Ralston, A., & Rabinowitz, P. (1978) in *A First Course in Numerical Analysis*, Section 8.12, McGraw-Hill, New York.
Regen, D. M., & Tarpley, H. L. (1974) *Biochim. Biophys. Acta 339*, 218-233.
Rosenberg, T., Vestergaard, B., & Wilbrandt, W. (1956) *Helv. Physiol. Pharmacol. Acta 14*, 334-241.
Sen, A. K., & Widdas, W. F. (1962) *J. Physiol. (London) 160*, 392-403.
Sogin, D. C., & Hinkle, P. C. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5725-5729.
Sogin, D. C., & Hinkle, P. C. (1980b) *Biochemistry 19*, 5417-5420.
Stein, W. D. (1986) in *Transport and Diffusion across Cell Membranes*, pp 231-306, Academic Press, New York.
Stineman, R. W. (1980) *Creative Comput. July*, 54-57.
Wheeler, T. J. (1986) *Biochim. Biophys. Acta 862*, 387-398.
Widdas, W. F. (1952) *J. Physiol. (London) 118*, 23-39.

In Vitro Characterization of Tissue-Specific Nuclear Proteins Preferentially Bound to the Mouse β-Globin Gene during MEL Cell Terminal Differentiation†

Jean-Claude Lelong,* Grégoire Prevost, Kyong-il Lee, and Michel Crepin
Institut d'Oncologie Cellulaire et Moléculaire Humaine, Université de Paris-Nord, 129 route de Stalingrad, 93000-Bobigny, France

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ABSTRACT: Using DNA restriction fragments of the mouse β-globin gene and other promoter-containing DNA fragments (LTR-MMTV and pBR322) as controls, we have characterized by protein blotting, in extracts of mouse erythroleukemia (MEL) cells, specific nuclear DNA binding proteins with a preferential affinity for the β-globin DNA. Some proteins (110 and 75 kDa) appear in differentiated MEL cells while others (100, 95, and 35 kDa) are present in immature MEL and normal erythroblast cells and bind selectively to the far-upstream region of the gene. These proteins could modulate either positively or negatively the expression of the β-globin gene and maybe, of other genes, during the terminal differentiation of erythroid cells.

Expression of the β-globin gene family in erythroid cells appears to be both tissue specifically determined (Collins et al., 1984; Groudine et al., 1981) and inducible (Ross et al., 1974; Wright et al., 1983). Mouse erythroleukemia (MEL) cells are Friend virus transformed erythroid cells, arrested at the proerythroblast stage of differentiation, that are easy to cultivate and may be induced with a variety of chemicals, including dimethyl sulfoxide (DMSO) (Ross et al., 1974). The accumulation of globin transcripts results from both transcriptional activation of the mouse globin genes and increased relative stability of their mRNAs (Hofer et al., 1982). Therefore, this system is a very suitable model to study the tissue-specific factors that are activated in order to promote the selectivity of transcription, leading to a particular type of terminal phenotypic expression. Cis-acting DNA sequences that control human, murine, and chicken β-globin gene transcription have been analyzed by deletion, point mutation, and chimeric gene constructions, using mainly so far either in vivo assays of transient expression of introduced genes (Charnay et al., 1984, 1985; Dierks et al., 1983; Myers et al., 1986; Chao et al., 1983), in vitro transcription systems (Hofer et al., 1982), or transgenic mice (Kollias et al., 1987). These are usually located at chromatin hypersensitive sites [for a review, see Galson and Housman (1988)]. The function of these control DNA elements in the developmental activation and tissue specificity of β-globin transcription requires the coordinate binding of both general and promoter-specific transcription factors. The binding sites of several such sequence-specific DNA binding proteins have been identified in the 5′ upstream flanking region from −300 to −70 bp, in the intragenic sequences, and in 3′ end of the gene [for a review, see Galson and Housman, (1988)].

Although a promoter-specific transcription factor need not be a sequence-specific DNA binding protein and vice versa, an important approach to the understanding of the tissue-specific regulation of gene expression might be to analyze variations during differentiation of cell- and sequence-specific nuclear DNA binding proteins, probed with cloned fragments of a regulated gene.

Because protein blotting (Bowen et al., 1980) is particularly suitable for a rapid and highly resolutive screening of cell crude extracts, we have used this technique to analyze MEL cell nuclear proteins, which have been tested for (1) preferential binding to a cloned mouse β-globin gene, (2) erythroid cell specificity, and (3) modulation of the DNA binding activity after induction of β-globin expression.

We have characterized several DNA binding proteins which satisfy these criteria and have also described results suggesting that three of them may interact with specific sequences located far upstream and downstream from the transcription start.

Experimental Procedures

Cell Lines. Mouse erythroleukemia cells, clone 707-1-C (from Dr. N. Affara), were grown at 37 °C in a 5% CO_2 atmosphere in Ham's F12 medium containing glutamine, 15% horse serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were collected at a density of 1.5×10^6/mL

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* To whom correspondence should be addressed.

0006-2960/89/0428-4594$01.50/0

at 1500 rpm, washed once in phosphate-buffered saline, and pelleted. Induction of globin synthesis was obtained by addition of 1.5% dimethyl sulfoxide in the culture medium at a density of 10^5 cells/mL. After 3–4 days, the cells reached a density of 10^6/mL, were 90% benzidine positive, and were collected as a dark red pellet. Untreated cells remained benzidine negative, giving a white pellet. The GR mouse mammary tumor cell line was cultivated in MEM with 10% fetal calf serum. Cells were scrapped from petri dishes before confluence, collected, and washed as above. Purified erythroblasts from rat embryonic liver were provided by P. Mayeux. Myoblast cell line clone 10 T 984 (Jakob et al., 1978) was obtained from M. Buckingham. The mink lung cell line was obtained from J. C. Chermann.

Cell Fractionation and Nuclear Extracts. All procedures were performed at 4 °C, and all buffers contained 0.5 mM dithiothreithol (DTT) and 0.5 mM phenylmethanesulfonyl fluoride (PMSF). Proteins were prepared either from total cells (Manley et al., 1980) or from purified nuclei.

For the purification of nuclei (Dastugue & Crépin, 1979), cells were resuspended and lysed in 9× packed cell volumes (PCV) of buffer I (10 mM Hepes, pH 8, 2 mM $MgCl_2$, 3 mM $CaCl_2$, and 0.2% NP 40) with a Dounce homogeneizer (B pestle, 20 strokes). To the broken cells was immediately added 2 × PCV of buffer II (10 mM Hepes, pH 8, 4 mM $MgCl_2$, and 2 M sucrose) followed by 6–10 × PCV of buffer III (10 mM Hepes, pH 8, 4 mM $MgCl_2$, and 0.33 M sucrose). The crude nuclear suspension was centrifuged through a 10-mL cushion of buffer II at 15 000 rpm for 60 min in an SW 27 rotor. The resulting pellet of purified nuclei was gently resuspended in buffer IV (10 mM Hepes, pH 8, 4 mM $MgCl_2$, 25% sucrose, and 50% glycerol) (Manley et al., 1980). The resulting suspension was brought dropwise to 10% saturation of ammonium sulfate, with continuous slow magnetic stirring for 30–60 min. The semiviscous solution was centrifuged at 50 000 rpm for 3 h in a 60 Ti rotor. The supernatant containing the bulk of non-histone nuclear proteins was 10× concentrated by ammonium sulfate precipitation (0.33 g/mL), dialyzed against buffer V [20 mM Hepes, pH 8, 6 mM $MgCl_2$, 40 mM $(NH_4)_2SO_4$, 0.2 mM EDTA, and 15% glycerol], and stored after clarification as aliquots in liquid nitrogen. Cytosol proteins were concentrated by ammonium sulfate precipitation of the crude nuclear supernatant.

Protein Blotting for Detection of Specific DNA Binding Proteins. The procedure of Bowen et al. (1980) was used with the following modifications: Protein samples for SDS–PAGE were prepared in running buffer (25 mM Tris–192 mM glycine, pH 8–8, and 0.1% SDS) and contained 5% β-mercaptoethanol.

The heating step was omitted. The stacking gel [5% acrylamide–0.13% bis(acrylamide), pH 6.8] and the separating gel [12.5% acrylamide–0.1% bis(acrylamide), pH 8.8] each contained 0.1% SDS without urea. Electrophoresis was performed overnight at 8 mA/gel. All slab gels were 0.9 mm thick. The renaturing step was reduced to 90 min with two changes of buffer, to avoid a progressive washout of proteins. Omitting this step increased the yield and number of proteins transferred but resulted in a complete loss of DNA sequence specificity of the DNA binding proteins. Varying the pH of transfer buffer from 3.7 to 8.9 influences the nature of the DNA binding proteins being transferred: a pH 7.5 was routinely used in all experiments. The protein concentrations were determined by UV absorbance at 280 and 260 nm. Protein blots were stained overnight by China ink (Pelikan 17.black) in phosphate-buffered saline containing 0.2% Tween 20.

DNA Fragments. Mouse β-globin DNA fragments were prepared from the λgt WES β m G 2 recombinant (Tilghman et al., 1977), kindly provided by Dr. P. Leder. The 7-kb β-globin *Eco*RI–*Eco*RI insert was cleaved by *Bam*HI, *Hin*dIII, *Sac*I, *Hpa*I, and *Bgl*II. The *Eco*RI–*Bam*HI fragment (1.9 kb), containing the 5′ upstream, the first exon, the first intron, and the second exon sequences, was subcloned in pBR322 and, in some experiments, further cleaved by *Pst*I, *Sau*3A, and *Fok*I. The resulting DNA fragments were isolated by agarose gel electrophoresis. Either isolated or mixed purified DNA fragments were ^{32}P labeled by nick-translation (Maniatis et al., 1982) omitting DNase I, or by the end-filling method using the Klenow large fragment of DNA polymerase I (Maniatis et al., 1982). The specific activity of the probes ranged from 10^7 to 5×10^7 cmp/μg of DNA.

The 5′ LTR-MMTV *Pst*I–*Pst*I DNA fragment (1.45 kb) originated from H. Diggelmann (Fasel et al., 1982). Poly-[d(AC)]–poly[d(GT)] was purchased from Boehringer.

DNA Binding. Protein nitrocellulose blots were incubated first for 30 min at room temperature in binding buffer: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.02% BSA, 0.02% Ficoll, and 0.02% poly(vinylpyrrolidone) containing 30 μg/mL poly(I)–poly(C).

The filters were then washed in three changes of binding buffer without poly(I)–poly(C) for 30 min and incubated in the same buffer containing the labeled DNA probe (2.5×10^4–1.2×10^5 cpm/mL) and various amounts of unlabeled nonspecific competitor DNA, either sonicated salmon sperm DNA, *Escherichia coli* DNA, or poly(I)–poly(C), for 60 min at room temperature. The filters were exhaustively washed in three changes of binding buffer until no radioactivity was detectable in the wash (usually overnight), then dried, and autoradiographed.

Results

Specific Binding of β-Globin Genomic DNA to MEL Cell Non-Histone Proteins. Increasing concentrations of a crude MEL cell non-histone protein preparation (Manley et al., 1980) were fractionated by SDS gel electrophoresis and then transferred, after SDS removal in the presence of urea, to nitrocellulose membranes which were incubated with ^{32}P-labeled β-globin DNA in the presence of an excess amount of unlabeled competitor, either poly(I)–poly(C) or salmon sperm DNA. According to previous observations (Jack et al., 1981), both protein dilution and increasing competition by nonspecific DNA progressively abolish the radioactive signals given by the various DNA binding proteins of the nuclear extract (data not shown) and improve the specificity of the protein–DNA complexes until an appropriate choice of protein/specific DNA ratio and excess amount of unlabeled competitor DNA allows the selection of a restricted number of high-affinity DNA binding proteins (Figure 1B, lanes 2 and 3).

The DNA sequence specificity of at least some of these non-histone proteins is suggested by the fact that preincubation of the nitrocellulose-bound proteins with an excess of unlabeled native sonicated salmon sperm DNA completely abolishes the binding of ^{32}P β-globin DNA to the residual histones, but not to these DNA binding proteins (data not shown).

In order to further investigate the DNA sequence specificity of these β-globin DNA-bound proteins, identical nitrocellulose blots of increasing concentrations of SDS–PAGE-fractionated undifferentiated MEL cell nuclear non-histone proteins (Figure 1A) have been incubated separately with various labeled double-stranded DNAs: under the same experimental conditions, we observed a preferential binding of at least three polypeptides (100, 95, and 35 kDa) to the β-globin *Eco*RI–

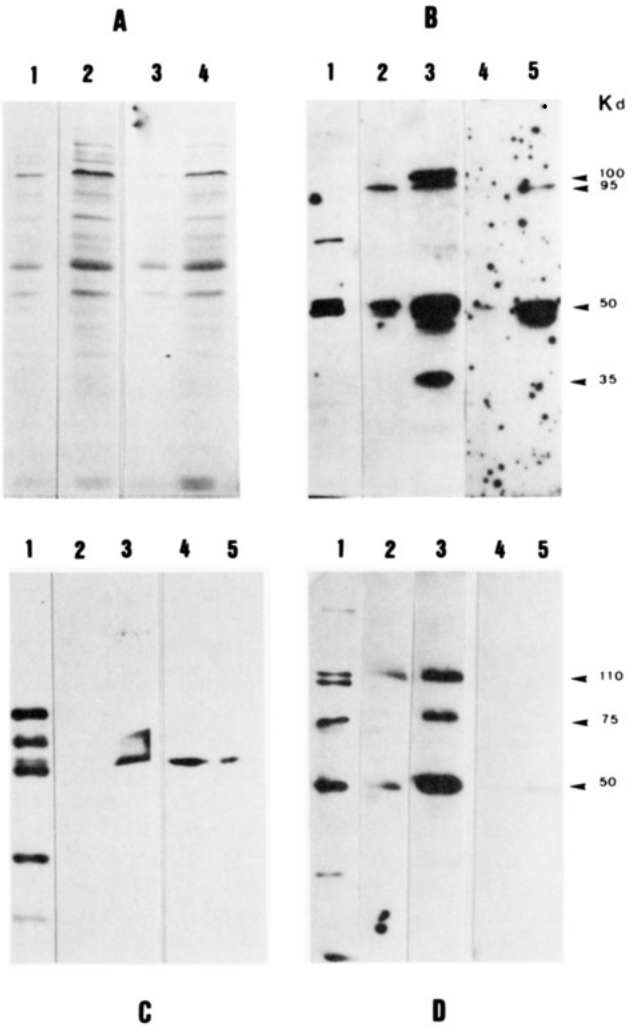


FIGURE 1: β-Globin specificity of uninduced MEL cell DNA binding proteins. (A) China ink stained protein blots from the same SDS–polyacrylamide gel loaded with (1 and 3) 15 μg or (2 and 4) 50 μg of uninduced MEL cell nuclear proteins. (B) Autoradiography of the same nitrocellulose protein blots probed with (2 and 3) 100 ng of ^{32}P-labeled β-globin DNA *Eco*RI–*Bam*HI fragment or (4 and 5) 60 ng of ^{32}P-labeled poly(AC)–poly(GT). The blots were preincubated with 20 μg/mL poly(I)–poly(C). Lane 1 contained ^{14}C-labeled marker proteins. (C) Autoradiography of the same protein blots probed with (2 and 3) 100 ng of ^{32}P-labeled MMTV-LTR DNA *Pst*I–*Pst*I fragment or (4 and 5) 140 ng of ^{32}P-labeled linear pBR322 DNA. The blots were preincubated with 50 μg/mL cold salmon sperm DNA. Lane 1 contained ^{14}C-labeled marker proteins. (D) Autoradiography of protein blots from (2 and 4) 20 μg or (3 and 5) 60 μg of DMSO-induced MEL cell nuclear proteins, probed with (2 and 3) 100 ng of ^{32}P-labeled β-globin DNA *Eco*RI fragment or (4 and 5) 200 ng of ^{32}P-labeled LTR DNA *Pst*I–*Pst*I fragment. Lane 1 contained ^{14}C-labeled marker proteins.

*Bam*HI β-globin DNA fragment (Figure 1B, lanes 2 and 3); nearly no binding of these polypeptides was observed to the synthetic repetitive double-stranded copolymer poly[d(AC)]–poly[d(GT)] (Figure 1B, lanes 4 and 5) nor to the LTR-MMTV 1.5-kb *Pst*I–*Pst*I fragment (Figure 1C, lanes 2 and 3) nor to the pBR322 DNA (Figure 1C, lanes 4 and 5). Thus, under stringent conditions, a group of high-affinity DNA binding proteins from undifferentiated MEL cells specifically recognizes sequences in the β-globin genomic DNA but has affinity neither for a synthetic copolymer nor for a prokaryote of even another type of eukaryotic promoter-containing DNA, the MMTV-LTR.

This is confirmed by the unspecific binding of a 50-kDa polypeptide to all of these DNAs undiscriminately that can be taken as a useful internal control. We found the latter

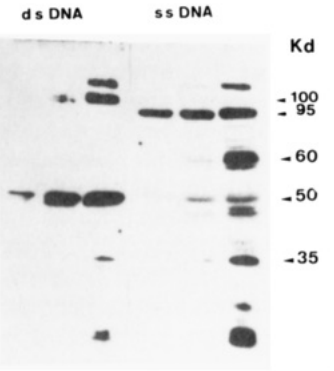


FIGURE 2: Differential affinity of β-globin DNA binding proteins for single- or double-stranded DNA. The same polyacrylamide gel loaded with increasing amounts of an uninduced MEL cell extract was equally transferred onto two nitrocellulose filters: one (left) was subsequently probed with native ^{32}P-labeled β-globin DNA, and the other (right) was probed with the same amount of heat-denatured ^{32}P-labeled β-globin DNA (*Eco*RI fragment).

unspecific DNA binding activity both in nuclear and in cytoplasmic extracts, while the three β-globin DNA binding proteins were exclusively nuclear (data not shown).

In addition, particular properties clearly differentiate the 100-kDa from the two other 95- and 35-kDa β-globin-specific MEL DNA binding proteins: the 100-kDa protein binds only to the native double-stranded form of β-globin DNA while the 95- and 35-kDa proteins and an additional 60-kDa protein bind with more affinity to heat-denatured, single-stranded β-globin DNA (Figure 2).

In contrast with previously reported results (Bowen et al., 1980), we found that protein transfer to nitrocellulose by diffusion after SDS removal was highly selective: proteins with no affinity for nitrocellulose are clearly not transferred while those which have a high affinity for it may be considerably enriched by transfer. This is the case of the 100- and 95-kDa proteins since they become heavily stained on nitrocellulose, while hardly detected on Coomassie blue colored gels before transfer (compare Figures 1A and 3, top panel). In addition, the ability for a protein to be transferred appears to be dependent on the pH of the transfer buffer: At pH 8.9, only the negatively charged acidic proteins are transferred and thus selectively enriched on nitrocellulose. Among these are the 100-kDa protein and several other acidic DNA binding proteins. By contrast, at pH 3.7, the latter proteins are not transferred, while the negatively charged basic proteins appear to be. The 95- and 35-kDa single-stranded DNA binding proteins belong to this class as well as the residual histones. This property might be used for selectively picking up rare polypeptides (data not shown) from a crude mixture of various proteins separated by SDS–polyacrylamide gel electrophoresis (unpublished results).

Cell Specificity of β-Globin DNA Binding Proteins. In order to analyze the cell specificity of β-globin DNA binding to these proteins, we have compared crude non-histone protein preparations from various rodent cell types including immature or DMSO-differentiated MEL cells and epithelial mammary cells (Figure 3). Total non-histone proteins (top) and β-globin DNA binding proteins (bottom) from MEL cells, either uninduced (lanes 2–4) or DMSO induced (lanes 5–7), are shown after SDS gel electrophoresis (top) and incubation with ^{32}P-labeled 7-kb β-globin DNA following nitrocellulose transfer (bottom). Total non-histone proteins stained with Coomassie blue are very similar in these three cell types (Figure 3, top). By contrast, β-globin DNA binding proteins are different in the three extracts (Figure 3, bottom). At least five DNA

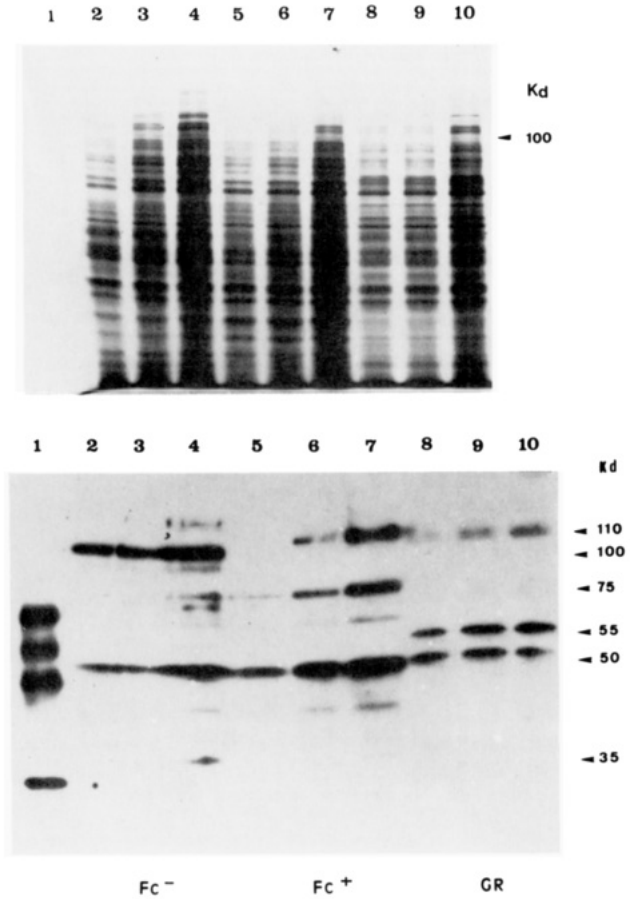


FIGURE 3: Tissue specificity of β-globin DNA binding proteins. (Top) SDS–PAGE pattern of non-histone proteins from erythroid and nonerythroid cell extracts (Coomassie blue staining): (1) ^{14}C-labeled molecular mass marker proteins BSA (69 kDa), IgG (55 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa); (2) 22 μg, (3) 33 μg, (4) 56 μg of noninduced MEL cell NHP; (5) 24 μg, (6) 37 μg, and (7) 63 μg of induced MEL cell NHP; (8) 28 μg, (9) 39 μg, and (10) 56 μg of GR mammary cell line NHP. (Bottom), Autoradiograph of the same proteins transferred to a nitrocellulose filter incubated in binding buffer, first with 40 μg/mL cold, native, sonicated salmon sperm DNA and then with 200 ng of ^{32}P-labeled globin DNA (*Eco*RI fragment) (specific activity 7.5×10^6 cpm/μg).

binding proteins seem to be MEL cell specific (110, 100, 95, 75, and 35 kDa). The major one (100 kDa) is also labeled in immature erythroblasts from rat embryonic liver (Figure 4, lane 3) but hardly detectable in mammary cells (Figure 3, bottom, lanes 8–10) as well as in various other cultured cells including myoblast clone 10 T 984 or mink lung cells (data not shown). One 55-kDa polypeptide binds DNA only in GR epithelial mammary cell extracts. Under the stringent conditions used in these experiments (high DNA/protein ratio, 0.1 M NaCl), only the 50-kDa polypeptide binds DNA indiscriminately in all cell types tested, according to its unspecific DNA binding (Figure 1).

Effect of DMSO-Induced Terminal Differentiation on the Pattern of MEL Cell β-Globin DNA Binding Proteins. When MEL cells differentiate after 4 days of DMSO treatment, and the synthesis of β-globin mRNA is maximum, the major DNA polypeptide of 100 kDa from immature cells no longer binds β-globin DNA (Figure 3, bottom, lanes 5–7).

By contrast, two other protein–DNA complexes appear preferentially in differentiated MEL cells, which have been designated 110 and 75 kDa according to their apparent molecular mass determined by reference to comigrating and cotransferred ^{14}C-labeled marker proteins (Figure 3, lane 1). The 110-kDa protein is clearly distinct from the major 100-kDa

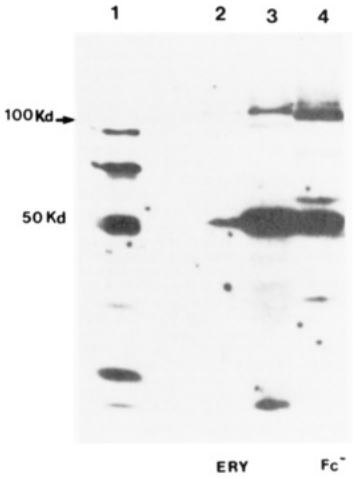
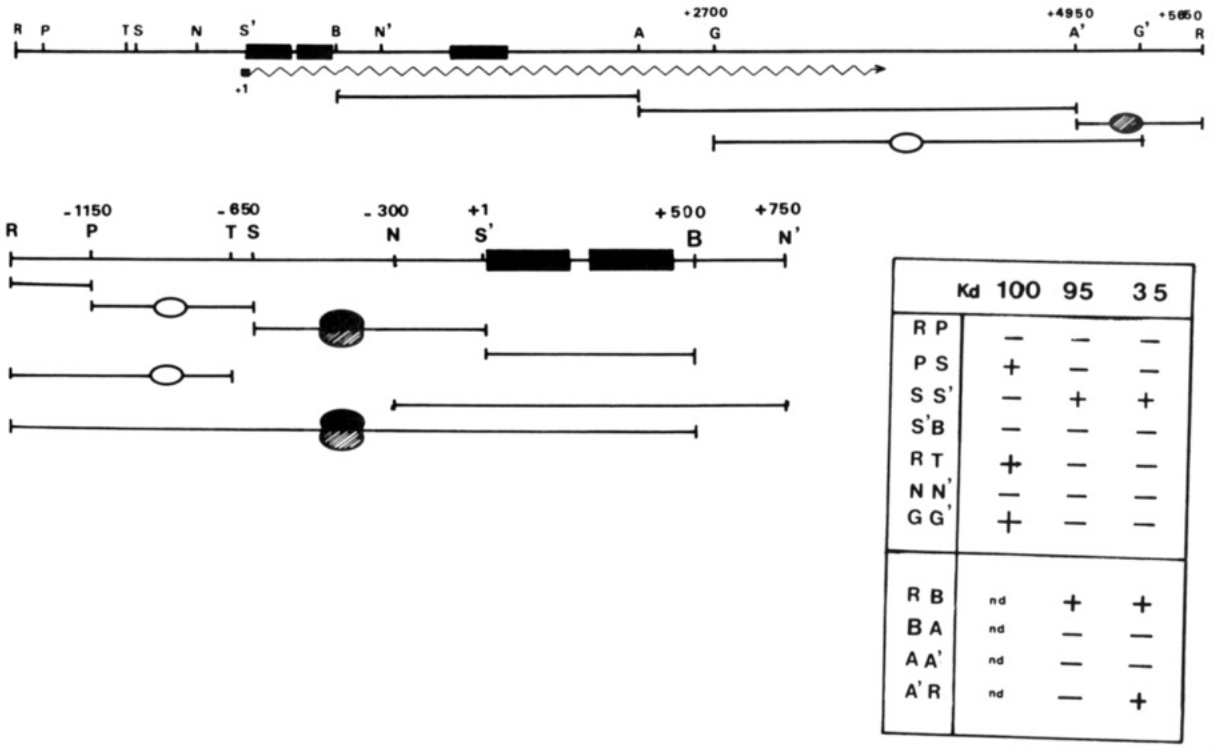


FIGURE 4: Tissue specificity of β-globin DNA binding proteins. Autoradiograph of a nitrocellulose filter containing the following proteins transferred from a SDS–polyacrylamide gel loaded with: (1) ^{14}C-labeled molecular mass marker proteins; (2 and 3) 10 μg and 30 μg of nuclear non-histone proteins from embryonic liver erythroblasts; (4) 60 μg of nuclear non-histone proteins from uninduced MEL cells. The blot was probed with 200 ng (1.5×10^6 cpm) of ^{32}P-labeled Klenow end-labeled β-globin *Eco*RI–*Bam*HI DNA fragment.

protein bound to β-globin DNA in undifferentiated cells (lanes 2–4) by its slightly different molecular mass and its immunoreactivity (unpublished results).

The DNA binding activities of the 110- and 75-kDa proteins in differentiated MEL cells appeared also β-globin specific since they did not bind to the LTR-MMTV DNA probe (Figure 1D).

Selective Affinity of β-Globin DNA Binding Proteins for Upstream Flanking Sequences. By using restriction fragments from the 7-kb genomic β-globin DNA, we have tentatively localized the specific binding sites of the best characterized nuclear DNA binding proteins from undifferentiated MEL cells. To perform these experiments, we have digested the *Eco*RI DNA fragment with *Pst*I, *Sst*I, *Sau*3A, *Hin*dIII, *Bam*HI, *Hpa*I, *Hpa*I, and/or *Bgl*II (Figure 5). The ^{32}P-labeled DNA subfragments were used as probes either alone or in combination. The Autoradiograph shows which protein binds the corresponding sequence using a single labeled subfragment. Using a mixture, we have eluted the unique labeled DNA fragment or fragments bound to a single polypeptide onto nitrocellulose and analyzed the eluted DNA by agarose gel electrophoresis: only the DNA fragments which have affinity for the individual protein are retained and will appear on an agarose gel autoradiograph (Lelong et al., 1989). Figures 6 and 7 show the pattern of the specific DNA–protein complexes obtained by probing with various isolated ^{32}P-labeled β-globin DNA subfragments identical nitrocellulose blots from polyacrylamide gel electrophoresis of an undifferentiated MEL cell non-histone protein preparation: the 100-kDa protein binds to the overlapping most upstream fragments *Eco*RI–*Sst*I (RT) (Figure 6) and *Pst*I–*Sau*3A (PS) (Figure 7). The absence of interaction with fragments *Eco*RI–*Pst*I (RP), *Sau*3A–Sau3A (SS′), and *Sau*3A–*Bam*HI (S′B) (Figure 7) indicates that the 100-kDa protein may recognize a nucleotide sequence located between −1150 and −655 bp (Figure 5). Surprisingly enough, we observed an additional interaction of this protein downstream to +2700 bp (*Bgl*II–*Bgl*II, GG′) (Figure 6). By contrast, the 95- and 35-kDa proteins appeared to bind specifically to the fragment *Sau*3A–*Sau*3A (SS′) (Figure 7). Since they apparently did not bind to any other fragment tested, particularly fragments corresponding to the proximal promoter region (NN′) (Figure



	Kd	100	95	35
R P		−	−	−
P S		+	−	−
S S'		−	+	+
S'B		−	−	−
R T		+	−	−
N N'		−	−	−
G G'		+	−	−
R B		nd	+	+
B A		nd	−	−
A A'		nd	−	−
A' R		nd	−	+

FIGURE 5: Positions of the various β-globin DNA restriction fragments bound to the 100-, 95-, and 35-kDa proteins from uninduced MEL cells. (Upper panel) Schematic representation of the *Eco*RI β-globin DNA fragment (7 kb) showing the restriction sites (R = *Bam*HI, A = *Hpa*I, T = *Sst*I, S = *Sau*3A, N = *Hin*dIII, B = *Bam*HI, and G = *Bgl*II), the exons (solid rectangles), the noncoding regions (continuous thick line), and the distances in base pairs from the transcription initiation site (+1). The latter is indicated by a black square followed by a zig-zag line covering the presumed length of the transcribed DNA according to Hofer et al. (1982). The DNA fragments used for the binding experiments are delimited below. Those fragments occupied by an ovale are bound to the corresponding protein: empty ovals represent the 100-kDa protein; solid ovals, the 95-kDa protein; dashed ovals, the 35-kDa protein. Oval pairs on the SN fragment are only a schematic representation suggesting that the two proteins might bind near each other. For clarity, the 5′ upstream region of the gene (fragment RN′) was scaled up twice. The table summarizes the position of the binding sites of the three proteins using isolated DNA fragments (upper) or mixed DNA fragments (lower).

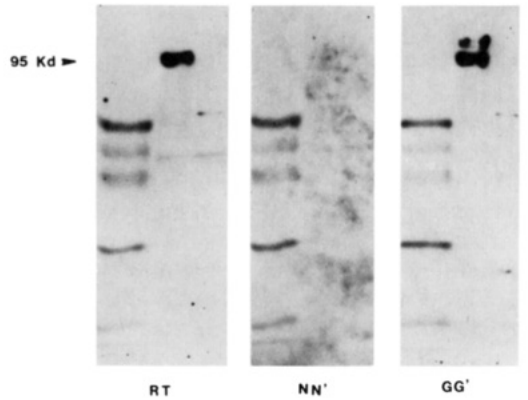


FIGURE 6: Selective affinity of the β-globin DNA binding proteins for isolated genomic DNA subfragments. Autoradiograph of identical nitrocellulose replicates obtained by transfer of uninduced MEL cell NHP extracts fractionated by SDS–PAGE. The filters were probed with the ^{32}P-labeled β-globin restriction fragments *Eco*RI–*Sst*I (RT), *Hin*dIII–*Hin*dIII (NN′), and *Bgl*II (GG′) (Figure 5). The slot on the left of each filter contains ^{14}C-labeled molecular mass marker proteins: BSA, 69 kDa; IgG, 55 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; lactoglobulin A, 18 kDa; lysozyme, 14.5 kDa. The slot on the right contains MEL cell NHP extract heated at 70 °C for 10 min prior to SDS–PAGE (Jack et al., 1981).

6) and (S′B) (Figure 7), we conclude that the latter two proteins recognize nucleotide sequences located between the two *Sau*3A restriction sites (SS′) and upstream from the *Hin*dIII site (N), therefore, in between −650 and −300 bp (SN) from the transcription initiation site (Figure 5). The labeled DNA retained on the nitrocellulose filter by the 95- and 35-kDa proteins from a *Bam*HI + *Hpa*I digest mixture of the entire 7-kb β-globin DNA fragment was eluted after excision of the corresponding radioactive spots and analyzed by agarose gel electrophoresis. The results of such experiments are shown in Figure 8: among the four DNA subfragments covering the entire length of the β-globin genomic fragment, and one additional subfragment resulting from partial digestion at the 3′-*Hpa*I sites (Figure 5), only the 1.9-kb *Eco*RI–*Bam*HI subfragment (RB) including the 5′ upstream flanking sequences and the two first exons was retained in the eluate from the excised 95-kDa radioactive nitrocellulose spot. Moreover, it was selectively bound to this protein, since DNA fragments including the second intron, the third exon, and the 3′ flanking sequences were not bound (Figure 8, lanes 3 and 4). A similar result was obtained with the DNA fragments bound to the 35-kDa protein. The major fragment retained was again the 5′-*Eco*RI–*Bam*HI 1.9-kb fragment (RB) (Figure 8, lanes 1 and 2). There was no retention of the 3′-*Hpa*I–*Hpa*I large fragment (AA′) nor of the *Bam*HI–*Hpa*I (BA) fragment. Interestingly, the small 3′ extreme distal 0.7-kb *Hpa*I–*Eco*RI fragment (A′R) which was previously shown to contain an in vitro initiation site of transcription is also retained by the 35-kDa protein. When isolated DNA fragments were used another protein, 100 kDa, has been shown to interact also with the 3′ region of the gene (Figure 6). Figure 5 summarizes the β-globin genomic DNA fragments found by protein blotting to bind specific MEL cell nuclear proteins.

Since the most striking result was the MEL cell specific binding of both the 95- and 35-kDa polypeptides to the β-globin 650 bp DNA fragment SS′, we further investigated these protein–DNA interactions using as an alternative method the gel retardation assay (Garner & Revzin, 1981). Among

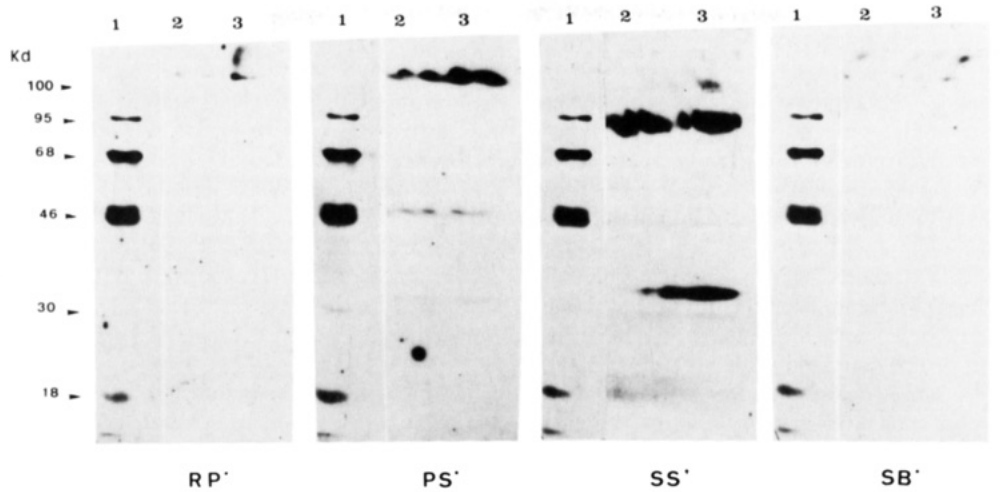


FIGURE 7: Selective affinity of β-globin DNA binding proteins for isolated genomic DNA subfragments. Autoradiograph of identical nitrocellulose transfer replicates of two increasing concentrations of uninduced MEL cell NHP extracts fractionated by SDS–PAGE. The filters were probed with the ^{32}P Klenow end-labeled β-globin restriction fragments *Eco*RI–*Pst*I (RP), *Pst*I–*Sau*3A (PS), *Sau*3A–*Sau*3A (SS′), and *Sau*3A–*Bam*HI (SB) (Figure 5). (1) ^{14}C-Labeled molecular mass marker proteins: phosphorylase B, 92.5 kDa; BSA, 68 kDa, ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; lactoglobulin A, 18 kDa; cytochrome *c*, 12 kDa. Lanes 2 and 3 show increasing concentrations of uninduced MEL cell NHP extracts.

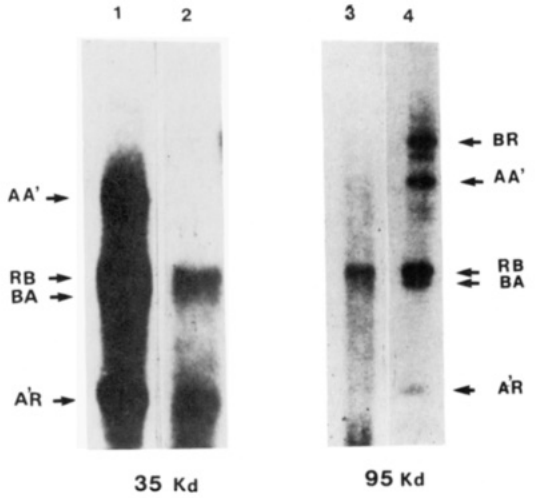


FIGURE 8: Selective affinity of the 95- and 35-kDa DNA binding proteins for β-globin upstream flanking sequences: discrimination between mixed genomic β-globin DNA subfragments. Uninduced MEL cell NHP transferred to nitrocellulose after SDS–PAGE were probed by incubation with a mixture of ^{32}P Klenow end-labeled β-globin DNA restriction fragments used as a probe indicated by arrows (see also Figure 5). Autoradiograph of 1.2% agarose gels loaded (1 and 4) with the mixture of ^{32}P-labeled β-globin DNA fragments used as a probe and (2 and 3) with the ^{32}P-labeled DNA fragments eluted from the 35-kDa (left panel) and 95-kDa (right panel) radioactive spots of the nitrocellulose filter (not shown).

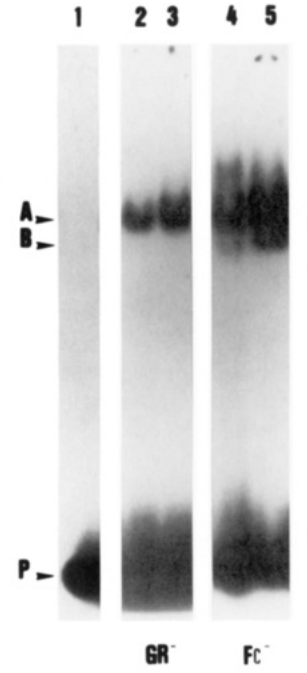


FIGURE 9: Gel retardation analyses of the binding of nuclear proteins from GR mammary and MEL cells to β-globin DNA. The 173 bp *Fok*I–*Fok*I-labeled β-globin DNA fragment spanning from −404 to −231 bp (2 ng) was incubated with 0.4 M NaCl extract GR or MEL cell nuclear proteins and increasing amounts of unspecific competitor poly(I)–poly(C) in binding buffer [10 mM Hepes, pH 8, 100 mM NaCl, 0.1 mM EDTA, and 15% glycerol] in a final volume of 30 μL, 30 min at 25 °C before electrophoresis at 150 V in a 5% polyacrylamide gel using recirculating TAE buffer (Maniatis, 1982). Autoradiograph of the dried gel: (1) 2 ng (15000 cpm) of *Fok*I–*Fok*I ^{32}P-labeled β-globin DNA fragment without protein; (2 and 3) the same amount of labeled DNA fragment with 12 μg of GR nuclear proteins and 5 and 10 ng, respectively, of poly(I)–poly(C); (4 and 5) the same amount of labeled DNA fragment with 12 μg of MEL nuclear proteins and 5 and 10 ng, respectively, of poly(I)–poly(C). The specific protein–DNA complexes (A and B) are indicated by arrows. P indicates the free DNA probes.

the series of restriction fragments covering the entire length of the 650 bp SS′ fragment, only a *Fok*I–*Fok*I 173 bp DNA fragment extending from −404 to −231 bp from the transcription initiation site was able to form a MEL cell specific protein complex (Figure 9, lane 5).

Discussion

The advantages of the protein blotting procedure for the detection of DNA binding proteins have already been extensively discussed in previous reports (Bowen et al., 1980; Silva et al., 1987; Hübscher, 1987; Jack et al., 1981; Miskimins et al., 1985). We find this technique particularly useful to screen various differentiation cell types, of normal or tumoral origin, for tissue-specific DNA binding proteins harboring an in vitro preferential affinity for a given type of purified genomic DNA fragment. No prior purification steps are necessary, and the size of the protein is directly observable (Lelong et al., 1989). However one has to be aware of the existence of nonspecific DNA–protein interactions that may be reduced by carefully optimizing the DNA/protein ratio as well as the amount and nature of the competitor DNA used. Our results (summarized

in Figure 5) suggest the existence of several high-affinity DNA binding proteins interacting specifically with upstream sequences of the β-globin gene. Noteworthy is the fact that the protein binding sites we have observed in vitro with nuclear extracts of uninduced MEL cells reside in a region previously shown to possess a transcriptional "dehancer" activity in vivo (Gilmour et al., 1984) and are clearly distinct from all those previously described [for a review, see Galson and Housman (1988)].

In addition, the fact that the 100-kDa polypeptide binds both far upstream and far downstream from the transcribed portion of the gene might suggest a role of these interactions in nuclear matrix attachment of the repressed gene.

Although there is no evidence that the 95- and 35-kDa polypeptides bind near each other in the 650 bp SS′ fragment, it might be the case in view of other common physical properties: unlike the 100-kDa polypeptide, the 95- and 35-kDa polypeptides are basic and possess an enhanced affinity for single-stranded DNA.

Moreover gel retardation experiments confirm the presence of only one MEL-specific protein–DNA complex on a 173 bp *Fok*I–*Fok*I fragment between −404 and −231 bp from the transcription start. Interestingly, this fragment contains two octameric sequences, ATTTGCAT, found in the heavy- and light-chain promoters (Singh et al., 1986; Wang et al., 1987; Scheidereit et al., 1988; Müller et al., 1988) but also in various other promoters or enhancers [for a review, see Wingender (1988)] and reported to bind important and sometimes cell-specific transcription factors.

The observed erythroid cell specificity and the variations of the specific β-globin DNA binding activity of these proteins during terminal differentiation suggest a role of these interactions in the modulation of β-globin gene expression, by positive and/or negative regulatory mechanisms. However, further experiments are needed to correlate the in vitro DNA binding of these polypeptides once purified with an effect on initiation of in vivo or in vitro transcription.

Although there is no evidence as yet that the sites bound by these proteins in vitro are occupied in vivo or reflect a physiologically relevant association, the use of two independent methods, such South–Western blot and mobility shift assay, obviously reinforces the biologic meaning of the observed binding. In fact, although the β-globin DNA binding proteins we have detected show an obvious degree of DNA sequence specificity, they may recognize DNA sequences present in multiple copies in the genome, which could be involved in regulation of a class of genes that are coordinately expressed: In this respect, they might also be analogous to the 85–105-kDa DNA binding proteins reported to bind to the promoter of the transferrin receptor gene (Miskimins et al., 1985), which is expressed at high levels in erythroid cells (Hu et al., 1977).

References

Bowen, B., Steinberg, J., Laemmli, U. K., & Weintraub, H. (1980) *Nucleic Acids Res. 8*, 1.

Briggs, M. R., Kadonaga, J. T., Bell, S. P., & Tjian, R. (1986) *Science 234*, 47.

Chao, M. V., Mellon, P., Charnay, P., Maniatis, T., & Axel, R. (1983) *Cell 32*, 483.

Charnay, P., Treisman, R., Mellon, P., Chao, M., Axel, R., & Maniatis, T. (1984) *Cell 38*, 251.

Charnay, P., Mellon, P., & Maniatis, T. (1985) *Mol. Cell. Biol. 5*, 1498.

Collins, F. S., & Weismann, S. M. (1984) *Prog. Nucleic Acid Res. Mol. Biol. 31*, 315–462.

Dastugue, B., & Crêpin, M. (1979) *Eur. J. Biochem. 99*, 491.

Dierks, P., Van Ooyen, A., Cochran, M. D., Dobkin, L., Reiser, J., & Weismann, C. (1983) *Cell 32*, 695.

Fasel, N., Pearson, K., Buetti, E., & Diggelmann, H. (1982) *EMBO J. 1*, 3.

Galson, D. L., & Housman, D. E. (1988) *Mol. Cell. Biol. 8*, 381.

Garner, M. M., & Revzin, A. (1981) *Nucleic Acids Res. 9*, 3047.

Gilmour, R. S., Spandidos, D. A., Vass, K., Gow, J. W., & Paul, J. (1984) *EMBO J. 3*, 1263.

Groudine, M., & Weintraub, H. (1981) *Cell 24*, 393.

Hofer, E., Hofer-Warbinek, R., & Darnell, J. E. (1982) *Cell 29*, 887.

Hu, H. Y., Gardner, J., Aisen, P., & Skoultchi, A. I. (1977) *Science 197*, 559.

Hübscher, U. (1987) *Nucleic Acids Res. 15*, 5486.

Jack, R. S., Gehring, W. J., & Brack, C. (1981) *Cell 11*, 321.

Jakob, H., Buckingham, M. E., Cohen, A., Dupont, L., Fiszman, M., & Jacob, F. (1978) *Exp. Cell Res. 114*, 403.

Jones, K. A., Kadonaga, J. T., Rosenfeld, P. J., Kelly, T. J., & Gjian, R. (1987) *Cell 48*, 79.

Kollias, G., Hurst, J., de Boer, E., & Grosveld, F. (1987) *Nucleic Acids Res. 15*, 5739.

Lelong, J. C., Prêvost, G., Lee, K., & Crêpin, M. (1989) *Anal. Biochem.* (in press).

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular cloning, a laboratory manuel*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Manley, J. L., Fire, A., Cano, A., Sharp, P. A., & Gefter, M. L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3855.

Miskimins, W. K., Roberts, M. P., McClelland, A., & Ruddle, F. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6744.

Müller, M. M., Ruppert, S., Schaffner, W., & Mathias, P. (1988) *Nature 336*, 544.

Myers, R. M., Tilly, K., & Maniatis, T. (1986) *Science 232*, 613.

Riggs, A. D., Suzuki, H., & Bourgeois, S. (1970) *J. Mol. Biol. 48*, 67.

Ross, J., Gielen, J., Packman, S., Ikawa, Y., & Leder, P. (1974) *J. Mol. Biol. 87*, 697.

Scheidereit, C., Cromlish, J. A., Gerster, T., Kawakami, K., Balmaceda, C. G., Currie, R. A., & Roeder, R. G. (1988) *Nature 336*, 551.

Silva, C. M., Tully, D. B., Petch, L. A., Jewell, C. M., & Cidlowski, J. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1744.

Singh, H., Sen, R., Baltimore, D., & Sharp, P. A. (1986) *Nature 319*, 154.

Tilghman, S. M., Tiemeier, D. C., Polsky, F., Edgell, M. H., Seidmann, J. G., Leder, A., Enquist, L. W., Norman, B., & Leder, P. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4406.

Wang, J., Nishiyama, K., Araki, K., Kitamura, D., & Watanabe, T. (1988) *Nucleic Acids Res. 15*, 10105.

Wingender, E. (1988) *Nucleic Acids Res. 16*, 1879.

Wright, S., de Boer, E., Grosveld, F. G., & Flavell, R. A. (1983) *Nature 395*, 333.