not transcribed. Because of the way in which the genetic experiments were conducted, we were able to demonstrate that the β -globin promoter was a necessary element in stage selection but not that it was (exclusively) sufficient for definitive cell stage-specific gene activation. Thus, different *trans*-acting factors responsible for definitive stage-specific activation of the β -globin gene could interact with either the promoter alone or with both the promoter and the enhancer. Therefore, we initiated biochemical analysis of both regulatory regions of the gene to ask whether or not unique factors, present only in definitive cells, might associate with one regulatory region or the other.

To determine the presence of β -globin enhancer- and promoter-binding proteins, we performed DNase I footprint analysis using basic extracts prepared from mature definitive red blood cells (RBC), from primitive RBC, from the immature definitive erythroid progenitor cell line, HD3 (Beug et al. 1982), and from perfused chicken liver cells. Figure 1 shows the footprints detected with these extracts within the β -globin promoter. Four β -globin footprints are seen when definitive extracts are allowed to associate with this β -globin promoter probe (Fig. 1A; lanes 1 and 2); β P-F1, a weak sequence protection centered at nucleotide -30 , containing a consensus TATAA sequence; β P-F2, a purine-rich sequence (AA-GAGGAGGGG) protection centered at nucleotide -50 ; β P-F3, a protection centered at nucleotide position -70 , containing a consensus CCAAT element; and β P-F4, a

G-rich (GGCTGGGG) protection centered at nucleotide position -95 . The patterns of protection derived from both primitive (Fig. 1A, lanes 3–5) and HD3 (Fig. 1B, lanes 2–4) erythroid cells are similar to those seen with definitive extracts, with a notable exception; there is a conspicuous absence of the purine-rich β P-F2 protection (centered at -50 bp) when using either the primitive or immature definitive (HD3) cell extracts. In addition, we (perhaps surprisingly) fail to detect binding of a CCAAT transcription factor from embryonic erythroid cells to the adult β -globin CCAAT box. Similarly, when examining extracts prepared from liver cells (Fig. 1C, lanes 4–6), no protection is observed at the β P-F2 position. On the basis of this preliminary analysis, β P-F2 appears to fulfill the requirements expected for a presumptive SSE factor taking part in β -globin stage-specific activation: It is present only in mature definitive erythroid cells, it binds to a specific sequence within the genetically defined SSE element, and it is not present in primitive or immature definitive erythroid cells nor in nonerythroid cells.

Figure 1 also shows the DNase I footprint pattern observed with these same extracts using a β -globin enhancer probe. As reported earlier (Emerson et al. 1987), five β -globin enhancer protections are seen with mature definitive erythrocyte basic extracts (Fig. 1D, lane 2); β E-F1, a protection centered at nucleotide position $+1835$, containing a consensus CTF/NF-1 sequence (Jones et al. 1987); β E-F2, a protection centered at nucleotide position $+1860$, containing consensus AP-1 and AP-2 sequences (Emerson et al. 1987; Evans et al. 1988); β E-F3, a weak protection centered at nucleotide position $+1883$, containing a site for an unidentified factor; β E-F4, a protection centered at nucleotide $+1918$, containing NF-E1 binding sites, hallmarked by a strong hypersensitive site at nucleotide position $+1922$ (see Discussion); and β E-F5 (sequence GAGAGGGGT-TAATCCTG), a protection centered at nucleotide position $+1995$. In addition to these five footprints, a sixth weak protection centered at nucleotide $+1938$ in the β -globin enhancer is often observed with definitive erythroid extracts. We have designated this region as β E-F4.5 because of its location between β E-F4 and β E-F5. This region contains a nearly perfect match to the consensus NF-E1 sequence R(A/T)GAT(A/T)R(A/C) (Evans et al. 1988; Wall et al. 1988), with the exception of an inserted C residue at nucleotide position $+1939$, leaving a modified NF-E1 sequence, GTGCATAAA.

The β -globin enhancer footprint protection patterns derived from the use of either primitive or HD3 cell extracts (Fig. 1E) are similar to those observed with definitive extracts, with two exceptions. The protein resulting in β E-F1 (presumably CTF/NF-1; Emerson et al. 1987) is not evident in primitive cell extracts (lanes 4–6), the pattern being identical to that of the negative control (lane 1). Furthermore, close examination of the β E-F5 region reveals a subtle but distinct difference between both the primitive (lanes 4–6) and HD3 (lanes 7 and 8) cell extracts when compared to the definitive cell extracts (lanes 2 and 3): in definitive extracts, a clear hy-

Figure 1. DNase I footprint analysis of the chicken β -globin promoter and enhancer. (A–C) A 32 P-labeled promoter probe was used, (D and E) a 32 P-labeled enhancer probe was used (Materials and methods). Various amounts of protein from definitive cells [D], 4.5-day-old primitive cells [P], the tsAEV-transformed erythroid cell line HD3 [HD3], and perfused liver [L] were examined. (–) Control reactions containing no protein. Numbers at left of A–E represent nucleotide positions relative to the β -globin mRNA cap site. Footprint designations are shown at right of the lanes. An arrow (D and E) highlights the β E-F5-hypersensitive site observed in definitive erythroid cell basic extract footprints.

persensitive site is seen within β E-F5 (denoted by an arrow, lanes 2 and 3) that is not present when primitive, HD3, or nonerythroid cell extracts are used in the footprinting assay. These results show that stage-specific differences also exist within the population of enhancer-binding proteins present during erythroid development (see Discussion).

We noted a strong similarity between the β P-F2 and β E-F5 sequences within the β -globin promoter and enhancer. Therefore, we were interested in determining whether or not the same protein might be capable of binding to both regulatory regions. To address this question, definitive proteins that specifically recognize the β E-F5 sequence were purified by DNA affinity chromatography (Kadonaga and Tjian 1986) and subsequently examined by DNase I footprint analysis, using both the β -globin promoter and enhancer probes. The results demonstrate that the affinity-purified proteins protect both β P-F2 (Fig. 2A), and β E-F5 (Fig. 2A, right), initially suggesting that a common factor can bind to either sequence. Similarly, when DNase I footprint analysis was performed with proteins selected on a β P-F2 oligonucleotide affinity matrix, both β P-F2 and β E-F5 regions were again protected (data not shown).

In addition to the selection of proteins that recognize β P-F2 and β E-F5 by the β E-F5 affinity column, Figure 2A also shows that proteins that protect β P-F4 and β E-F3 are coselected. We therefore addressed the question of whether this was due to the presence of a single DNA-binding protein that recognizes multiple, degenerately related DNA-binding sites or to selection of multiple proteins by the β E-F5 oligonucleotide column. As shown in Figure 2B, the β P-F2 protection generated, using unfractionated definitive erythrocyte basic extracts, is spe-

cifically competed by a 50-fold excess (50 ng) of unlabeled β P-F2 oligonucleotide (lane 3) or by a 50-fold excess of unlabeled β E-F5 oligonucleotide (lane 5), suggesting that the β P-F2 factor is capable of binding to either sequence (but perhaps with a somewhat higher affinity to the β P-F2 region). In contrast to the β P-F2-specific competition, no competition for binding to either β P-F2 or β E-F5 was observed with a 50-fold excess of either unlabeled β E-F4 or Sp1 oligonucleotides. Taken together, these data (Figs. 1 and 2), strongly imply that a single protein, present only in definitive cells, binds to both β P-F2 and β E-F5 with high avidity.

β P-F2 is necessary for abundant β -globin transcription

To determine whether or not the definitive cell-specific protein that binds to β P-F2 in vitro also confers transcriptional activation to the β -globin gene, a clustered substitution mutation of the entire footprint was created using synthetic oligonucleotides. The double-stranded oligonucleotide was used to replace natural β -globin promoter sequences -43 to -54 relative to the mRNA cap site, creating mutant β^* P-F2⁻; in every other respect, the wild-type and mutant constructs are identical (Choi and Engel 1988).

The marked β^* and β^* P-F2⁻ genes were individually transfected into HD3 cells and shifted to elevated temperature and high pH in the presence of anemic chicken serum to allow the cells to partially differentiate (Beug et al. 1982; Choi and Engel 1986). After 36 hr under differentiation conditions, cells were counted and lysed for RNA preparation (Materials and methods). Equivalent amounts of RNA were hybridized to the β^* (marked

Figure 2. Footprint analysis and oligonucleotide competition using DNA affinity-purified β E-F5. (A) β -globin promoter (lanes 1–4) and enhancer (lanes 5 and 6) footprint analysis with β E-F5 oligonucleotide affinity-purified proteins. (B) Footprint competition between the β -globin promoter probe and a 50-fold excess (50x) of oligonucleotides corresponding to β P-F2 (lane 3), β E-F5 (lane 5), β E-F4 (lane 7), and Sp1 (lane 9). Definitive basic extracts were used in the competition experiments (Materials and methods).

gene) probe, treated with S1 nuclease, and finally resolved on a 5% denaturing polyacrylamide gel, as described previously (Choi and Engel 1986, 1988).

As shown in Figure 3, introduction of the β P-F2⁻ mutation into the promoter reduces β -globin transcription by three- to fourfold relative to the native promoter sequence. Whereas the magnitude of this effect appears to be smaller than might have been anticipated, deletion of a single transcription factor-binding site from a complex regulatory locus (such as the β -globin promoter) might allow compensatory changes to partially mitigate the effects of such a mutation (see Discussion). Nonetheless, the transfection experiment shown in Figure 3 demonstrates that the *cis*-regulatory sequence recognized by the β P-F2-binding protein is required for abundant β -globin transcription.

Reverse radioimmunoassay model studies

Because we were interested in identifying antibodies to the *trans*-acting factors that interact with the β -globin

Figure 3. Transfection of a β -globin promoter mutant missing β P-F2. β -globin construct $\beta^*\epsilon^*(+)$ (Choi and Engel 1988) was mutated in β P-F2 by insertion of a double-stranded oligonucleotide that replaced the sequence from the promoter *Sma*I site (at nucleotide -59) to an artificial *Pvu*I site (centered at position -37; Dolan et al. 1983). The nucleotides replaced were wild type (5'-AGAGGAGGGGCC-3') to mutant (5'-CATAC-CACACGA-3'). The wild-type and mutant marked β -globin genes were separately transfected into HD3 cells and allowed to differentiate for 36 hr. RNA was prepared, and equal quantities were hybridized to the β^* S1 probe, treated with S1 nuclease, and run on a 5% polyacrylamide-50% urea gel, as described (Choi and Engel 1986). (Lane A) One microgram of anemic adult hen RBC RNA; (lane B), 2×10^6 cell equivalents of RNA from HD3 cells transfected with the wild-type β^* globin gene; (lane C), 2×10^6 cell equivalents of RNA from HD3 cells transfected with the β^* P-F2⁻ mutant gene. Exposure time was 17 hr.

promoter and enhancer elements, we were initially concerned that the low abundance of these factors would preclude the use of conventional antibody-screening assays (which require relatively large amounts of homogeneous preparations of antigen). Figure 4 illustrates the rationale of the reverse radioimmunoassay (rRIA) developed to circumvent this problem.

The rRIA is initiated by first coating 96-well polyvinylchloride (PVC) plates with goat anti-mouse IgG. After blocking potential nonspecific binding sites with calf serum protein, culture supernatants from hybridomas are allowed to bind to the anti-mouse antibody-coated wells. Simultaneously, solution-binding reactions are initiated in which sequence-specific DNA-binding proteins are complexed with a 32 P-labeled oligonucleotide probe containing the protein-binding site, in direct analogy to the binding reaction used in a gel-shift assay (Garner and Revzin 1981). After removing the unassociated hybridoma supernatant from the PVC plates, the oligonucleotide-binding reactions are added to the antibody-coated wells. Antibodies that recognize a particular protein-oligonucleotide complex will bind to the native protein antigen and allow a specific radiolabeled oligonucleotide probe to remain associated with the wells.

To investigate the general utility and sensitivity of the rRIA to detect antibodies that recognize low-abundance, sequence-specific DNA-binding proteins, pilot studies were initiated using the well-established T antigen/SV40 origin of replication interaction (Tjian 1978; Ariga and Sugano 1983) as a model. As detailed in Materials and methods, binding reactions [containing a 32 P-labeled SV40 origin of replication probe, together with various amounts of COS whole-cell extracts (containing T antigen) and parental CV-1 whole-cell extracts (containing no T antigen)] were allowed to react with the anti-T antigen monoclonal antibody (mAb) PAb419 (Harlow et al. 1981), which had been bound to goat anti-mouse IgG-coated PVC plates.

As illustrated in Table 1, significant counts were retained only in those wells in which COS cell extracts (wells 1-5) and the SV40 origin probe were included. The control wells, containing either blocking protein (well 7) or 50 μ g of total protein from CV-1 cells (well 6), retained only very low background counts. Based on the specific activity and quantity of the SV40 ori probe used, we calculate that as little as 5-50 pg of T antigen is required to verify the presence of the solid-phase bound T antigen monoclonal antibody. This calculation corresponds well with the total amount of T antigen present in these crude extracts, as determined by quantitative ELISA assay [2-4 μ g/ml in the COS extracts (Y. Gluzman, pers. comm.)]. When nonspecific antibodies were used in place of the anti-T antigen monoclonal, once again, only background counts were retained in the rRIA (data not shown). Thus, for the case of the T antigen/SV40 origin model system, the rRIA represents an extremely sensitive means to detect antibodies using crude cellular extracts containing DNA-binding proteins of low abundance.

Figure 4. Schematic diagram of rRIA. Standard 96-well PVC plates are used; each of the cups represents a different well on one plate. Probes can be either ^{32}P -labeled (*) restriction fragments or oligonucleotides (for description see Results).

Isolation of antibodies that recognize β -globin enhancer-binding proteins

The basic rRIA methodology developed in the T antigen pilot studies was employed to screen for antibodies to β -globin enhancer-binding proteins. Tissue-culture supernatants from ~3000 hybridomas were bound to goat anti-mouse IgG-coated PVC plates, and the antibody-

Table 1. rRIA: T antigen in whole cell extracts of COS and CV-1 cells

rRIA well number	Protein/well (μg)		^{32}P -labeled cpm retained per well
	COS-1 extract	CV-1 extract	
1	50	0	12,672
2	25	25	14,258
3	5	45	6,976
4	0.5	50	490
5	0.05	50	240
6	0	50	36
7	0	0	58

rRIA was performed as described in Materials and methods. For rRIA procedure, see Fig. 4 schematic diagram.

coated wells were subsequently allowed to react with ^{32}P -labeled enhancer probes complexed with binding protein(s) present in mature definitive erythroid basic extracts. The binding reactions for the initial screening consisted of a ^{32}P -labeled restriction fragment containing β -globin enhancer footprints 3–5 (Choi and Engel 1986; Emerson et al. 1987). For negative and positive controls, antibodies from preimmune and immune sera, respectively, were bound to the goat anti-mouse IgG-coated wells. As shown in Table 2, supernatants from eight primary macrowell hybridoma cultures (see Materials and methods) retained counts twofold or higher above background when compared to the negative control.

The primary hybridoma culture supernatants contained a mixture of antibodies derived from multiple hybridomas (an average of 60) present in a single macrowell. Thus, it was anticipated that only a fraction of the total murine antibody bound to the wells (by the goat anti-mouse IgG) would represent a monoclonal antibody specific for a given enhancer-binding protein. We reasoned that the number of counts retained in the rRIA should increase if the hybridoma responsible for the specific antibody was isolated as a monoclonal line. Upon cloning the microwell-partitioned hybridoma mixtures by limiting dilution, this was indeed found to be the case, because supernatants derived from isolated subclones demonstrated a dramatic increase in counts retained as compared to supernatants from the original mixed hybridoma populations (Table 2). Thus, the rRIA has allowed us to isolate several monoclonal antibodies

Table 2. Reverse RIA for hybridomas producing antibodies to erythroid basic whole cell extracts

^{32}P -labeled cpm retained per well ^a			
macrowell ^b		microwell ^c	
I.7	574	I.7OP	11,660
I.9	1270	—	—
I.17	462	I.17GH	1,530
I.19	338	I.19GH	808
II.6	400	—	—
II.16	682	II.16EF	27,121
		II.16OP	39,023
II.17	382	II.17EF	24,573
II.24	382	II.24MN	1,286
+ control ^d	800		
— control ^d	154		

rRIA was performed as described in Materials and methods.

^aThe probe was a ^{32}P -labeled restriction fragment containing β -globin enhancer sequences $\beta\text{E-F3}$ – $\beta\text{E-F5}$ (Emerson et al. 1987).

^bMacrowells represent tissue-culture supernatants containing multiple hybridomas; the initial macrowell designation is shown on the left, and the cpm of radiolabeled enhancer probe retained are shown on the right.

^cMicrowells represent tissue-culture supernatants of cloned hybridomas (derived from the corresponding macrowell) producing the specific antibody being assayed.

^d+ control and — control represent immune and preimmune sera, respectively.

that have high affinity for β -globin enhancer-binding proteins.

Because the monoclonal antibodies isolated in rRIA screening were detected using a β -globin enhancer restriction fragment containing multiple *trans*-acting factor-binding sites as the probe, we were unable to immediately discern which of the enhancer-binding proteins was being recognized by individual monoclonal antibodies. To determine whether or not any of these antibodies recognize proteins having affinity for the β E-F5 sequence, we repeated the rRIA with β E-F5 oligonucleotide affinity-purified protein complexed with a 32 P-labeled, ligated β E-F5 oligonucleotide as probe. In addition, we added various amounts of poly[d(I-C)]/[d(A-T)], ligated β E-F5 oligonucleotide, or ligated β E-F4 oligonucleotide as unlabeled competitors. As shown in Figure 5 (for mAb I.7OP) reductions of 12% and 13% of counts retained were seen when a 25-fold excess of poly[d(I-C)]/[d(A-T)] and β E-F4 competitors were included in the reaction, respectively. However, a 60% reduction was achieved in this competition assay, using a 25-fold excess of β E-F5 oligonucleotide competitor. Similar results were recorded for monoclonal lines II.16EF and II.16OP. Thus, a significant reduction in counts per minute retained was achieved for all three lines only with a β E-F5 oligonucleotide competitor, indicating that these three monoclonal antibodies all recognize a protein that has high affinity for β E-F5 (or closely related) sequences.

Figure 5. Competitive rRIA. A standard rRIA was initiated (see Materials and methods; and Fig. 4 schematic diagram) with β E-F5 oligonucleotide affinity-purified protein (Fig. 2A), mixed with a 32 P-labeled oligonucleotide probe corresponding to β E-F5, together with increasing amounts of unlabeled competitors corresponding to either the β -globin enhancer sequence β E-F4 (dashed line) or β E-F5 (solid line). The percentage of counts retained per well are plotted against nanograms of unlabeled competitor.

Footprint selection of β -globin promoter and enhancer-binding proteins

Although the β E-F5 oligonucleotide affinity column appears to select a protein that can recognize both β E-F5 within the enhancer and β P-F2 within the promoter, the DNase I footprint experiments (Fig. 2A) allow for the possibility that β P-F2 or β E-F5 DNA affinity columns coselected two or more proteins that were responsible for the observed results. We therefore developed a modified DNase I footprint assay (which we refer to as footprint selection) in which solid-phase monoclonal antibody-selected proteins are allowed to protect specific DNA-binding sites from DNase I digestion *in situ*. The footprint selection results using the monoclonal antibody II.16EF are shown in Figure 6.

After washing the solid-phase antibody/DNA-binding protein complexes with increasing amounts of salt (to remove nonspecifically associated proteins), and after having allowed the β -globin promoter probe to bind to the complex, subsequent DNase I footprinting reveals that only footprint β P-F2, at position -50 bp, is selected (Fig. 6A, lanes 3–5); all other footprints disappear when reactions are compared to a mock selection performed with the anti-T antigen monoclonal antibody (Fig. 6A, lane 6). When the selection was repeated with the β -globin enhancer probe, the definitive β E-F5 footprint (including the definitive erythroid-specific hypersensitive site marked by the arrow; see Fig. 1) is selected with increasingly stringent salt washes of the II.16EF antibody-antigen complex (Fig. 6B, lanes 3–5). With the possible exception of the β E-F3 region, all other footprints disappear, the pattern again becoming identical to that seen with the anti-T antigen-antibody negative control (Fig. 6B, lane 6). These data show that the antigen recognized by mAb II.16EF has highest affinity for β P-F2 and β E-F5.

Analysis of the II.16EF antigen

To determine the tissue specificity of the protein recognized by mAb II.16EF, Western blot analysis was performed using basic extracts from several cell types; the results are shown in Figure 7. In the lane containing basic definitive erythroid cell extracts (lane 1), a prominent 65-kD band is clearly detected. In contrast, no antigen is detected in equal amounts of total protein (2.5 μ g) in basic extracts prepared from primitive erythroid cells (lane 2), from HD3 cells grown at 35°C (conditions under which β -globin is not transcribed; lane 3), or from liver cells (lane 4). Thus, the protein antigen recognized by mAb II.16EF is restricted to mature cells of the definitive erythroid lineage.

Discussion

*Positive β -globin transcriptional regulation by an erythroid cell developmental stage- and tissue-specific *trans*-acting factor*

We have shown previously that a small region of the β -

Figure 6. Footprint selection with the II.16EF antigen. Basic definitive extracts were bound by immobilized mAbs II.16EF (A and B, lanes 3–5) or PAb419 [anti-T antigen; A and B, lane 6], as described in Materials and methods. Antibody–definitive erythroid cell basic extract protein complexes were washed with footprint buffer (Materials and methods) containing 100 mM KCl (lane 3), 200 mM KCl (lane 4), or 400 mM KCl (lane 5), and allowed to bind with either a 32 P-labeled β -globin promoter probe (A) or a 32 P-labeled β -globin enhancer probe (B). The PAb419 (anti-T antigen) monoclonal antibody–protein complex was washed with buffer containing 200 mM KCl only. Footprint analysis was performed as described (Materials and methods). Positive and negative controls are shown in lanes 1 and 2, respectively.

globin promoter (nucleotides -112 to -20) is responsible for the preferential activation of the adult β -globin gene in definitive cells and for the concomitant suppression of the embryonic ϵ -globin gene in the same cells (Choi and Engel 1988). That DNA sequence was called the β -globin developmental SSE. In that report we also demonstrated that β -globin activation was a positive regulatory event and proposed a model in which the *trans*-acting factors interacting with this regulatory region confer (with the required enhancer element) the preferential expression of the β -globin gene over that of the ϵ -globin gene in definitive erythroid cells.

In the present study we have examined the DNase I footprint patterns of the β -globin promoter region (containing the SSE), using proteins extracted from stage-specific erythroid, as well as nonerythroid, cells. These experiments demonstrate that in basic extracts derived from terminally differentiated adult definitive cells, a unique footprint, centered at nucleotide position -50 , is generated in the β -globin promoter. The protein factor responsible for this footprint is not found in extracts prepared from primitive, immature definitive, and nonerythroid cell types (Fig. 1). The active factor responsible for the observed protection is therefore an erythroid-specific *trans*-acting promoter-binding protein that is exclusively expressed during the latter stages of definitive erythropoiesis. Thus, in keeping with the model proposed earlier (Choi and Engel 1988), it appears from the biochemical evidence presented here that transcriptional activation of chicken β -globin gene expression is due to a positive regulatory mechanism in which a definitive erythroid cell stage-specific factor interacts with a specific β -globin promoter element (β P-F2).

Mutation of the β P-F2 element leads to reduced transcriptional efficiency in transfected β -globin genes (Fig. 3). Although this effect appears to be small for deletion of an element presumed to be vital for accurate, abundant β -globin transcription, it has now been demonstrated in a number of complex regulatory elements (promoters and enhancers that are bound by multiple regulatory proteins) that deletion of only a single binding site frequently has a less dramatic effect than anticipated. These observations suggest that such regulatory complexes may suffer a single mutation without complete abrogation of the overall regulatory effect, rendering the complex less efficient but still active. Whether such compensation is achieved by the binding of multiple, redundant factors that accomplish the same

Figure 7. II.16EF antigen is present only in mature definitive erythroid cells. Two and one-half micrograms of basic extract from mature definitive cells (D), 4.5-day-old primitive cells (P), tsAEV-transformed erythroid precursor cells (HD3), and liver cells (L) were electrophoresed on standard 10% SDS–polyacrylamide gels, transferred to nitrocellulose and, after blocking, bound to mAb II.16EF (Ausubel et al. 1989). The antibody–antigen reactions were detected using 125 I-labeled secondary goat anti-mouse F(Ab')₂ and exposure to X-ray film.

regulatory effect within the element or whether the protein–protein contacts within the complex are sufficient to specify less effective binding of the correct factor (augmented by its nonspecific affinity for DNA) is not yet clear.

Deletion of the analogous sequence to which this protein binds within the enhancer (β E-F5) appears to have only minimal effect on adult β -globin transcription (Emerson et al. 1987; Reitman and Felsenfeld 1988). However, with the discovery that the enhancer is also required for ϵ -globin transcription (Choi and Engel 1988; Nickol and Felsenfeld 1988), it may be true that deletion of this sequence has an unusual phenotype only when both genes are tested for tissue and stage specificity. A simple prediction from the model for hemoglobin switching (presented below) would suggest that a β E-F5 deletion might have only a modest effect on adult β -globin transcription (which is, in fact, observed) but would allow a higher level of 'leaky' transcription of a *cis*-linked embryonic ϵ -globin gene in definitive erythrocytes.

Identification of the β -globin-activating protein NF-E4

In this analysis we have presented a general, antibody-based strategy to identify and characterize *trans*-acting factors that interact with sequence-specific regulatory regions and have applied it to the study of the chicken β -globin promoter- and enhancer-binding proteins. Using an extremely sensitive rRIA, several thousand hybridomas (derived from B cells of mice immunized with definitive basic erythroid cell extracts) were screened for antibodies that react with proteins that specifically bind to a β -globin enhancer DNA probe.

Having identified several hybridomas that produce antibodies to enhancer-binding proteins, subsequent immunochemical analysis revealed the principal antigen to be a 65-kD definitive erythroid-specific protein. Footprint selection shows that this factor has high affinity for homologous DNA sequences present in both the β -globin enhancer (β E-F5) and in the β -globin promoter (β P-F2), where β P-F2 and β E-F5 each share the conserved purine-rich sequence, RAGAGGRGG. Most importantly, the tissue distribution of the antigen recognized by the mAb II.16EF corresponds precisely to that of the definitive cell stage-specific erythroid factor (as determined by DNase I footprint analysis of basic extracts from a variety of cells; Fig. 1), which is responsible for the β P-F2 footprint within the region of the promoter shown to contain the SSE (Choi and Engel 1988). Because there is only one consistent difference in the promoter DNase I footprint patterns within the SSE region between cells that either do or do not express the β -globin gene, the data strongly imply that the 65-kD protein recognized by mAb II.16EF is the factor required for late erythroid stimulation of β -globin transcription, which we will name and subsequently refer to as NF-E4.

Several reports have shown that there are multiple *trans*-acting factors capable of binding β -globin *cis*-regulatory elements. One of these, NF-E1 [also called Eryf1

and GF-1 (Evans et al. 1988; Wall et al. 1988; Martin et al. 1989)], has been shown to bind to β E-F4 and to its analog in mammalian globin regulatory sequences. By DNase I footprint analysis, methylation interference, and gel shift assay, it appears that NF-E1 is RBC specific but not specific for a particular developmental stage of erythroid cells (see also Fig. 1). A second erythrocyte-specific factor, NF-E2, was reported to have affinity for the consensus AP-1 sequence found in β E-F2 and in the porphobilinogen deaminase gene promoter. This protein is also found in both embryonic and adult RBC (Mignotte et al. 1989). A third erythroid-specific factor, BGP1, which recognizes the G-string sequence of the strong chicken β -globin 5' hypersensitive site, is also expressed in both embryonic and adult erythrocytes (Lewis et al. 1988). A fourth factor, which is associated with the CCAAT modification in HPFH syndrome, has been reported recently (Mantovani et al. 1989); the factor (NF-E3) has not been characterized fully. In contrast, NF-E4, reported here, represents an erythroid-specific *trans*-acting factor whose expression is tightly coupled to the definitive erythroid lineage in which the adult β -globin gene becomes transcriptionally activated. Recently, we identified monoclonal antibodies recognizing a second, definitive erythrocyte stage-specific enhancer binding protein, NF-E5. NF-E5 footprints β E-F4.5 *in vitro* (see Results); its physiological role in β -globin transcriptional regulation remains unclear (Gallarda et al., *in prep.*). Final confirmation that NF-E4 is indeed a promoter factor required for stage-specific β -globin transcriptional activation will rely on *in vitro* transcription or genetic experiments in which the function of this protein can be conclusively demonstrated.

Multiple factors bind to similar sequence elements within the β -globin promoter and enhancer

The footprint selection data (presented in Fig. 6) demonstrate that the G-rich β P-F4 region of the promoter (nucleotide sequence GGCTGGGG) is not protected by the II.16EF antibody-selected factor when compared to the anti-T antigen antibody negative control (Fig. 6A, lanes 5 and 6); the antibody-selected protein may, however, have low affinity for β E-F3 (nucleotide sequence GGGTGGGG) of the enhancer (Fig. 6B, cf. lanes 5 and 6). In this assay it appears that NF-E4 has the highest affinity for β P-F2 and β E-F5, which share RAGAGGRGG as a consensus sequence.

The data presented in Figure 2A show that β E-F5 affinity-purified proteins protect both β P-F2 and β E-F5. In addition, Figure 2B shows that the β P-F2 protection can be competed either by the homologous β P-F2 oligonucleotide or by a β E-F5 oligonucleotide but not by oligonucleotides corresponding to Sp1- or NF-E1-binding sites. These data also imply that a common factor can bind to both the β P-F2 and β E-F5 sequences. However, as shown in Figure 2A, the G-rich β P-F4 and β E-F3 regions are also protected by β E-F5 oligonucleotide affinity-purified proteins. This result could be explained in one of two ways: Either a single factor has the ability to bind all four se-

quences or multiple factors are selected by the β E-F5 affinity matrix, which bind the four sequences. We favor the latter explanation for the following reasons (1) SDS–gel electrophoresis of total protein purified by two cycles of β E-F5 DNA affinity chromatography yields multiple bands (data not shown), (2) Sequence comparisons of the four protected regions reveal that although all have limited similarity, β E-F5 (nucleotide sequence GAGAGGGG) and β P-F2 (nucleotide sequence AAGAGGAGG) are more similar to each other than they are to β E-F3 and β P-F4. Conversely, β E-F3 (nucleotide sequence GGGTGGGG) and β P-F4 (GGCTGGGG) are more similar to each other than they are to β E-F5 or β P-F2, implying that different factors might bind these two sets of sequences. (3) A similar β -globin enhancer β E-F5 protection is obtained when using extracts prepared from HD3, primitive, or liver cells (none of which contain NF-E4; Fig. 7); this pattern is quite distinct from the pattern obtained with extracts from definitive erythroid cells (Figs. 1D,E and 6B). These comparative data argue that in addition to NF-E4 (present only in definitive cells), other factors (which are not erythroid specific) are present in cells that have an affinity for this region and would consequently be selected by a β E-F5 oligonucleotide affinity column. The factor(s) responsible for the β P-F4 and β E-F3 footprints may therefore, because of limited sequence similarity to the β E-F5 sequence, be coselected with NF-E4 on this column. Taken together with the footprint selection data, these data indicate that NF-E4 preferentially binds the consensus sequence RAGAGGRGG common to β E-F5 and β P-F2 and that other ubiquitous factors, present in all cells, have lower affinity for this, or a closely related DNA sequence.

NF-E4 function in stable transcription complex formation during erythroid cell maturation

The β E-F5 sequence does not appear to play an important role in enhancer activity, because it has been shown in erythroid cell transfection studies that deletion of this sequence does not reduce the level of transcription of a *cis*-linked reporter gene (Emerson et al. 1987; Reitman and Felsenfeld 1988; K.P. Foley, unpubl.). Nonetheless, the identical tissue- and stage-specific patterns of expression of the β P-F2-binding protein (as determined by footprint analysis; Fig. 1) and the 65-kD protein (which is shown to associate strongly with β P-F2 and β E-F5 by footprint selection with mAb II.16EF; Fig. 6) argues that NF-E4 is the SSE factor and binds to β P-F2 and β E-F5 sequences with similar affinity. We can therefore only speculate about the DNA-binding activity exhibited by this factor for β E-F5, which is seemingly unnecessary for β -globin transcription. One attractive possibility is that because the adult β - and embryonic ϵ -globin genes both require the enhancer for activity (Choi and Engel 1988; Nickol and Felsenfeld 1988), NF-E4 binding to β E-F5 might inhibit enhancer interaction with the ϵ -globin promoter in definitive cells.

The appearance of NF-E4 in the terminal stages of definitive erythrocyte maturation could certainly account

for one property attributed to the SSE, that of promoting preferential β -globin transcriptional activation in definitive erythroid cells. What of the other property previously attributed to this *cis*-regulatory element—that of suppressing the embryonic ϵ -globin gene? In a previous communication, we showed (genetically) that this suppressive effect on the closely linked embryonic gene was attributable to the lack of sufficient enhancer activity and that this suppression could be reverted by introduction of a tandem repeat of the β -globin enhancer (Choi and Engel 1988). We proposed a model to explain these data, which suggested that *trans*-acting factors binding to the promoter and the enhancer interact with one another by formation of a DNA loop, thereby allowing these distal regulatory sequences (~2000 bp apart) to interact physically to form a stable transcription complex.

The biochemical analyses undertaken in the present studies allow refinement of that model and, in addition, permit a possible explanation for the ϵ -globin suppression effect. If one physically aligns the double-stranded DNA sequences corresponding to the β -globin promoter and enhancer, one is immediately struck by the apposition of binding sites for *trans*-acting factors within the two regulatory regions: binding sites for footprint factors (Fig. 8, boxes) align almost perfectly on the two *cis*-linked regulatory elements. If we superimpose the information from the erythroid cell stage-specific footprinting (Fig. 1) on the aligned sequences, an obvious explanation for ϵ -globin suppression becomes apparent (Fig. 8). The ability of the β -globin promoter to suppress ϵ -globin transcription in definitive cells is not a result of the absence or presence of NF-E4 but, rather, of the formation of a thermodynamically stable structure in definitive cells in which β -globin is, or soon will be, transcribed.

The model shown in Figure 8 predicts that the structure of the stable transcription complex for the adult β -globin gene is already formed in immature definitive (HD3) cells, and because of favorable (enhancer–promoter) protein–protein contacts (Fig. 8, center), ϵ -globin cannot be transcribed (the shared enhancer is already sequestered in these immature definitive cells by interaction with the β -globin promoter). However, because NF-E4 has not yet been temporally activated, the β -globin gene is transcriptionally inert. Activation of β -globin transcription simply requires reaching the proper maturation stage of erythropoiesis [presumably later than CFU-E, the maturation stage most closely approximated by HD3 cells (Beug et al. 1982)] so that NF-E4 is expressed. The binding of this factor is then presumed to be sufficient for overt β -globin transcription (Fig. 8, right).

In primitive erythroid cell extracts, β E-F1-, β P-F2-, and β P-F3-binding proteins are not found (Fig. 1; Emerson et al. 1987). The absence of these three factors in primitive cells (in comparison to HD3 cells) would then preclude the formation of a stable β -globin promoter–enhancer structure. The transcription ‘lollipop’ shown (Fig. 8, right) would not be formed in primitive erythrocytes because critical protein–protein contacts

Figure 8. 'Duelling lollipops' as a mechanism for stage-specific β -globin regulation. (*Left*) Physical alignment of the β -globin promoter and β -globin enhancer. Sequences are ordered numerically relative to the β -globin cap site. Boxes indicate DNase I footprints present in definitive erythroid basic extracts (Fig. 1). These binding sites correspond to consensus sequences in the promoter for β P-F1 (TFIID: TATA; Nakajima et al. 1988), β P-F2 (NF-E4: RAGAGGRGG; see Results and Discussion), β P-F3 (CTF/NF-1: CCAAT; Efstratiadis et al. 1980; Santoro et al. 1988), and β P-F4 (factor unknown: GGNTGGGG; see Results and Discussion); and in the enhancer for β E-F1 (CTF/NF-1: TGGNNNNNGCCAA; Emerson et al. 1987; Jones et al. 1987), β E-F2 (proximal = AP-1 or NF-E2: GTGAGT(C/A), and distal = AP-2: CCC(C/G)CNGGC; Angel et al. 1987; Imagawa et al. 1987; Mitchell et al. 1987; Mignotte et al. 1989), β E-F3 (identical to β P-F4?; see Discussion), and an inverted repeat of the consensus for β E-F4 [NF-E1: (A/C)Y(T/A)ATC(A/T)Y; Evans et al. 1988; Wall et al. 1988; Martin et al. 1989]. (*Right*) Superposition of the *trans*-acting factors (hatched figures) shown to exist in different stages of erythroid cells on their respective binding sites, and predicted factor-factor interactions in primitive (embryonic) cells (*left*), HD3 (immature definitive) erythroid cells (*center*) and mature definitive (adult) cells (*right*). The binding of NF-E4 to the promoter is depicted as a filled figure. DNase I protection data are taken from Figs. 1, 2, and 6. The model predicts that the structures in the *center* and *right* are stable in definitive cells, whereas the structure on the *left* (primitive RBCs) is unstable and would therefore be predicted to lead to stable complex formation only between the β -globin enhancer and the ϵ -globin promoter (Choi and Engel 1988).

would be missing in these embryonic cells.

Several experimentally testable predictions immediately follow from such a precise model: *Trans*-acting factors that bind within the promoter should associate strongly with specific enhancer-binding proteins; novel factors should exist in primitive cells that allow preferential interaction of the enhancer with the ϵ -globin promoter; supplementation of undifferentiated HD3 cells with exogenous NF-E4 should prematurely activate β -globin transcription. Testing these several predictions using the antibodies described here (for analysis of presumptive protein-protein interactions and for cloning of NF-E4 and other factors) should allow refutation or verification and further refinement of this model.

A general method for generation and identification of monoclonal antibodies to trans-acting factors

At present, there are two basic methods that have been successfully developed for studying cellular *trans*-acting factors and for cloning the genes that encode them. The first method requires multistep biochemical purification of the factor to homogeneity and determination of the amino acid sequence of the proteins. One may then prepare reagents (either a deduced coding oligonucleotide or antipeptide antibody) to use in the screening of cDNA libraries. Although this methodology is both expensive and labor-intensive, it has been successfully applied to the characterization and cloning of most of the *trans*-acting factors identified to date (Walter et al. 1985; Kadonaga et al. 1987; Bodner et al. 1988). The second method, first reported by Singh et al. (1988), is technically simpler than the first: Double-stranded DNA oligonucleotide probes, corresponding to DNA sequences recognized by a specific *trans*-acting factor, are used to screen expression libraries for clones encoding this factor. Although this methodology has been successfully applied to the cloning of several genes encoding *trans*-acting factors (Clerc et al. 1988; Muller et al. 1988; Sturm et al. 1988; Murre et al. 1989), as it relies on the expression of a functional DNA-binding motif in *Escherichia coli*, factors requiring eukaryotic post-translational processing or heterologous subunits for high affinity binding to the sequence will probably not be detected.

We developed an alternative strategy to identify and characterize such *trans*-acting factors. The methodology relies on the production of monoclonal antibodies to the native factors. Although these proteins are presumably present in cells only in extremely low abundance, two features of the system have allowed us to isolate multiple monoclonal antibodies to several low-abundance erythroid-specific DNA-binding proteins, including NF-E4: (1) We made use of a high frequency myeloma fusion partner to generate the hybridomas, and (2) we developed an extremely sensitive rRIA screening assay to detect those hybridomas secreting monoclonal antibodies to sequence-specific DNA-binding proteins.

The advantages of this methodology are that extensive biochemical purification of a given protein is unnecessary and that the rRIA screening assay requires only ex-

remely small amounts of the factor as a component of quite crude extracts. Furthermore, one may generate monoclonal antibodies to different determinants on the same protein. This is significant for two reasons: The first is that besides having an immediately useful reagent for studying the expression of the *trans*-acting factor (see Fig. 7), monoclonal antibodies to different determinants should facilitate the study of important protein-protein associations that may be involved in transcriptional regulation. The second reason is that having several monoclonal antibodies to a particular factor may greatly facilitate the successful screening of expression libraries for a cDNA encoding that factor. As mentioned above, because recombinant fusion proteins are expressed in *E. coli*, potentially important post-translational modifications may be lacking. Such domains may involve either the DNA-binding site or potential sites of association with other proteins. However, because the methodology presented here allows for the isolation of monoclonal antibodies to different epitopes in the same protein antigen, one does not rely on the structural integrity of the entire protein for the successful isolation of cDNA clones encoding a factor. Because the method depends on the isolation of monoclonal antibodies to the native structure of sequence-specific DNA-binding proteins, it should be of general use in studying any *trans*-acting factor for which the DNA recognition sequence is known.

Materials and methods

Preparation of DNA-binding protein extracts

Whole-cell extracts were prepared from primitive (embryonic) 4.5-day-old chicken erythroid cells, mature definitive (adult) erythroid cells, HD3 cells [a cell line representing approximately the CFU-E stage of erythroid cellular maturation (Beug, et al. 1982)], and adult chicken (perfused) liver, as described previously (Emerson et al. 1985). DNA-binding proteins were purified by adsorption of the whole-cell extracts to, and subsequent elution from, double-stranded calf thymus DNA cellulose. This preparation of total DNA-binding proteins from whole cells is subsequently referred to as a basic extract (to distinguish it from unenriched whole-cell or crude nuclear extracts). COS and CV-1 whole-cell extracts were prepared by the method of Dixon and Nathans (1985). Protein concentrations for these preparations were determined by A_{280} absorption and by Bradford assay (Ausubel et al. 1989).

Oligonucleotides and affinity purification of trans-acting factors

Double-stranded oligonucleotides used in these analyses were prepared on an Applied Biosystems 4 DNA synthesizer and purified on Applied Biosystems oligonucleotide purification cartridges. The oligonucleotides used and sequences referred to throughout this paper have been abbreviated in keeping with previous studies, e.g., β -globin enhancer footprint 4 oligonucleotide abbreviated β E-F4; (footprint IV of Emerson et al. 1987; Gallarda et al. 1989). DNA sequences of oligonucleotides used in this study are as follows:

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 $\beta$ P-F2:  AGCTTGGGAAGAGGAGGGCCCCGCGA
        ACCCTTCTCCTCCCCGGGACGTTCCGA
 $\beta$ P-F2': GGGGACATACCACGACGCGCGA
        CCCCTGTATGGTGTGCTCCCGCT
  
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βE-F4: AAAAGGTTGCAGATAAACATTTTGCTATCAAGACTTGCA
 CCAACGCTCTATTGTAAACGATAGTTCTGAACGTTTTT
 βE-F5: AAAAGGAAGAGAGGGGGTAAATCCTGTCAA
 CCTTCTCTCCCCCAATTAGGACAGTTTTTT
 Spl: GATCTGAAAAGCGGGGTCTCCA
 ACTTTTCGCCAGAGGTCTAG

DNA sequences for the β-globin gene promoter and enhancer are from the original reports (Dolan et al. 1983; Choi and Engel 1986; Hesse et al. 1986).

Affinity purification of β-globin enhancer-specific DNA-binding proteins was performed according to the method of Kadonaga and Tjian (1986), with only minor modifications. Double-stranded oligonucleotides were ligated and then covalently attached to CNBr-activated Sepharose 4B. Basic erythroid cell extracts (between 500 μg and 2.5 mg of total protein) were absorbed to the matrix in the presence of an equimolar mixture of poly[d(I-C)] and poly[d(A-T)] [poly[d(I-C)]/[d(A-T)]] for several hours in buffer Z [20 mM HEPES (pH 7.9), 5 mM MgCl₂, 100 mM KCl, 10% glycerol, 1 mM DTT, 0.3 mM PMSF, 0.2 mM EDTA, and 0.1% Brij-35]; unbound proteins were washed from the affinity matrix with the equivalent of 20 bed volumes of buffer Z. Bound proteins were then eluted with buffer Z containing 1.5 M KCl. Fractions of 0.5 ml were collected during the high salt wash, and the eluted protein was precipitated by the addition of 1 ml per fraction of 2.6 M ammonium sulfate, 0.1 M HEPES (pH 7.9). Precipitated protein was then resuspended in 100 μl of buffer Z, dialyzed overnight against buffer Z, and frozen at -80°C until it was used.

In vitro DNase I footprint analysis

DNase I footprint analysis was performed as described (Galas and Schmitz 1978; Emerson et al. 1987). In general, 0.5–2.0 μg of basic extract was added to 0.25 ng of a β-globin promoter probe containing promoter footprint sequences 1–4 [labeled at the NcoI site at nucleotide +75 (Dolan et al. 1983)] or 1 ng of a 460-bp β-globin enhancer probe containing footprint sequences 1–5 (Emerson et al. 1987) in the presence of an empirically determined amount of competitor [poly[d(I-C)]/[d(A-T)]]]. The final buffer conditions for the binding reaction were 25 mM HEPES (pH 7.9), 80 mM NaCl, 5 mM MgCl₂, 10 mM DTT, 10% glycerol, and 100 μg/ml BSA in 50 μl. Protein–DNA complexes were allowed to form for 60 min at 0°C, at which time DNase I (Worthington Biochemicals) was added to a final concentration of 2.0 μg/ml (1.8 units/μg). The DNase I reactions were carried out at 22°C for 90 sec and terminated by the addition of 300 μl of footprint stop buffer containing 0.25 M NaCl, 10 mM EDTA (pH 7.5), 0.1% SDS, and 20 μg/ml denatured salmon sperm DNA. The DNA was extracted by the addition of an equal volume of phenol–sevag (50% phenol/48% chloroform/2% isoamyl alcohol), precipitated with 3 volumes of ethanol, and subsequently electrophoresed on 6% polyacrylamide–50% urea wedge sequencing gels. DNase I footprint analysis of proteins recovered from the oligonucleotide affinity columns was carried out in similar fashion.

Transfections

Transfection of the ts34-AEV-transformed cell line HD3 (Beug et al. 1982) was performed as detailed previously (Choi and Engel 1986). Thirty-six hours after differentiation induction, the cells were counted, collected, and lysed using the RNA isolation procedure of Chomczynski and Sacchi (1987). Hybridization, S1 nuclease treatment, and gel analysis were also performed as described (Choi and Engel 1986).

SIs were normalized for equivalent RNA concentrations by using the RNA recovered from an equal number of cells from

each transfection experiment. The reproducibility within a single set of transfections (all performed at the same time) is very high [see Choi and Engel (1988); Fig. 2]. Furthermore, we have found that cotransfection control substrate DNAs can clearly compete for *trans*-acting factors (Trainor and Engel 1989). We have therefore purposely omitted cotransfection positive controls to obviate the possibility of misinterpretation of transcriptional activities because of plasmid competition for factors; instead, we currently rely on multiple independent transfections, which lead to the same conclusions.

Preparation of monoclonal antibodies

BALB/c mice were given a series of intraperitoneal injections every 2 weeks with 200 μg of definitive erythroid cell basic extracts over a period of 2 months. One week prior to fusion, the mice were injected intravenously with 50 μg of basic erythroid extract. The spleen cells from these mice were subsequently fused to a subline of the myeloma NSO-1, which yields 5- to 10-fold the number of hybridomas as our previous Sp2/0 line (NSO-1 cells kindly supplied by C. Lovell, University of Iowa Hybridoma Facility), and plated in two Bellco 384 plates. These plates have 24 square macrowells, into which 50–100 hybridoma clones are seeded (each clone sharing a common medium supernatant). The bottoms of the macrowells have 16 microwell chambers, into which the 50–100 hybridomas are partitioned, therefore allowing physical separation of mixtures of hybridomas. Macrowell culture supernatants were assayed for antibodies to specific β-globin enhancer-binding proteins by rRIA (described below). Subcloning is subsequently facilitated by direct picking of clones from each microwell of a macrowell (scored as positive for antibodies to enhancer-binding proteins) and by retesting with rRIA.

rRIA

Standard 96-well PVC plates (Falcon) were coated with 150 μl/well of goat anti-mouse IgG in PBS (total protein concentration: 1 mg/ml, Cappel) for 3 hr at 37°C. The anti-mouse antibody was removed, and the wells were then blocked for 30 min at room temperature in PBT (phosphate-buffered saline containing 10% calf serum, 0.05% Tween 20, and 0.01% thimerosol).

For the rRIA model studies in which we examined the T antigen/SV40 origin interaction, the PBT was removed and 150 μl of culture supernatant from the anti-T antigen hybridoma PAb419 (Harlow et al. 1981) was allowed to bind to the wells overnight at 4°C. The supernatants were removed and the wells washed once with PBT. Each well was then incubated with 100 μl of a binding solution containing 50 μg of total protein by adding various amounts of a stock preparation of COS cell crude extracts (containing 2–4 μg/ml T antigen; Y. Gluzman, pers. comm.) and CV-1 crude extracts (containing no T antigen) together with 1 ng of a ³²P-labeled *Hind*III fragment containing the SV40 origin of replication. The binding reaction was carried out in 0°C rRIA-binding buffer [10 mM HEPES (pH 7.9), 10 mM NaCl, 0.1 mM EDTA, 0.05% NP-40, 2 mM DTT, and 0.1 mg/ml BSA]. After allowing the binding reactions to proceed in the antibody-coated plates overnight at 0°C, the reactions were aspirated and the wells washed four times each with 200 μl of 0°C rRIA wash buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% NP-40]. The wells were subsequently cut apart and counted individually in scintillation cocktail.

For the rRIA experiments to identify antibodies to β-globin enhancer-binding proteins, 150 μl of the primary hybridoma culture supernatants was allowed to bind to the goat anti-mouse immunoglobulin-coated wells overnight at 4°C. After

washing the wells once with PBT, 100 μ l of rRIA-binding buffer was added, which included 1 ng of a 190-bp 32 P-labeled β -globin enhancer probe [containing β -globin enhancer footprint sequences 3, 4, and 5 (Choi and Engel 1986; Emerson et al. 1987)], together with $\sim$ 10–20 ng of definitive erythroid basic extract protein. After allowing the binding reactions to proceed in the antibody-coated plates overnight at 0°C, the reactions were aspirated and the wells were washed four times each with 200 μ l of 0°C rRIA wash buffer. The wells were subsequently cut and counted individually in scintillation cocktail.

Competitive rRIA

An rRIA experiment was set up with several of the isolated monoclonal lines recovered as described above, but the rRIA-binding buffer contained $\sim$ 10 pg of β E-F5 affinity-purified protein and 2 ng of a 32 P-labeled, ligated DNA oligonucleotide corresponding to the β E-F5 sequence (Emerson et al. 1987), together with various amounts of poly[d(I-C)]/[d(A-T)], an unlabeled, ligated DNA oligonucleotide corresponding to β E-F4 (Emerson et al. 1987) or an unlabeled, ligated DNA oligonucleotide corresponding to β E-F5. The reactions were carried out as described above.

Footprint selection

Monoclonal antibodies that reacted with β -globin enhancer-binding proteins in the rRIA were attached to goat anti-mouse immunoglobulin-coated Sepharose 4B beads (Pharmacia). Hybridoma culture supernatant (1.5 ml) was added to 50 μ l of the goat anti-mouse immunoglobulin beads in a microcentrifuge tube and rotated for 3 hr at room temperature. The beads were pelleted at low speed (6000 rpm for 2 min at ambient temperature in an Eppendorf microcentrifuge) and washed four times with 1 ml of PBS at room temperature, followed by one wash in 4°C rRIA-binding buffer. Fifty microliters of rRIA-binding buffer [containing 2–10 μ g of basic erythroid extracts and 1 μ g of poly[d(I-C)]/[d(A-T)] was added to the beads and allowed to react overnight at 0°C. The beads were then washed four times with 1 ml of 4°C rRIA wash buffer and once with 1 ml of 4°C footprint buffer [25 mM HEPES (pH 7.9), 80 mM NaCl, 5 mM MgCl_2 , 0.1 mg/ml BSA]. After aspirating the buffer completely from the beads, DTT was added to the bead slurry to a final concentration of 10 mM, with either 1 ng of a 32 P-labeled β -globin enhancer probe or 0.25 ng of a 32 P-labeled β -globin promoter probe. After binding for 2 hr at 0°C, 10 μ l of footprint buffer [containing 0.015 mg/ml DNase I] was mixed into the bead slurry at 0°C and transferred to a 22°C water bath. The DNase I reaction was terminated after 90 sec by the addition of 300 μ l of footprint stop buffer. The reaction was heated to 65°C for 10 min and briefly centrifuged, and the DNA was then processed as described above for the standard DNase I footprint analysis.

Protein blot analysis

Proteins were electrophoresed in 10% SDS–polyacrylamide gels, as described (Ausubel et al. 1989). Pyronin Y was added in the sample buffer to allow delineation of individual lanes after transfer to nitrocellulose. Individual nitrocellulose strips were cut and allowed to react with tissue-culture supernatants from the cloned hybridomas. Antigen was detected using 125 I-labeled secondary antibody.

Acknowledgments

We thank Beverly Emerson (Salk Institute) for initially showing us how to prepare basic erythroid cell extracts, Yasha Gluzman (Cold Spring Harbor Laboratory) for quantitative T antigen de-

termination, Debra Endean for help and encouragement in the early parts of this work, and Carla Hofland for preparing the figures. This work was supported by a National Institutes of Health (NIH) NRSA Fellowship (to J.L.G.), a U.S. Army Research Fellowship (DAA L03-86-G-0033 to K.P.F.), and NIH grants (to J.D.E.).

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The beta-globin stage selector element factor is erythroid-specific promoter/enhancer binding protein NF-E4.

J L Gallarda, K P Foley, Z Y Yang, et al.

Genes Dev. 1989, **3**:

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