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The −6.1-Kilobase Chicken Lysozyme Enhancer Is a Multifactorial Complex Containing Several Cell-Type-Specific Elements

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In the chromatin domain of the chicken lysozyme gene of myeloid and oviduct cells, which both have the potential to activate the gene, a developmentally stable DNase I-hypersensitive site is formed around 6.1 kb upstream of the gene. This implies that this DNA region, which has previously been demonstrated to function as a transcriptional enhancer element in myeloid cells, is intimately involved in the cell-type-specific activation of the lysozyme gene locus. Deletion analysis identifies a 157-bp minimal fragment that confers the same promacrophage-specific enhancer activity as the originally described 562-bp −6.1-kb enhancer fragment. By introducing specific point mutations, we demonstrate in transient gene transfer experiments that the minimal fragment consists of at least six adjacent elements, each substantially contributing to enhancer function. The compact multifactorial enhancer complex includes a nuclear factor I (NF-I)/TGGCA binding site, homologies to AP1, and octanucleotide or enhancer core consensus motifs. Point mutation of the NF-I binding site results in the loss of NF-I binding in vitro and enhancer activity in vivo after gene transfer. Surprisingly, four overlapping oligonucleotides, each consisting of at least two elements of the −6.1-kb enhancer, confer myeloid-cell-specific enhancer activity. We found several myeloid-cell-specific DNA-binding proteins interacting with the −6.1-kb enhancer, a result consistent with that described above. Therefore, we suggest that more than a single *trans*-acting factor mediates the cell type specificity of the −6.1-kb enhancer.

Enhancers are *cis*-acting DNA elements known to stimulate transcription in an orientation- and basically distance-independent manner (39, 42). Many viral enhancers, such as the simian virus 40 (SV40) enhancer, activate transcription in a large variety of cells (16), whereas the activity of most known cellular enhancers is restricted to particular cell types (23, 24, 65; for a review, see reference 39).

Detailed mutational analysis of enhancers revealed that they are composed of multiple short sequence motifs (8 to 20 bp) (30, 67, 69). In addition to transcription factors, which confer constitutive (15, 58) or inducible (1; for reviews, see references 14 and 17) activity in almost every cell type studied, DNA motifs interacting with cell-type-specific factors have been identified. The B-cell-specific Oct-2 protein is believed to confer the cell type specificity of the IgH enhancer (22, 55). Other cell-type-specific factors are, for example, the erythroid-cell-specific GATA-1 (previously known as Eryf-1 [45]) and the pituitary-cell-specific Pit-1 (9, 31) proteins. Such findings imply that the cell-type-specific activity of regulatory elements might be due to the cell-type-specific expression of a particular *trans*-acting factor.

The chicken lysozyme gene is an attractive model to study differential expression of genes in different tissues of the same organism: its expression is induced by steroid hormones in the tubular gland cells of the oviduct (57), whereas it is constitutively expressed in macrophages (27, 61). The actively transcribed gene is located in the center of a chromatin domain displaying general DNase I sensitivity which is approximately 20 kb in size (33) (Fig. 1C). The DNA regions at the 5′ and the 3′ borders of general DNase I sensitivity coincide with matrix attachment regions (46). Recently it has been demonstrated that the 5′ domain border collocates with a novel type of *cis* element for gene expression, a so-called A element (Fig. 1D), which together with the −6.1-kb chicken lysozyme gene enhancer confers high-level and position-independent expression of a reporter gene (64). Within the chromatin domain several silencer and enhancer elements have been identified (3, 29, 63, 65), all located within the DNase I-sensitive region of the gene. The position of each regulatory element is demarcated by a DNase I-hypersensitive site (DHS) in chromatin of cell types in which the respective element is functional (Fig. 1B).

The DHS located 6.1 kb upstream of the transcriptional initiation site of the gene is present only in cell types which actively transcribe the lysozyme gene or have the potential for transcriptional activation. This region has previously been demonstrated to harbor an enhancer element which functions with cell type specificity in the myeloid cell line HD11 (HD11 = HBC1 [65]). By transiently transfecting hematopoietic and nonhematopoietic chicken cells, we extended this analysis and demonstrated that the −6.1-kb lysozyme gene enhancer displays macrophage-specific enhancer function. Deletion analysis shows that the −6.1-kb enhancer can be reduced to a minimal 157-bp DNA fragment that confers the same level and range of tissue-specific activity as the originally identified 562-bp enhancer fragment. Mutational analysis reveals that like other enhancers, the −6.1-kb lysozyme gene enhancer possesses a modular structure. It consists of at least six elements with a binding site for nuclear factor I (NF-I)/TGGCA proteins (11, 52), a putative binding site for a member of the Fos/Jun (AP1)

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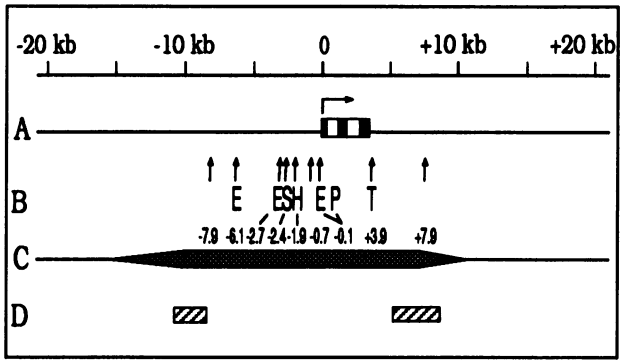

FIG. 1. Chromatin domain of the chicken lysozyme gene. The diagram schematically shows the position of the chicken lysozyme gene (A) with its four exons (filled bars) and three introns (open bars) relative to the region of general DNase I sensitivity (stippled bar; C). The direction of transcription is indicated by an arrow; the location of nine DHSs (vertical arrows; B), their functional characteristics [E = enhancer, S = silencer, H = hormone responsive element, P = promoter, T = poly(A) point], and their locations (in kilobases) relative to the transcriptional start site are given. Matrix attachment regions and location of A elements at the distal ends of the DNase I-sensitive domain are marked by hatched bars (D).

protein family (1, 37), and other yet unknown factors. Surprisingly, the myeloid-cell-specific enhancer activity is not restricted to a single element; rather, we identified several distinct subfragments of the −6.1-kb enhancer, each with the same cell type specificity in hematopoietic cells as the minimal 157-bp enhancer. DNA-protein interaction studies identify several myeloid-cell-specific DNA-binding proteins interacting with the minimal enhancer fragment. This indicates that the lysozyme −6.1-kb enhancer functions as a multifactorial complex in which more than a single element and *trans*-acting factor contribute to the myeloid-cell-specific enhancer activity.

(The larger part of this work was done in partial fulfillment of thesis requirements for T. Grewal.)

MATERIALS AND METHODS

Plasmids. All plasmids were constructed and isolated by standard recombinant DNA techniques (54).

Deletion mutants. Appropriate fragments were isolated from a 593-bp *Bam*HI-*Hin*dIII −6.1-kb chicken lysozyme enhancer fragment. All fragments were cloned blunt end into a unique *Xba*I restriction site upstream of the lysozyme promoter (−579 to +14) fused to the chloramphenicol acetyltransferase (CAT) reporter gene of plasmid plysPCAT (=pLYSCAT2000 [65]).

plysE/TKCAT, plysEsTKCAT, plysEs3′BLCAT2. The original 562-bp *Bam*HI-*Sau*3A (E*l*) and 157-bp *Stu*I-*Bsm*I (E*s*) blunt-end enhancer fragments were cloned into the unique *Bam*HI restriction site upstream of the thymidine kinase (TK) promoter (−105 to +52 [12]) or downstream of the CAT reporter gene into a unique *Sma*I restriction site of plasmid pBLCAT2 (38).

plysE/SVCAT (=pLE1CAT) carries the 562-bp enhancer fragment (E*l*) upstream of the SV40 early promoter from *Sph*I (133) to *Hin*dIII (5171; BBB system [66]) fused to the CAT reporter gene and has been published previously (65).

Element T2 mutants. T2/C and T2/P mutants were introduced into the enhancer sequences by substituting an *Hgi*AI-*Hin*fI fragment (positions −6014 to −5981) with double-stranded oligonucleotides corresponding to wild-type sequences, except from positions −6000 to −6003 (CTA to AGC) in mutant T2/C and positions −6005 and −5695 (G to C; T to A) in mutant T2/P. Both mutations were introduced into the 157-bp *Stu*I-*Bsm*I (E*s*) and 410-bp *Bam*HI-*Bsm*I (E*m*) enhancer fragments and cloned into the *Xba*I restriction site of plysPCAT.

Element C mutants. Mutants C/UP1, C/UP2, C/DOWN1, and C/DOWN2 were constructed according to the oligonucleotide-directed in vitro mutagenesis system of Amersham. Double-stranded DNA from M13 phages carrying mutations C/UP1 at position −5976 (T to A), C/UP2 at positions −5983 and −5976 (G to C; T to A), C/DOWN1 at position −5982 (T to G), and C/DOWN2 at position −5983 (G to T) within the 157-bp *Stu*I-*Bsm*I enhancer fragment and a wild-type 157-bp fragment were cloned into a unique *Hin*dIII restriction site upstream of the lysozyme promoter (−579 to +14) fused to the luciferase reporter gene of plasmid pCL2001 (28).

Element D mutants. To construct the D/DOWN3 mutant, chicken lysozyme enhancer sequences from positions −5981 (*Hin*fI) to −5921 (*Bsm*I) were substituted with double-stranded oligonucleotides corresponding to wild-type sequences, except at position −5956 (A to C) and position −5960 (G to T). A 410-bp *Bam*HI-*Bsm*I and a 157-bp *Stu*I-*Bsm*I enhancer fragment carrying the double point mutations were cloned blunt end into the unique *Xba*I restriction site of plysPCAT. Mutants D/DOWN1 (5′-CTT TGGAACTGACAG-3′), D/DOWN2 (5′-CTTTTGAAATGA CAG-3′), D/OCT (5′-CTATGCAAATTACAG-3′), D/CORE (5′-CTGTGGAAAGGACAG-3′), and D/NF-I (5′-CTTTGG CAATGACAG-3′) were introduced into the enhancer sequences by substituting wild-type sequences from positions −5964 to −5950 with 15-bp double-stranded oligonucleotides (which are listed above in parentheses) representing wild-type sequences, except for the nucleotides underlined. All mutations were introduced in the 157-bp (E*s*) *Stu*I-*Bsm*I fragment and cloned blunt end in the restriction site *Xba*I of plysPCAT.

Element E mutant. In mutation E/1, the wild-type sequences from positions −5981 (*Hin*fI) to −5921 (*Bsm*I) were substituted with double-stranded oligonucleotides corresponding to the wild type, except positions −5944 (G to A), −5942 and −5941 (TG to AT), and −5939 (C to T). The mutation was introduced in the 157-bp *Stu*I-*Bsm*I enhancer fragment and was then cloned blunt end into the *Xba*I recognition site of plysPCAT.

Oligonucleotides of 60, 58, 46, and 68 bp. Double-stranded oligonucleotides containing wild-type enhancer sequences from positions −6075 to −6015, −6047 to −5989, −6014 to −5968, and −5989 to −5921 were cloned as monomers and dimers into the unique *Sma*I restriction site downstream of the CAT reporter gene fused to the TK promoter (−105 to +52) of plasmid pBLCAT2 (38). The dimers were cloned in a head-to-tail orientation according to a protocol described previously by Rosenfeld and Kelly (49).

Cell growth and transfection. Primary macrophage cultures were obtained as described previously (65). Cell lines HD11 (=HBC1 [7]), DU249 (36), and MSB1 (5) and primary chicken embryo fibroblasts and E26-transformed myeloblasts (4) were grown in 150-cm^2 tissue culture flasks (Costar) in Iscove's modified Dulbecco's medium (GIBCO) supplemented with 8% fetal calf serum and 2% chicken serum at 37°C and 5% CO_2. BM2 (40) cells were grown in RPMI 1640 (GIBCO) supplemented with 10% tryptose phosphate broth, 5% fetal calf serum, and 5% chicken serum at 37°C and 5% CO_2. I 3 (6) cells were grown in RPMI 1640

containing 10% fetal calf serum and 2% chicken serum at 37°C and 5% CO_2.

DNA was introduced into HD11 and DU249 cells, primary macrophages, and chicken embryo fibroblasts by the calcium phosphate coprecipitation method (65), with the following modifications: 24 h before transfection, 3×10^6 HD11 and DU249 cells, 2×10^6 primary macrophages, and 1.5×10^6 chicken embryo fibroblasts were plated on a 10-cm tissue culture dish (Falcon). Four hours before transfection, the cells were fed with 5 ml of fresh medium. Twenty-five micrograms of the test plasmid and 2 μg of pRSVlacZII (a gift from W. Ankenbauer) as an internal control for transfection efficiency were suspended in 500 μl of 10 mM Tris-HCl (pH 7.6)–250 mM $CaCl_2$. After mixing, the DNA-$CaCl_2$ suspension was added dropwise to 500 μl of 2× HBS (280 mM NaCl; 1.5 mM Na_2HPO_4; 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.13). Thirty minutes later, the DNA–calcium phosphate suspension was added to the cells. Cells were incubated for 8 to 10 h at 37°C and 5% CO_2. After being washed three times with phosphate-buffered saline (PBS), transfected cells were fed with 10 ml of fresh medium and incubated for 48 h at 37°C and 5% CO_2. Cells were harvested as described previously (65).

BM2 cells were transfected by the DEAE-dextran method (2). Cells (5×10^6) were harvested in a Minifuge GL (Heraeus) at 1,200 rpm for 5 min. Cells were washed twice with 10 ml of TBS, pH 7.5 (0.5 mM $MgCl_2$; 0.7 mM $CaCl_2$; 137 mM NaCl; 5 mM KCl; 0.6 mM Na_2HPO_4; 25 mM Tris, pH 7.5). The final cell pellet was resuspended in 600 μl of TBS containing 10 μg of DNA and 0.5 mg of DEAE-dextran (Pharmacia) per ml and incubated for 30 min at room temperature. Cells were collected, washed with TBS, resuspended in 5 ml of medium containing 0.1 mM chloroquine-diphosphate (Sigma) and transferred into 10-cm tissue culture dishes (Falcon). After incubation for 2 to 3 h at 37°C and 5% CO_2 cells were again collected, washed, resuspended in fresh medium, and incubated at 37°C and 5% CO_2. Cells were harvested as described previously (65).

HD3 and MSB1 cells were electroporated according to the method of Chu et al. (13), with the following modifications: 10^7 cells were once washed with PBS, and the cell pellet was resuspended in 1 ml of HeBS buffer (20 mM HEPES, pH 7.05; 137 mM NaCl; 5 mM KCl; 0.7 mM Na_2HPO_4; 6 mM glucose) containing 50 μg of test plasmid DNA per ml (25 μg/ml for MSB1) and 4 μg of RSVlacZII as an internal control. After a 380-V, 960-μF pulse with a Genepulser (Bio-Rad), cells were incubated at room temperature for 10 min, fed with 10 ml of fresh medium, and incubated for 24 h at 37°C and 5% CO_2. Cells were harvested as previously described (65).

β-Galactosidase assay. As an internal control for transfection efficiency, the cells were cotransfected with the plasmid pRSVlacZII, which contains the Rous sarcoma virus long terminal repeat fused to the β-galactosidase reporter gene. Ten to forty microliters of extract of transfected cells was incubated in a total volume of 300 μl of 100 mM sodium phosphate (pH 7.0)–10 mM KCl–4 mg of *ortho*-nitrophenyl-β-D-galactopyranoside per ml–1 mM $MgSO_4$–50 mM β-mercaptoethanol at 37°C. The reaction was stopped by adding 600 μl of 1 M Na_2CO_3. The optical density at 420 nm was determined by using a spectrophotometer, and the absolute values of the CAT and luciferase assays were corrected according to their β-galactosidase values.

CAT assay. The activity of CAT was determined as described previously (65). The values given represent the average of at least three independent transfections with a deviation of less than 10% and were corrected according their β-galactosidase values (standard deviations are given for the deletion analysis in Fig. 2, as no internal control was performed).

Luciferase assay. Cells were harvested as described previously (65) and resuspended in 150 μl of extraction buffer (100 mM potassium buffer, pH 7.8; 1 mM dithiothreitol). Ten to thirty microliters of extract was mixed with reaction buffer (25 mM glycylglycine, pH 7.8; 2 mM ATP; 10 mM $MgSO_4$) to a total volume of 350 μl, and the luciferase activity was determined by using the Biolumat LB 9500 T (Berthold) after automatic injection of 100 μl of injection buffer (20 mM glycylglycine, pH 7.8; 0.2 mM luciferin). The luciferase activity of each cell extract was measured several times, and average values were determined.

Preparation of RNA and S1 mapping. Total cellular RNA was prepared and lysozyme mRNA levels were determined by S1 mapping analysis as described previously (65).

Preparation of nuclear extracts. Nuclear extracts were prepared according to the method described by Shapiro et al. (59). The crude nuclear extract of 10^9 cells was precipitated with 0.33 g of ammonium sulfate per ml and resuspended after centrifugation for 20 min at 85,000 × *g* in 1 ml of extract buffer (20 mM HEPES, pH 8.0; 40 mM KCl; 0.2 mM EDTA; 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid [EGTA]; 2 mM dithiothreitol; 20% glycerine). Extracts were dialyzed twice against the same buffer and finally cleared by centrifugation for 5 min at 10,000 × *g*.

DNase I footprinting. DNA fragments were selectively end labeled by filling in with DNA polymerase I (Klenow). Four-microliter nuclear extracts (approximately 30 μg of protein) or serial dilutions of a phosphocellulose fraction of purified NF-I/TGGCA protein (53) were incubated in 20 μl of 10 mM HEPES (pH 8.0)–30 mM NaCl–0.1 mM EDTA–5 mM dithiothreitol–100 μg of bovine serum per ml–1,000 ng of poly(dI-dC) (Pharmacia) with end-labeled DNA (1 to 2 fmol) for 15 min at room temperature. After addition of 50 to 150 ng of DNase I (Cooper) in 10 mM HEPES (pH 8.0)–25 mM $CaCl_2$–50 mM $MgCl_2$, DNA was digested for 3 min on ice. Reactions were terminated by the addition of 80 μl of 20 mM Tris-HCl (pH 8.0)–20 mM EDTA–250 mM NaCl–10 μg of glycogen per ml–0.5% sodium dodecyl sulfate–125 μg of proteinase K per ml, and samples were extracted with phenol and precipitated. DNA fragments were analyzed on denaturing 8% polyacrylamide-urea gels as described previously (11).

RESULTS

A 157-bp DNA fragment comprises the −6.1-kb enhancer function. We have previously reported the identification of a 562-bp enhancer fragment (E*l*; E = enhancer, *l* = large) located 6.1 kb upstream of the transcriptional start site of the chicken lysozyme gene, which is active in the lysozyme-producing myeloid cell line HD11 (65). To identify DNA sequence elements which are important for enhancer function within the E*l* fragment, we constructed a series of deletion mutants (Fig. 2A). The resulting E*l* subfragments were cloned 5′ of the 579-bp lysozyme promoter fused to the CAT gene in the plasmid plysPCAT (65). The results shown in Fig. 2B document the enhancer activity of each fragment relative to the 562-bp E*l* enhancer fragment after gene transfer in HD11 monocytes (7). Deletions at the 3′ end of the E*l* enhancer to position −5889 or −5918 (E*m*; *m* = medium) retain full enhancer activity. Deletions to position

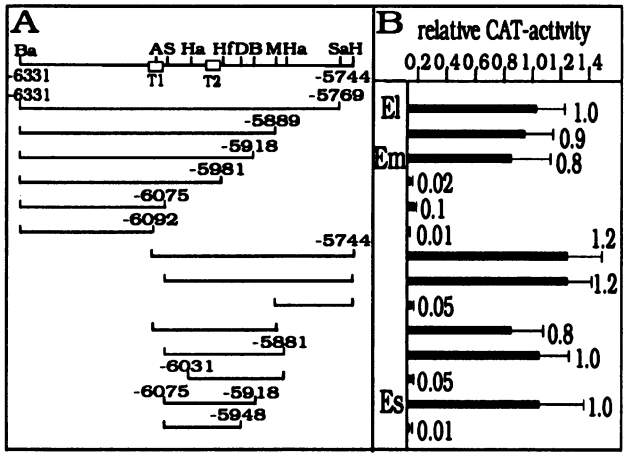

FIG. 2. Relative activity of −6.1-kb chicken lysozyme enhancer deletion mutants. (A) Restriction map of the 562-bp E*l* fragment and the deletion mutants. The positions relative to the transcriptional start site and the size of fragments are shown. Recognition sites for restriction endonucleases are *Bam*HI (Ba), *Alu*I (A), *Stu*I (S), *Hae*III (Ha), *Hin*fI (Hf), *Dra*III (D), *Bsm*I (B), *Mae*I (M), *Sau*3A (Sa), and *Hin*dIII (H). The locations of the NF-I/TGGCA protein binding sites T1 and T2 are shown. (B) Deletion mutants of the 562-bp *Bam*HI-*Sau*3A enhancer fragment were cloned into the unique *Xba*I restriction site of plysPCAT that contains chicken lysozyme promoter sequences from −579 to +14 fused to the CAT gene (65). HD11 cells (3×10^6) were transfected with 25 μg of plasmid DNA. Cells were harvested after 48 h, and the activity of CAT was determined as described previously (65). The indicated values are relative to the CAT activity of cells transfected with plysE*l*PCAT (1.0) and represent the average of at least three independent transfections. The standard deviations are given, and the E*l*, E*m*, and E*s* enhancer fragments are pointed out. The absolute value for the enhancerless construct plysPCAT is 7.7 pmol converted per hour per 10^6 cells, that for the enhancer construct plysE*l*PCAT is 114.2 pmol converted per hour per 10^6 cells, and that for plysE*s*PCAT is 112.5 pmol converted per hour per 10^6 cells.

−5981 and farther (−6075 or −6092) resulted in constructs that show no or only minimal stimulation of the CAT reporter gene expression. 5′ deletions of the E*l* enhancer element to position −6092 or −6075 have no influence on enhancer activity in HD11 cells. But further deletion of sequences to position −5889 results in the complete loss of enhancer activity. This suggests that the −6.1-kb chicken lysozyme gene enhancer is located within the region from positions −6075 to −5889. To test this possibility, we analyzed several E*l* subfragments covering the DNA region from positions −6092 to −5881 with respect to their enhancer properties. Fragments from positions −6092 to −5889 or −6075 to −5881 mediate the full activity of the E*l* enhancer. Deleting the fragment from −6075 to −5881 at its 5′ end to position −6031 or at its 3′ end to position −5948 results in a 95 or a 99% loss of enhancer activity, respectively, whereas a 157-bp fragment from positions −6075 to −5918 has the same stimulatory capacity as the E*l* fragment. Therefore, the −6.1-kb lysozyme gene enhancer can be reduced to a 157-bp minimal enhancer (E*s*; *s* = small) that is sufficient to confer enhancer activity in the myeloid cell line HD11. This is surprising since in vitro DNA footprinting experiments with nuclear protein extracts and purified transcription factor NF-I (11) (see also Fig. 5 and 7) suggest that the *cis*-active region for enhancer activity would extend farther upstream.

The transfected −6.1-kb chicken lysozyme enhancer is active only in cells that transcribe the endogenous lysozyme gene. The −6.1-kb chicken lysozyme enhancer stimulates transcription of a transiently transfected CAT reporter gene fused to the lysozyme promoter from the correct transcriptional start site in the lysozyme-expressing myeloid cell line HD11 but not in fibroblasts (65). To further elucidate the cell-type-specific function of the −6.1-kb enhancer, we analyzed the stimulatory effect of the 562-bp E*l* and the 157-bp E*s* enhancer fragments in primary macrophages, primary chicken embryo fibroblasts, and several cell lines representing different hematopoietic lineages and nonhematopoietic cell types of chickens (Table 1). To exclude possible cell-type-specific promoter effects by the homologous lysozyme promoter, we also studied the E*l* and E*s* enhancers for the ability to activate the heterologous herpes simplex virus TK gene promoter from positions −105 to +52 and the SV40 early promoter from *Sph*I (133) to *Hin*dIII (5171; BBB system [66]).

After transfection into primary macrophages or the myeloid cell lines HD11 and BM2 (40), both fragments E*l* and E*s* are able to stimulate expression of the lysozyme promoter-CAT construct. The plysE*l*PCAT construct stimulates

TABLE 1. Cell-type-specific activation of homologous and heterologous promoters by the −6.1-kb enhancer in transient transfections[a]

Enhancer and/or promoter[b]	Relative activity per cell type						
	BM2 myeloblasts	HD11 monocytes	Primary macrophages	HD3 erythroblasts	MSB1 T lymphocytes	DU249 hepatocytes	Chicken embryo fibroblasts
lys P	1.0	1.0	1.0	1.0	1.0	1.0	1.0
lys El + lys P	14.3	15.0	5.3	1.3	0.9	0.8	0.9
lys Es + lys P	ND[c]	14.5	ND	1.0	1.0	1.1	0.9
TK P	ND	1.0	ND	1.0	1.0	1.0	1.0
lys El + TK P	ND	2.3	ND	1.5	0.8	1.2	0.3
lys Es + TK P	ND	3.1	ND	1.1	1.7	0.8	0.9
lys Es3′ + TK P	ND	7.4	ND	1.0	1.0	0.8	1.1
SV P	ND	1.0	ND	ND	ND	1.0	1.0
lys El + SV P	ND	6.0	ND	ND	ND	1.3	0.9

[a] Numbers of endogenous lysozyme transcripts per cell were as follows: 150, 150, 300, <5, <5, <5, and <5 for BM2 and HD11 cells; primary macrophages; HD3, MSB1, and DU249 cells; and chicken embryo fibroblasts, respectively. Values were determined by quantitative S1 mapping (see also Fig. 3).

[b] Abbreviations: lys P, lysozyme promoter (−579 to +14); lys El, 562-bp *Bam*HI-*Sau*3A lysozyme enhancer fragment; lys Es, 157-bp *Stu*I-*Bsm*I lysozyme enhancer fragment; TK P, TK promoter (−105 to +52); lys Es3′, 157-bp E*s* enhancer fragment cloned 3′ the TK promoter fused to the CAT reporter gene; SV P, SV40 early promoter from *Sph*I (133) to *Hin*dIII (5171; BBB system [see reference 66]).

[c] ND, not determined.

CAT activity 15.0-fold in HD11 cells, 14.3-fold in BM2 cells, and 5.3-fold in primary macrophages relative to the enhancerless control plysPCAT. The construct plysE*s*PCAT as well shows a 14.5-fold stimulation in HD11 cells. No stimulation of CAT gene activity by the E*l* or E*s* enhancer element is detected in avian erythroblastosis virus-transformed erythroblasts (HD3 [6]) and the lymphoid cell line MSB1 (5). In addition, the E*l* and E*s* enhancers fail to activate the lysozyme promoter in the nonhematopoietic MC29-transformed hepatic cell line DU249 (36) and primary chicken embryo fibroblasts.

Furthermore, the E*l* enhancer stimulates the heterologous herpes simplex virus TK promoter 2.3-fold and stimulates the SV40 promoter 6.0-fold in myeloid HD11 cells (Table 1). The minimal enhancer also activates the TK promoter independently of orientation and distance in the constructs plysE*s*TKCAT and plysE*s*3′BLCAT2. Again, no stimulation of reporter gene activity by the E*s* element fused to the herpes simplex virus TK promoter compared with the enhancerless TK promoter constructs is observed in any other cell line transfected. These results demonstrate that the enhancer activity of the −6.1-kb enhancer is restricted to the myeloid lineage of the hematopoietic system of the chicken. Furthermore, the 157-bp E*s* enhancer fragment has the same stimulatory potential and range of cell type specificity as the 562-bp E*l* element.

The cell-type-specific stimulation of the lysozyme, TK, and SV40 promoters by the −6.1-kb enhancer in transient transfection experiments correlates with the cell-type-specific activity of the endogenous lysozyme gene. Figure 3 shows a quantitative S1 mapping analysis of lysozyme transcripts with total RNA from different hematopoietic and nonhematopoietic cells and tissues of chickens. In addition to oviduct tissue (lane 2) and terminally differentiated macrophages (lane 3), lysozyme-specific transcripts can also be found in monocytes (HD11, lane 7), myeloblasts (BM2, lane 6) and E26-transformed early myeloblasts (lane 5) (9). The concentration of lysozyme mRNA increases from 15 to 20 molecules per cell in E26-transformed early myeloblasts to 150 molecules per cell in HD11 and BM2 cells and 300 molecules per cell in unstimulated primary macrophages (see also Table 1, footnote *a*). No lysozyme transcripts could be detected in erythroid or lymphoid cells (HD3, lane 8; MSB1, lane 9) or in nonhematopoietic tissues such as laying-hen liver (lane 4), hepatocytes (DU249; lane 10), and chicken embryo fibroblasts (lane 11). These results imply that the −6.1-kb enhancer is intimately involved in the activation of the cell-type-specific transcription of the chicken lysozyme gene in the myeloid lineage of the hematopoietic system.

The chicken lysozyme enhancer consists of multiple elements that contribute to its stimulatory function. The analysis of DNA-protein interaction in vitro and in vivo (60) (see also Fig. 7) and sequence comparison suggest a modular structure of the −6.1-kb lysozyme gene enhancer. The putative modules of the E*s* enhancer and the DNA sequence from positions −6014 to −5929 are shown in Fig. 4.

Element T1, which is located just upstream of the 5′ border of the E*s* enhancer, and element T2 both contain binding sites for the NF-I/TGGCA proteins (11, 53). Module C shares a sequence homology with the Fos/Jun (AP1) consensus sequence (37), and element D contains a homology to the enhancer core (67) as well as to the octa-/decanucleotide consensus sequence (18) and to one half of the palindromic recognition sequence for the NF-I/TGGCA proteins (11), which is sufficient to bind NF-I/TGGCA in vitro (51). The NF-I/TGGCA protein binding site in T2 and a palindrome that spans D and E are indicated by arrows. The Fos/Jun binding site and enhancer core/octanucleotide homologies in C and D are underlined. No obvious homologies to any yet known DNA recognition sequence exists for the putative elements A, B, and E.

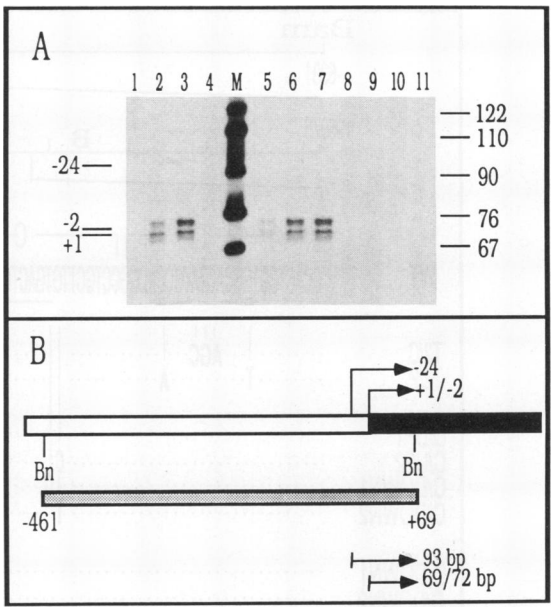

FIG. 3. Analysis of cell-type-specific expression of the chicken lysozyme gene by S1 mapping. (A) Total cellular RNA was hybridized against an at least 40-fold molar excess of 5′-end-labeled *Bst*NI promoter fragment (panel B). After S1 digestion, S1-resistant DNA fragments were separated on an 8% polyacrylamide-urea gel (62). The radioactive probe was hybridized against no RNA (lane 1), 0.1 μg of RNA from laying-hen oviduct (lane 2), 20 μg of RNA from primary blood-derived macrophages (lane 3), 150 μg of RNA from laying-hen liver (lane 4), 100 μg of RNA from E26-transformed myeloblasts (lane 5), 60 μg of RNA from myeloblasts (BM2; lane 6), 60 μg of RNA from monocytes (HD11; lane 7), 150 μg of RNA from erythroblasts (HD3; lane 8), 150 μg of RNA from T lymphocytes (MSB1; lane 9), 150 μg of RNA from hepatocytes (DU249; lane 10), and 150 μg of RNA from primary chicken embryo fibroblasts (lane 11). The numbers on the left indicate the positions of the start sites of lysozyme transcripts (65). The lengths of size marker DNA fragments (in base pairs) are indicated on the right. (B) The diagram shows the chicken lysozyme promoter region. The solid box indicates the first exon. The arrows point to the multiple start sites of transcription at −24, −2, and +1. The radioactive probe (stippled box) and the positions of the *Bst*NI restriction sites (Bn) relative to the transcriptional start site are shown. The positions and lengths of the protected fragments are given at the bottom of the diagram.

In order to elucidate the functional relevance of the presumptive lysozyme enhancer modules, we introduced sequence-specific mutations and tested their influence on the activity in the 157-bp minimal enhancer (E*s*) or in a 410-bp enhancer fragment (E*m*) after gene transfer into HD11 monocytes (Fig. 4).

Element C. Element C contains the sequence 5′-GTGAC TCT-3′ (Fig. 4), which has homology to the AP1 consensus (C/G)TGACT(C/A)A (1) in 7 of 8 bp. We constructed the mutations C/UP1 (5′-GTGACTCA-3′) and C/UP2 (5′-CT GACTCA-3′) by altering the wild-type sequence into a full homology to the Fos/Jun (AP1) consensus sequence ("up mutation approach"). Furthermore, we modified the wild-type sequence from 5′-GTGACTCT-3′ to 5′-GGGACTCT-3′ in the construct C/DOWN1 and to 5′-TTGACTCT-3′ in the

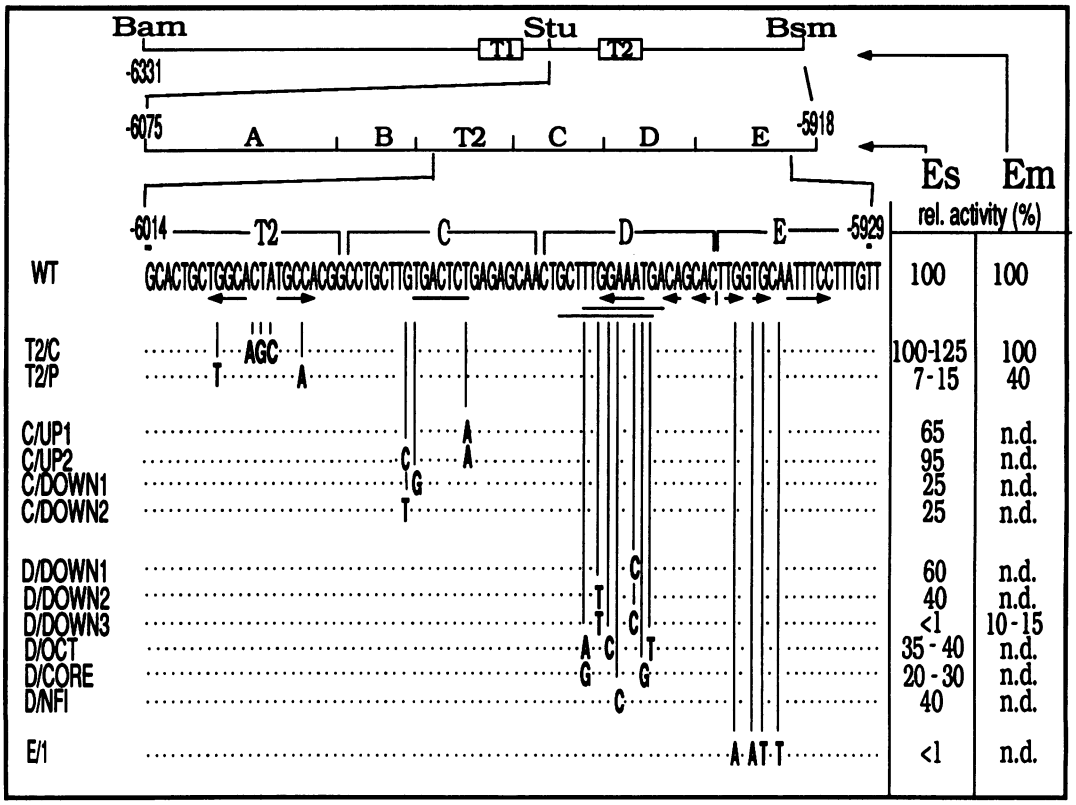

FIG. 4. Functional identification of elements and relative activity of the −6.1-kb lysozyme enhancer mutants. A part of the wild-type (WT) sequence of the 157-bp E*s* enhancer (−6075 to −5918) from positions −6014 to −5929 is shown. Modules T2, C, D, and E of the E*s* enhancer are indicated above the sequence. The E*s* enhancer is part of the E*m* enhancer from positions −6331 to −5918, as indicated in the top line. Recognition sites for restriction endonucleases are *Bam*HI (Bam), *Stu*I (Stu), and *Bsm*I (Bsm). The locations of T1 and T2 within the E*m* enhancer are shown. The mutations and the modified nucleotides of each mutation introduced in the various elements are listed. Arrows indicate the palindromic NF-I/TGGCA motif and a palindromic sequence spanning D and E. The AP1 consensus homology in module C, the overlapping homologies to the octa-/decanucleotide DNA motif, and the enhancer core consensus sequence in D are underlined. Functional analysis of mutants was performed by transfecting 3 × 10^6 HD11 cells with 25 μg of test DNA and 4 μg of pRSVlacZII as an internal control. Cells were harvested after 48 h (24 h for the luciferase reporter gene constructs used for the C mutants), and the activity of CAT (luciferase activity for C mutants) was determined (see Materials and Methods). The absolute values of the CAT and luciferase assays were corrected according their β-galactosidase values. The indicated values, relative to the fully active E*m* and E*s* enhancers, represent an average of at least four independent transfections. The absolute value for the E*s* enhancer construct is given in the legend to Fig. 2.

mutant C/DOWN2 ("down mutation approach"). These mutations would interfere with the binding of factors belonging to the Fos/Jun family (for a review, see reference 14). Whereas the mutations C/DOWN1 and C/DOWN2 lead to a 75% loss of enhancer activity, the mutations C/UP1 and C/UP2 show residual stimulations of approximately 65 and 95% compared with the wild-type enhancer (Fig. 4). The loss of enhancer function by introducing the mutations C/DOWN1 and C/DOWN2 and the ability to replace the wild-type sequence with a perfect AP1 homology without a significant loss of enhancer activity imply that a member of the Fos/Jun DNA-binding protein family can interact with module C of the E*s* enhancer.

Element D. For the down mutation approach, the transversions A to C in the construct D/DOWN1 (5′-TGCTTTG GAACTGA-3′) and G to T in D/DOWN2 (5′-TGCTTT TGAAATGA-3′) as well as both point mutations together in the plasmid D/DOWN3 were introduced into the sequence of the E*s* enhancer. These mutations were chosen since they inhibit binding of the Oct-1 protein in vitro (47). Mutating either of the two nucleotides leads to a partial loss of enhancer activity in HD11 cells, namely, 60% activity for the D/DOWN1 and 40% activity for the D/DOWN2 mutations relative to the activity of the wild-type enhancer (Fig. 4). The double mutation D/DOWN3 results in a enhancer activity complete loss of E*s* and an 85 to 90% loss of E*m* enhancer activity and points out that module D is essential for enhancer function (Fig. 4).

To find out which type of factor most likely binds to element D, we again used the up mutation approach and mutated the wild-type sequence into perfect octa-/decanucleotide (D/OCT; 5′-TGCTATGCAAATTA-3′), enhancer core (D/CORE; 5′-TGCTGTGGAAAGAG-3′), and NF-I/TGGCA (D/NF-1; 5′-TGCTTTGGCAATGA-3′) consensus motifs. As shown in Fig. 4, all three mutations retain an enhancer activity of only 20 to 40% of that of the wild-type element. Even though none of the three up mutations can replace the wild-type sequence, the experiments point out that region D plays a critical role in lysozyme enhancer function. However, they do not help to determine whether one of the factors mentioned above interacts with module D.

Element E. The deletion of 27 bp in element E (positions −5921 to −5948) of the 157-bp E*s* enhancer inactivates the minimal enhancer element (Fig. 2). This deletion was con-

structed by introducing an artificial *Bgl*II restriction site into the wild-type enhancer sequence. To test whether the 4-bp mutation from 5′-GGTGCA-3′ to 5′-AGATCT-3′ (E/1 in Fig. 4) within the palindromic sequence spanning D and E by itself has an effect on the activity of the minimal enhancer, we introduced the mutation E/1 within the 157-bp DNA fragment. In the transient transfection experiments performed with the myeloid HD11 cells, the E/1 mutation shows a complete loss of enhancer activity. This supports the result of the deletion mutation from positions −6075 to −5948 (Fig. 2) and underlines the importance of module E for the activity of the entire enhancer element.

Element T2. The 562-bp E*l* enhancer contains two NF-I binding sites within regions T1 and T2. While deletion of T1 from positions −6082 to −6016 has no influence on wild-type enhancer activity (Fig. 2) in transient transfection experiments, element T2 is located within the E*s* element. We generated mutations T2/C (C = central) and T2/P (P = peripheral) to examine the influence of NF-I/TGGCA protein binding to T2 on the chicken lysozyme enhancer. In mutation T2/C, the three central nucleotides of the palindromic NF-I motif 5′-CTGGCACTATGCCAC-3′ were modified from CTA to AGC. This mutation does not interfere with the recognition sequence of NF-I. By altering the wild-type sequence into 5′-CTTGCACTATGCAAC-3′ and thereby eliminating two essential GN7 contacts for NF-I, mutation T2/P was generated (53).

Figure 5 shows a DNase I footprint analysis of the NF-I/TGGCA protein binding to DNA of the 410-bp E*m* enhancer fragment containing wild-type sequences (lanes 1 to 7), mutation T2/P (lanes 8 to 14), and mutation T2/C (lanes 15 to 21). T1 harbors an NF-I binding site which serves as an internal control for the binding of NF-I to E*m* DNA with the mutated element T2. The modification of three central base pairs in T2/C has no effect on protein binding to T2, as expected. However, mutation T2/P destroys the NF-I/TG GCA recognition sequence and consequently no binding to T2 is detectable.

Both mutations were functionally analyzed in the context of the E*s* and E*m* enhancer fragments (Fig. 4). Results from transient transfections correlate with results from the NF-I/TGGCA in vitro binding experiments. Mutating the central 3 bp in T2/C leaves fully active E*m* and E*s* enhancer fragments, which is consistent with the fact that NF-I can still interact with this sequence in vitro. Destruction of the NF-I protein binding site in the element T2 in mutation T2/P reduces the activity of the minimal enhancer to 7 to 15% compared with that of the wild type. Interestingly, the negative effect of this mutation is less severe in the 410-bp E*m* enhancer, as it still retains 40% of the wild-type activity.

The functional analysis of point mutations introduced in the predicted DNA-binding motifs of elements T2, C, D, and E confirm assumptions from in vivo and in vitro DNA-protein interaction studies (see also Fig. 7) and demonstrate that like the viral SV40 enhancer (44, 56), the −6.1-kb lysozyme gene enhancer possesses a highly condensed modular structure.

The enhancer contains several subfragments acting in a cell-type-specific manner. Several cell-type-specific *trans*-acting factors are known to confer tissue-specific activity by interacting with *cis*-regulatory elements (39). Studies of the SV40 enhancer revealed that it is composed of multiple short elements that have intrinsic enhancer properties (44, 56). Some of these elements display unique patterns of cell-type-specific enhancer activities and can act on their own, while others need to interact functionally with different elements in order to activate transcription (21, 43). To identify elements of the −6.1-kb E*s* enhancer that confer macrophage specificity, we divided the −6.1-kb E*s* enhancer into four overlapping subfragments, each containing at least two modules of the minimal enhancer (Fig. 6A). A 60-bp oligonucleotide consists of A and B; the 58-bp oligonucleotide harbors B, T2, and a part of A; the 46-bp DNA fragment encompasses T2 and C; and the 68-bp oligonucleotide contains C, D, and E. We tested these synthetic oligonucleotides as monomers and head-to-tail dimers in a 3′ position relative to the CAT

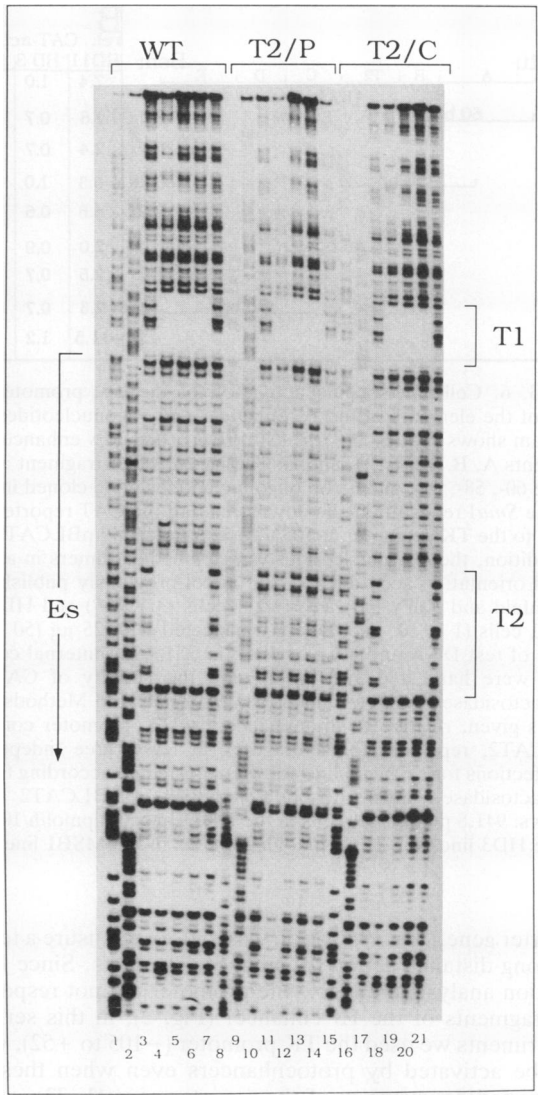

FIG. 5. DNase I footprint of NF-I/TGGCA protein binding to T1 and T2 of the −6.1-kb enhancer. The wild-type 410-bp (E*m*) *Bam*HI-*Bsm*I enhancer fragment (lanes 1 to 7; WT) and the E*m* enhancer fragments containing the mutations T2/P (lanes 8 to 14) and T2/C (lanes 15 to 21) were radioactively labeled at the 3′ end of the noncoding strand. Two femtomoles of DNA was used in sequencing reactions (lanes 1, 8, and 15, T+C; lanes 2, 9, and 16, A+G) or digested with DNase I after incubation with bovine serum albumin (lanes 3, 7, 10, 14, 17, and 21) or 4 μl (lanes 4, 11, and 18), 1 μl (lanes 5, 12, and 19) or 0.125 μl (lanes 6, 13, and 20) of purified NF-1/TGGCA protein (53). The NF-I/TGGCA binding sites T1 and T2 are indicated on the right. The 5′ border of the E*s* enhancer is shown on the left.

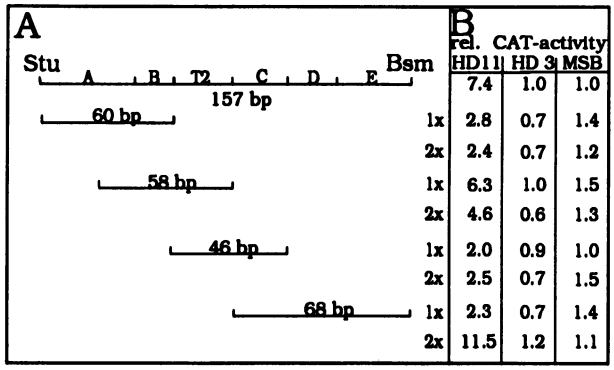

FIG. 6. Cell-type-specific activation of the TK promoter. (A) Map of the elements of the Es enhancer and oligonucleotides. The diagram shows the 157-bp *Stu*I (Stu)-*Bsm*I (Bsm) Es enhancer with elements A, B, T2, C, D, and E. The Es enhancer fragment as well as the 60-, 58-, 46-, and 68-bp oligonucleotides were cloned into the unique *Sma*I recognition site downstream of the CAT reporter gene fused to the TK promoter (−105 to +52) of plasmid pBLCAT2 (41). In addition, the oligonucleotides were cloned as dimers in a head-to-tail orientation according to a protocol previously published by Rosenfeld and Kelly (49). (B) HD11 cells (3×10^6) and HD3 and MSB1 cells (1×10^7 each) were transfected with 25 μg (50 μg for HD3) of test DNA and 4 μg of pRSVlacZII as an internal control. Cells were harvested after 48 h, and the activity of CAT and β-galactosidase was determined (see Materials and Methods). The values given, relative to the enhancerless TK promoter construct pBLCAT2, represent the average of at least three independent transfections for each cell line and were corrected according to their β-galactosidase values. Absolute activities of pBLCAT2 are as follows: 941.8 pmol/h/10^6 cells in the HD11 line, 3.4 pmol/h/10^6 cells in the HD3 line, and 12.75 pmol/h/10^6 cells in the MSB1 line.

reporter gene. The 3′ position was chosen to ensure a test for the long-distance action of enhancer elements. Since in our deletion analysis the lysozyme promoter did not respond to subfragments of the Es enhancer (Fig. 2), in this series of experiments we used the TK promoter (−105 to +52), which can be activated by protoenhancers even when these are positioned downstream of the reporter gene (1, 32).

All constructs together with the wild-type control plysEs3′BLCAT2 and the enhancerless plasmid pBLCAT2 (38) were transiently transfected in the hematopoietic cell lines HD11, HD3, and MSB1 (Fig. 6B). None of the oligonucleotides show a stimulatory effect on the TK promoter in the erythroid cell line HD3 and the lymphoid cell line MSB1. Unexpectedly, all four fragments are able to activate the promoter in the myeloid cell line HD11 compared with the enhancerless control. The 60-, 58-, and 46-bp oligonucleotides with the elements A and B; B, T2, and part of A; and T2 and C, respectively, stimulate the TK promoter two- to sixfold in monocytes (Fig. 6B). No significant difference in the ability to enhance transcription is seen between the monomers and dimers tested. In contrast, the 68-bp oligonucleotide, containing C, D, and E, shows a stimulation of the TK promoter that depends on the copy number of the enhancer subfragment. Enhancer activity of the CDE subfragment is increased from 2.3-fold when tested as a monomer to 11.5-fold when tested as a dimer. This indicates that C, D, and E are able to act cooperatively when tested as a multimer. Furthermore, the results show that each of the four different subfragments of the −6.1-kb lysozyme enhancer contains sufficient information to independently direct myeloid-cell-specific enhancer function.

Several cell-type-specific factors interact with the Es enhancer. In order to independently confirm our finding that all subfragments of the Es enhancer contain macrophage-specific elements, we performed DNase I footprinting experiments with nuclear extracts from the same three cell lines that were used for transient gene transfer experiments (Fig. 7). The upper and the lower strands of a 300-bp *Dde*I-*Bsm*I enhancer fragment were selectively 3′ end labeled and were incubated with nuclear extracts from HD11, HD3, and MSB1 cells.

As expected, elements T1 and T2 are both bound by members of the ubiquitous NF-I protein family in all three nuclear extracts (lanes 3, 4, 6, 7, 9, and 10 in Fig. 7A and B) and the protected regions are similar to purified NF-I (lanes 12 and 13 in 7A and B). Interestingly, the NF-I/TGGCA protein family members in the different nuclear extracts form different complexes at T1 and T2. In HD11 and MSB1 cells, two different sites of increased DNase I sensitivity appear upstream of T2 (Fig. 7A, lanes 3, 4, 9, and 10). HD3 cells form a characteristic hypersensitive site downstream of T1 (Fig. 7A, lanes 6 and 7). This seems to indicate a possible participation of different NF-I protein family members (35, 52) with the Es enhancer or an interaction with different cell-type-specific coactivators.

Only with nuclear extracts from HD11 monocytes are additional regions protected from nuclease digestion detectable, and these coincide with enhancer elements A, D, and E. No DNA binding signals to elements B and C are visible in these experiments. The first region protected in a cell-type-specific manner, around position −6050 (approximately −6055 to −6040 on the upper strand and −6068 to −6045 on the lower strand), corresponds to module A. A second region protected in a cell-type-specific manner, from approximately positions −6070 to −5920, covers elements D and E. The 3′ border of this protected region, not visible in Fig. 7, was determined by DNase I footprinting experiments with an appropriate DNA fragment covering additional DNA sequences downstream of the Es enhancer (25). Similar analysis of the mutants D/DOWN3 and E/1 allows the separation of footprint boxes D and E and reveals its junction around position −5951 (25).

DISCUSSION

The cell-type-specific function of the chicken lysozyme −6.1-kb enhancer. We have shown in transient transfection experiments that the chicken lysozyme gene enhancer located approximately 6.1 kb upstream of the transcriptional initiation site which was originally identified as a 562-bp DNA fragment (E*l* [65]) can be narrowed down to a 157-bp minimal enhancer element (Es). All further deletions tested result in a dramatic loss of enhancer activity. This 157-bp enhancer fragment displays the same cell-type-specific activity as the 562-bp E*l* fragment. Both enhancer fragments stimulate the lysozyme promoter in myeloid cell lines, whereas no stimulation is seen in lymphoid and erythroid cell lines, a chicken hepatoma cell line, or primary chicken embryo fibroblasts. The E*l* and Es enhancer fragments also stimulate the heterologous TK and SV40 promoters with the same cell type specificity, indicating that a possible contribution by the lysozyme promoter is not relevant for the observed restriction of enhancer activity to myeloid cells. Although the minimal enhancer reveals the same cell-type-specific activation potential as the E*l* enhancer, sequences upstream of the minimal element conditionally are involved in enhancer function in macrophages (discussed later).

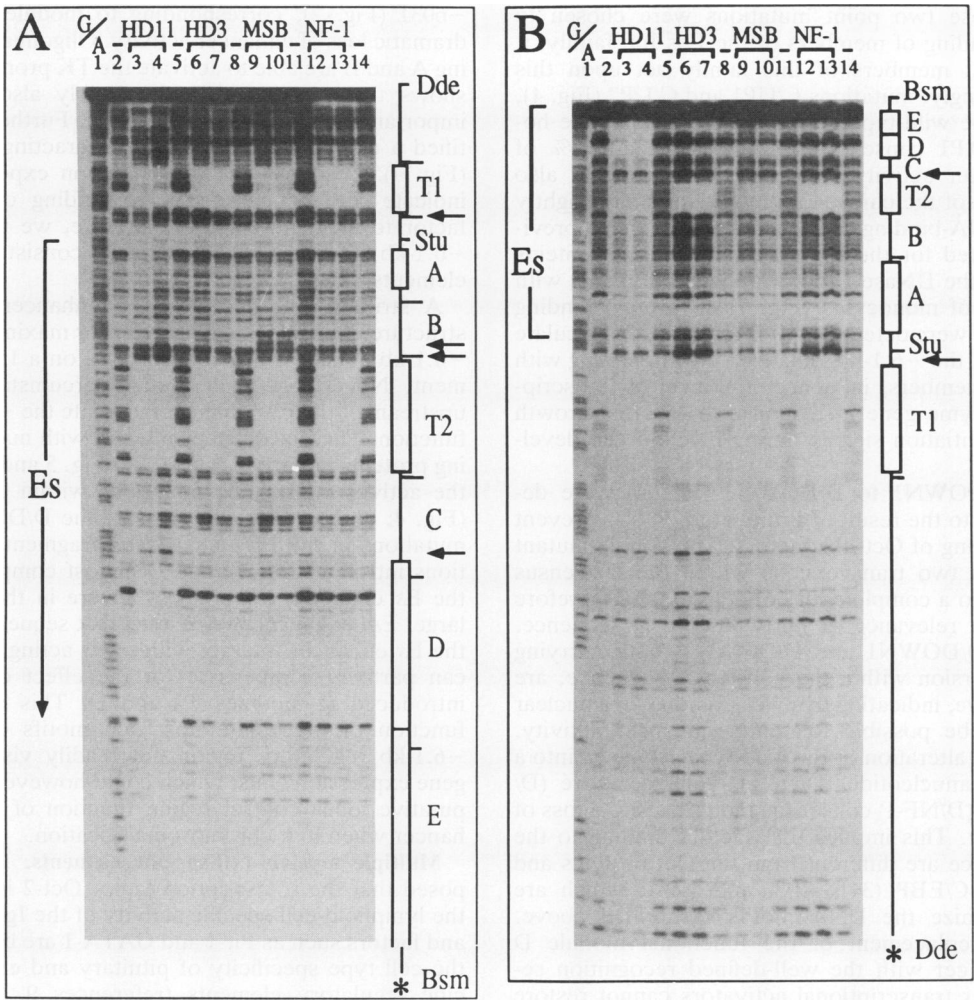

FIG. 7. DNase I footprint of nuclear extract proteins on the −6.1-kb enhancer. The upper (A) or the lower (B) strand of a 300-bp *Dde*I-*Bsm*I enhancer fragment was radioactively end labeled as indicated (asterisks). After incubation with extract buffer (lanes 2, 5, 8, 11, and 14); nuclear extracts from HD11 cells (lanes 3 and 4), HD3 cells (lanes 6 and 7), or MSB1 cells (lanes 9 and 10); or 0.125 μl of purified NF-I/TGGCA protein (53) (lanes 12 and 13), which was followed by DNase I digestion and purification, DNA samples were separated by denaturing gel electrophoresis and analyzed by autoradiography. Binding sites T1 and T2 and the regions of the enhancer protected by nuclear extracts from HD11 cells are indicated by open bars. The borders of elements A, B, C, D, and E are shown. Hypersensitive sites are marked by arrows. The 5′ end of the *Es* enhancer is shown on the left. G- and A-specific sequencing reactions are shown in lanes 1.

The cell type specificity of the enhancer correlates well with the activity of the endogenous lysozyme gene in chicken cells. Lysozyme gene transcripts are detected only in myelocytes (E26-transformed myeloblasts and BM2 cells), monocytes (HD11 cells), and primary macrophages of the hematopoietic system and in oviduct tissue. No transcripts of the chicken lysozyme gene are found in erythroblasts (HD3 cells) and lymphocytes (MSB1 cells) or in any nonhematopoietic cell line or tissue analyzed. The −6.1-kb lysozyme gene enhancer coincides with a DHS in uninduced and induced oviduct and in all myeloid cell types transcribing the lysozyme gene (19, 20, 62). In contrast, another macrophage-specific enhancer located 2.7 kb upstream of the gene (41, 63) is present as a DHS only in chromatin of nuclei from more mature myeloid cells. The appearance of the DHS at −2.7 kb in turn correlates with an increase in lysozyme mRNA level during monocyte differentiation. Thus, the −6.1-kb enhancer might function as the general enhancer for chicken lysozyme transcription in the two only tissues which are known to express the lysozyme gene, oviduct and macrophages (61). Therefore, we suggest that the −6.1-kb lysozyme gene enhancer plays a central role in the developmental and cell-type-specific activation of the gene and may have the general function of determining the potentially active state of the gene. According to this model, the −6.1-kb enhancer, coinciding with a DHS detectable throughout the development of myeloid cells, would correspond functionally to the locus control region of the human β-globin gene locus (26).

The structural organization of the −6.1-kb enhancer. Elements A, B, T2, C, D, and E have been deduced by binding studies in vitro and in vivo (11, 60) (see also Fig. 7) as well as by sequence analysis. By gene transfer analysis of point mutations, we demonstrate the functional relevance of T2, C, D, and E. Module C has been analyzed by several point mutations altering the homology to the AP1 recognition sequence (1, 37). The mutations C/DOWN1 and C/DOWN2 clearly show that element C is important for *Es* enhancer

function. As these two point mutations were chosen to interfere with binding of members of the Fos/Jun family, it seems likely that members of this family act upon this element. Surprisingly, mutations C/UP1 and C/UP2 (Fig. 4), which convert the wild-type sequence into a complete homology to the AP1 consensus, result in 65 and 95% of wild-type enhancer activity. One could assume that also various members of the chicken AP1 protein family slightly differ in their DNA-binding preferences, as has been previously demonstrated for the basic helix-loop-helix proteins (8). Although in the DNase I footprinting experiments with nuclear extracts of monocytes (Fig. 7) no protein binding signals to motif C were detectable, it is tempting to speculate that element C of the −6.1-kb enhancer, by interacting with Fos/Jun family members, influences the level of transcription of the lysozyme gene in response to external growth stimuli or differentiation signals during myeloid cell development.

Mutations D/DOWN1 to D/DOWN3 (Fig. 4) were designed according to the result of Pruijn et al. (47) to prevent the possible binding of Oct-like factors. The double mutant D/DOWN3, with two transversions within the consensus sequence, leads to a complete loss of activity and therefore demonstrates the relevance of the wild-type D sequence. The mutations D/DOWN1 and D/DOWN2, both carrying only one transversion within the consensus sequence, are still partially active, indicating that weak binding of a nuclear factor may still be possible to confer enhancer activity. Interestingly, the alteration of the wild-type sequence into a perfect octa-/decanucleotide (D/OCT), enhancer core (D/CORE), or NF-I (D/NF-I) consensus motif leads to a loss of enhancer activity. This implies that factors binding to the wild-type sequence are different from the Oct proteins and factors such as C/EBP (34), AP3, and NF-I, which are known to recognize the DNA motifs mentioned above. Therefore, the replacement of the functional module D within the enhancer with the well-defined recognition sequences for other transcriptional activators cannot restore the activity of the wild-type sequence and strongly argues for a different type of transactivator that acts upon element D.

Mutation E/1 of the E*s* enhancer and the deletion of this DNA region lead to a complete loss of activity. Besides D/DOWN3, the point mutation E/1 is the only sequence modification found that completely inactivates the E*s* enhancer, underlining the importance of module E for the E*s* enhancer. As both the D/DOWN3 and the E/1 mutations disrupt a palindromic sequence spanning elements D and E and having individually the same drastic effect on the enhancer activity, we assume that the same factor complex interacts cooperatively with the two elements (see also Fig. 7).

The best-understood interaction of regulatory proteins with the −6.1-kb enhancer is the binding of the NF-I/TGGCA proteins to elements T1 and T2. T1 is bound by NF-I/TGGCA in vitro but can be deleted in transient transfection experiments without losing enhancer activity. In contrast, there is a correlation between the binding of NF-I to element T2 in vitro and E*s* enhancer function. Eliminating two GN7 contacts essential for NF-I binding in vivo in mutation T2/P results in the inability of NF-I to bind in vitro (Fig. 5) and leads to a concomitant drop of enhancer function after gene transfer in HD11 cells. Therefore, the binding of NF-I to module T2 is important for full lysozyme enhancer activity.

No point mutations were introduced in modules A and B. However, the deletion of sequences from positions −6075 to −6031 (Fig. 2), corresponding to module A, results in a dramatic loss of enhancer activity. Oligonucleotides containing A and B are able to activate the TK promoter, which also shows that element A and possibly also module B are important for E*s* enhancer function. Furthermore, we identified a cell-type-specific factor interacting with module A (Fig. 7). Dimethyl sulfate protection experiments in vivo indicate the monocyte-specific binding of a *trans*-acting factor to element B (10). Therefore, we propose that the −6.1-kb lysozyme gene enhancer consists of at least six elements: A, B, T2, C, D, and E.

A striking feature of the E*s* enhancer is its compact structure. All elements necessary for maximal activity of the −6.1-kb enhancer are concentrated on a 157-bp DNA fragment. Nevertheless, in certain circumstances sequences upstream of the E*s* enhancer modulate the −6.1-kb enhancer function. These sequences interact with nuclear DNA-binding proteins in vitro (11) (see also Fig. 5 and 7) and influence the activity of specific mutations within the E*s* enhancer (Fig. 4; compare the activities of the D/DOWN3 and T2/P mutations in the 157- and 410-bp fragments). As the mutations introduced in T2 and D almost completely inactivate the E*s* enhancer but are less severe in the context of the larger E*m* enhancer, we assume that sequences upstream of the E*s* enhancer interact with *trans*-acting factors and thus can partially compensate for the effect of the mutations introduced in modules T2 and D. This could indicate a function of immediate upstream motifs of the wild-type −6.1-kb regulatory region not readily visible in transient gene expression tests, which could however be essential for putative locus control region function of the −6.1-kb enhancer when in a chromosomal location.

Multiple myeloid-cell-specific elements. It has been proposed that the transcription factor Oct-2 is responsible for the lymphoid-cell-specific activity of the IgH enhancer (55), and factors such as Pit-1 and GATA-1 are believed to confer the cell type specificity of pituitary and erythroid-cell-specific regulatory elements (references 9, 31, and 68 and references therein). In order to identify an element which confers the macrophage specificity of the chicken lysozyme −6.1-kb enhancer, we tested the four different overlapping subfragments of the E*s* enhancer in cell lines representing different lineages of the hematopoietic system. Surprisingly, none of the oligonucleotides, irrespective their copy number, is able to activate the TK promoter in the erythroid and lymphoid cell lines HD3 and MSB1. Instead, all oligonucleotides show an activation of the herpes simplex virus TK promoter specifically in the lysozyme-producing HD11 monocytes.

The DNA-protein interaction studies with nuclear extracts support the finding that all four tested enhancer subfragments act in a myeloid-cell-specific manner. Monocyte HD11-specific DNA-binding activities to modules A, D, and E could be detected. Further footprint analyses of the D/DOWN3 and E/1 mutant enhancer DNAs now demonstrate the binding of two separable cell-type-specific binding activities to D and E (25). Interestingly, the NF-I/TGGCA protein family members derived from cells of myeloid, erythroid, and lymphoid origin also form distinguishable patterns of DNase I-hypersensitive cutting sites at the borders of elements T1 and T2 (Fig. 7), indicating cell-type-specific differences in the NF-I protein family or a possible interaction with different cell-type-specific coactivators, as has been described for other ubiquitous proteins (48). As we have found that NF-I proteins are encoded by four different genes, at least three of which produce several alternatively

spliced mRNAs (35, 52), it is possible that certain subspecies of this protein family function as cell-type-specific activators, reminiscent of the action of individual members of other transactivator families, such as Oct proteins or retinoic acid receptors (reference 50 and references therein). In summary, the model of a single cell-type-specific DNA-binding protein in concert with constitutive factors determining the cell-type-specific function of a multifactorial enhancer does not hold true for the macrophage-specific enhancer complex. Clearly, multiple elements contribute to the myeloid-cell-specific function of the −6.1-kb enhancer of the chicken lysozyme gene.

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REFERENCES

1. **Angel, P., R. Imagawa, R. Chiu, B. Stein, R. J. Imbra, C. Rahmsdorf, C. Jonat, C. Herrlich, and M. Karin.** 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans acting factor. Cell **49**:729–739.
2. **Banerji, J., S. Rusconi, and W. Schaffner.** 1981. Expression of a globin gene is enhanced by remote SV40 DNA sequences. Cell **27**:299–308.
3. **Baniahmad, A., C. Steiner, A. C. Kühne, and R. Renkawitz.** 1990. Modular structure of a chicken lysozyme enhancer: involvement of an unusual thyroid hormone receptor binding site. Cell **61**:505–514.
4. **Beug, H., A. Leutz, P. Kahn, and T. Graf.** 1984. Ts mutants of E26 leukemia virus allow transformed myeloblasts, but not erythroblasts or fibroblasts, to differentiate at the nonpermissive temperature. Cell **39**:579–588.
5. **Beug, H., H. Müller, S. Grieser, G. Döderlein, and T. Graf.** 1981. Hematopoietic cells transformed in vitro by REV-T avian reticuloendotheliosis virus express characteristics of very immature lymphoid cells. Virology **115**:907–919.
6. **Beug, H., S. Palmieri, C. Freudenstein, H. Zentgraf, and T. Graf.** 1982. Hormone-dependent terminal differentiation in vitro of chicken erythroleukemia cells transformed by ts mutants of avian erythroblastosis virus. Cell **28**:907–919.
7. **Beug, H., A. von Kirchbach, G. Döderlein, J. F. Conscience, and T. Graf.** 1979. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three different phenotypes of differentiation. Cell **18**:375–390.
8. **Blackwell, T. K., and H. Weintraub.** 1990. Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science **250**:1104–1110.
9. **Bodner, M., J. L. Castrillo, L. E. Theill, T. Deerinck, M. Ellisman, and M. Karin.** 1988. The pituitary specific transcription factor GHF-1 is a homeobox containing protein. Cell **55**:505–518.
10. **Borgmeyer, U.** Unpublished data.
11. **Borgmeyer, U., J. Nowock, and A. E. Sippel.** 1984. The TGGCA-binding protein: a eucaryotic nuclear protein recognizing a symmetrical sequence on double-stranded linear DNA. Nucleic Acids Res. **12**:4295–4311.
12. **Cato, A. B., R. Miksicek, G. Schütz, J. Arnemann, and M. Beato.** 1986. The hormone regulatory element of mouse mammary tumor virus mediates progesterone induction. EMBO J. **5**:2237–2240.
13. **Chu, G., H. Hayakawa, and P. Berg.** 1987. Electroporation for the efficient transfection of mammalian cells with DNA. Nucleic Acids Res. **15**:1311–1326.
14. **Curran, T., and B. R. Franza.** 1988. Fos and Jun: the AP-1 connection. Cell **55**:395–397.
15. **Davidson, I., C. Fromental, P. Augereau, A. Wildeman, M. Zenke, and P. Chambon.** 1986. Cell-type specific protein binding to the enhancer of simian virus 40 in nuclear extracts. Nature (London) **323**:544–548.
16. **de Villiers, J., L. Olson, C. Tyndall, and W. Schaffner.** 1982. Transcriptional enhancers from SV40 and polyoma virus show a cell type preference. Nucleic Acids Res. **10**:7965–7976.
17. **Evans, R. M.** 1988. The steroid and thyroid hormone receptor superfamily. Science **240**:889–895.
18. **Falkner, F. G., and H. G. Zachau.** 1984. Correct transcription of an immunoglobulin gene requires an upstream fragment containing conserved sequence elements. Nature (London) **310**:71–74.
19. **Fritton, H. P., T. Igo-Kemenes, J. Nowock, U. Strech-Jurk, M. Theisen, and A. E. Sippel.** 1987. DNaseI-hypersensitive sites in the chromatin structure of the lysozyme gene in steroid hormone target and non-target cells. Biol. Chem. Hoppe-Seyler **368**:111–119.
20. **Fritton, H. P., A. E. Sippel, and T. Igo-Kemenes.** 1983. Nuclease hypersensitive sites in the chromatin domain of the chicken lysozyme gene. Nucleic Acids Res. **11**:3467–3485.
21. **Fromental, C., M. Kanno, H. Nomiyama, and P. Chambon.** 1988. Cooperativity and hierarchical levels of functional organisation in the SV40 enhancer. Cell **54**:943–953.
22. **Gerster, T., P. Matthias, M. Thali, J. Jiricny, and W. Schaffner.** 1987. Cell type-specificity elements of the immunoglobulin gene enhancer. EMBO J. **6**:1323–1330.
23. **Gillies, S. D., S. L. Morisson, V. T. Oi, and S. Tonegawa.** 1983. A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. Cell **33**:717–728.
24. **Godbout, R., R. Ingram, and S. Tilghman.** 1986. Multiple regulatory elements in the intergenic region between the α-fetoprotein and albumin genes. Mol. Cell. Biol. **6**:477–487.
25. **Grewal, T.** Unpublished data.
26. **Grosveld, F., G. Bloom van Assendelft, D. R. Greaves, and G. Kollias.** 1987. Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell **51**:975–985.
27. **Hauser, H., T. Graf, H. Beug, I. Greiser-Wilke, W. Lindenmaier, M. Grez, H. Land, K. Giesecke, and G. Schütz.** 1981. Structure of the lysozyme gene and expression in the oviduct and macrophages. Haematol. Blood Transfusion **26**:175–178.
28. **Hecht, A.** Unpublished data.
29. **Hecht, A., A. Berkenstam, P. E. Strömstedt, J. A. Gustaffsson, and A. E. Sippel.** 1988. A progesterone responsive element maps to the far upstream steroid dependent DNaseI hypersensitive site of chicken lysozyme chromatin. EMBO J. **7**:2063–2073.
30. **Herr, W., and J. Clarke.** 1986. The SV40 enhancer is composed of multiple functional elements that can compensate for one another. Cell **45**:461–470.
31. **Ingraham, H. A., R. Chen, H. J. Mangalam, H. P. Elsholtz, S. E. Flynn, C. R. Lin, D. M. Simmons, L. Swanson, and M. G. Rosenfeld.** 1988. A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. Cell **55**:519–529.
32. **Jantzen, H. M., U. Strähle, B. Gloss, F. Steward, W. Schmid, M. Boshard, R. Miksicek, and G. Schütz.** 1987. Cooperativity of glucocorticoid responsive elements located far upstream of the tyrosine aminotransferase gene. Cell **49**:29–38.
33. **Jantzen, K., H. P. Fritton, and T. Igo-Kemenes.** 1986. The DNaseI sensitive domain of the chicken lysozyme gene spans 24 kb. Nucleic Acids Res. **14**:6085–6099.
34. **Johnson, P. F., W. H. Landschulz, B. J. Graves, and S. L. McKnight.** 1987. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes Dev. **1**:133–146.
35. **Kruse, U., F. Quian, and A. E. Sippel.** 1991. Identification of a fourth nuclear factor I gene in chicken by cDNA cloning: NFI-X. Nucleic Acids Res. **19**:6641.
36. **Langlois, A. J., K. Lapis, R. Ishizaki, J. W. Beard, and D. P. Bolognesi.** 1974. Isolation of a transplantable cell-line induced by the MC29 avian leukosis virus. Cancer Res. **34**:1457–1464.

37. **Lee, W., A. Haslinger, M. Karin, and R. Tjian.** 1987. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. Nature (London) **325:**368–372.
38. **Luckow, B., and G. Schütz.** 1987. CAT constructions with multiple unique restriction sites for the functional analysis of eucaryotic promoters and regulatory elements. Nucleic Acids Res. **15:**5490.
39. **Maniatis, T., S. Goodbourn, and J. A. Fischer.** 1987. Regulation of inducible and tissue-specific gene expression. Science **236:** 1237–1245.
40. **Moscovici, C., M. G. Moscovici, H. Jiminez, M. M. C. Lai, M. J. Hayman, and P. K. Vogt.** 1977. Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. Cell **11:**95–103.
41. **Müller, A., T. Grussenmeyer, U. Strech-Jurk, M. Theisen, A. Stief, and A. E. Sippel.** 1990. Macrophage differentiation at the genome level: studies of lysozyme gene activation. Bone Marrow Transplant. **5:**13–14.
42. **Müller, M. M., T. Gerster, and W. Schaffner.** 1988. Enhancer sequences and the regulation of gene transcription. Eur. J. Biochem. **176:**485–495.
43. **Ondek, B., L. Gloss, and W. Herr.** 1988. The SV40 enhancer contains two distinct levels of organisation. Nature (London) **333:**40–45.
44. **Ondek, B., A. Shepard, and W. Herr.** 1987. Discrete elements within the SV40 enhancer region display different cell-specific enhancer activities. EMBO J. **6:**1017–1025.
45. **Orkin, S. H.** 1990. Globin gene regulation and switching: circa 1990. Cell **63:**665–672.
46. **Phi-Van, L., and W. H. Strätling.** 1988. The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain. EMBO J. **7:**655–664.
47. **Pruijn, G. J. M., W. van Driel, and P. C. van der Vliet.** 1986. Nuclear factor III, a novel sequence-specific DNA-binding protein from Hela cells stimulating adenovirus replication. Nature (London) **322:**656–659.
48. **Pugh, B. F., and R. Tjian.** 1990. Mechanism for transcriptional activation by SP1: evidence for coactivators. Cell **61:**1187–1197.
49. **Rosenfeld, P. J., and T. J. Kelly.** 1986. Purification of nuclear factor I by DNA recognition site affinity chromatography. J. Biol. Chem. **261:**1398–1408.
50. **Ruberte, E., P. Dolle, P. Chambon, and G. Morriss-Kay.** 1991. Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. Development **111:**45–60.
51. **Rupp, R. A. W.** Personal communication.
52. **Rupp, R. A. W., U. Kruse, G. Multhaup, U. Göbel, K. Beyreuther, and A. E. Sippel.** 1990. Chicken NFI/TGGCA proteins are encoded by at least three independent genes: NFI-A, NFI-B and NFI-C with homologues in mammalian genomes. Nucleic Acids Res. **9:**2607–2616.
53. **Rupp, R. A. W., and A. E. Sippel.** 1987. Chicken liver TGGCA-protein purified by preparative mobility shift electrophoresis shows a 29.8 to 36.8 kd microheterogeneity. Nucleic Acids Res. **15:**9707–9726.
54. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
55. **Schaffner, W.** 1989. How do different transcription factors binding the same DNA sequence sort out their jobs? Trends Genet. **5:**37–39.
56. **Schirm, S., J. Jiricny, and W. Schaffner.** 1987. The SV40 enhancer can be dissected into multiple segments, each with a different cell type specificity. Genes Dev. **1:**65–74.
57. **Schütz, G., M. C. Nguyen-Huu, K. Giesecke, N. E. Hynes, B. Groner, T. Wurtz, and A. E. Sippel.** 1978. Hormonal control of egg white protein messenger RNA synthesis in the chicken oviduct. Cold Spring Harbor Symp. Quant. Biol. **42:**617–624.
58. **Sen, R., and D. Baltimore.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46:**705–716.
59. **Shapiro, D. J., P. A. Sharp, W. W. Wahli, and M. J. Keller.** 1988. Laboratory methods: a high-efficiency HeLa cell nuclear transcription extract. DNA **7:**47–55.
60. **Sippel, A. E., A. Stief, A. Hecht, A. Müller, M. Theisen, U. Borgmeyer, R. A. W. Rupp, T. Grewal, and T. Grussenmeyer.** 1989. The structural and functional domain organisation of the chicken lysozyme gene locus. Nucleic Acids Mol. Biol. **3:**133–147.
61. **Sippel, A. E., M. Theisen, U. Borgmeyer, U. Strech-Jurk, and T. Igo-Kemenes.** 1986. The TGGCA protein binds in vitro to DNA contained in nuclease hypersensitive region that is present only in active chromatin of the lysozyme gene, p. 155–162. *In* T. Godzicker, P. A. Sharp, and M. R. Botchan (ed.), Cancer cells 4/DNA tumor viruses: gene expression and replication. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
62. **Sippel, A. E., M. Theisen, U. Borgmeyer, U. Strech-Jurk, R. A. W. Rupp, A. W. Püschel, A. Müller, A. Hecht, A. Stief, and T. Grussenmeyer.** 1988. Regulatory function and molecular structure of DNaseI-hypersensitive elements in the chromatin domain of a gene, p. 355–369. *In* G. Kahl (ed.), Architecture of eucaryotic genes. VCH Verlagsgesellschaft, Weinheim, Germany.
63. **Steiner, C., M. Müller, A. Baniahmad, and R. Renkawitz.** 1987. Lysozyme gene activity in chicken macrophages is controlled by positive and negative regulatory elements. Nucleic Acids Res. **15:**4163–4178.
64. **Stief, A., D. M. Winter, W. H. Strätling, and A. E. Sippel.** 1989. A nuclear DNA attachment element mediates elevated and position independent gene activity. Nature (London) **341:**343–345.
65. **Theisen, M., A. Stief, and A. E. Sippel.** 1986. The lysozyme enhancer: cell-specific activation of the chicken lysozyme gene by a far upstream DNA element. EMBO J. **5:**719–724.
66. **Tooze, J.** 1982. Molecular biology of tumor viruses, 2nd ed., part 2, revised. DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
67. **Weiher, H., M. König, and P. Gruss.** 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science **219:** 626–631.
68. **Yamamoto, M., L. J. Ko, M. W. Leonard, H. Beug, S. H. Orkin, and J. D. Engel.** 1990. Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. Genes Dev. **4:**1650–1662.
69. **Zenke, M., T. Grundström, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon.** 1986. Multiple sequence motifs are involved in SV40 enhancer function. EMBO J. **5:**387–397.