Requirement of an E1A-sensitive Coactivator for Long-range Transactivation by the β-Globin Locus Control Region*

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Four erythroid-specific DNase I-hypersensitive sites at the 5′-end of the β-globin locus confer high-level transcription to the β-globin genes. To identify coactivators that mediate long-range transactivation by this locus control region (LCR), we assessed the influence of E1A, an inhibitor of the CBP/p300 histone acetylase, on LCR function. E1A strongly inhibited transactivation of Aγ and β-globin promoters by the HS2, HS2-HS3, and HS1-HS4 subregions of the LCR in human K562 and mouse erythroleukemia cells. Short- and long-range transactivation mediated by the LCR were equally sensitive to E1A. The E1A sensitivity was apparent in transient and stable transfection assays, and E1A inhibited expression of the endogenous γ-globin genes. Only sites for NF-E2 within HS2 were required for E1A sensitivity in K562 cells, and E1A abolished transactivation mediated by the activation domain of NF-E2. E1A mutants defective in CBP/p300 binding only weakly inhibited HS2-mediated transactivation, whereas a mutant defective in retinoblastoma protein binding strongly inhibited transactivation. Expression of CBP/p300 potentiated HS2-mediated transactivation. Moreover, expression of GAL4-CBP strongly increased transactivation of a reporter containing HS2 with a GAL4 site substituted for the NF-E2 sites. Thus, we propose that a CBP/p300-containing coactivator complex is the E1A-sensitive factor important for LCR function.

The β-globin locus control region (LCR)† is a complex genetic element necessary for high-level transcription of the β-globin genes (1–3). The LCR was defined initially by its ability to confer copy number-dependent and position-independent expression to β-globin transgenes (1). The LCR also confers strong, erythroid-specific expression to linked genes in transfection assays, consistent with an intrinsic transcriptional enhancer function. Mutational studies have shown that multiple recognition sites for trans-acting factors are necessary for enhancer activity in stable transfection assays and to overcome position effects in transgenic mice (4–10).

Tandem binding sites for the hematopoietic transcription factor NF-E2 within the HS2 subregion of the LCR are important for strong transactivation of β-globin promoters in multiple systems (6, 11–14). NF-E2 binds to DNA as a heterodimer consisting of a 45-kDa hematopoietic subunit, p45 (15, 16), and an 18-kDa ubiquitous subunit, p18 (16, 17). Besides NF-E2, additional proteins such as NRF1 (18, 19), NRF2 (20), AP-1 (21), and Bach (22) proteins are known to interact with the NF-E2 sites, although the consequence of these interactions for LCR function is unresolved. The involvement of NF-E2 in functioning through these sites has been established in CB3 cells, murine erythroleukemia cells that lack p45 (23, 24). However, disruption of the murine p45 gene does not greatly impair β-globin synthesis (25), suggesting that there may be redundant factors functioning through the NF-E2 sites. The p45 gene disruption resulted in defective platelet formation, implicating NF-E2 as a critical regulator of megakaryopoiesis. Based on the requirement of NF-E2 for megakaryopoiesis and importance for globin synthesis in CB3 cells, it is of considerable interest to determine how NF-E2 activates transcription.

Sequences within the amino terminus of p45 are necessary for strong transactivation (26–28). Since these sequences are not required for DNA binding, and their deletion does not confer protein instability, they may engage in protein-protein interactions with coactivators. Several proteins have been suggested to be important for NF-E2-mediated transactivation, including WW domain-containing E3 ubiquitin ligases (28, 29), a HAT (CBP/p300) (30), and TAF110 (27), a component of the TFIID complex. We have shown that a PTY sequence within a 41-amino acid region of the amino terminus of p45, which is necessary for strong transactivation, mediates specific and high-affinity binding to WW domains from the WW1 ubiquitin ligase (28). Gavva et al. (29) also measured the binding of a GST-p45 fusion protein to several WW domains. WW domains are protein modules that mediate protein-protein interactions by binding to PXXY ligand sequences (31). The functional significance of a WW domain protein interaction was supported by mutagenesis studies in which PTY was mutated to AAA, resulting in a 73% reduction in transactivation (28). However, this p45 mutant retained weak but significant activity, suggesting that the protein binding to PTY is not the sole mediator of transactivation.

In this study, we asked whether the CBP/p300 interaction with NF-E2 is functionally important within the context of the β-globin LCR. It is possible that the interaction would be critical for the activity of a simple promoter driven exclusively by NF-E2, but not for a complex element containing multiple factor-binding sites such as the LCR. The requirements for transactivation from simple and complex activating elements
can differ, as it was exemplified recently by studies of glucocorticoid receptor phosphorylation (32). Phosphorylation of the receptor enhanced transactivation of a reporter gene containing a simple glucocorticoid-responsive promoter but not a complex glucocorticoid-responsive promoter with multiple transcription factor-binding sites. Here, we show that an inhibitor of CBP/p300, the adenoviral E1A protein, almost completely abolishes LCR-mediated transactivation of \( \gamma \)- and \( \beta \)-globin promoters. The implications of this result are discussed versus models of LCR function invoking the recruitment of HATs that remodel chromatin and regulate the activity of nonhistone components.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human erythroleukemia cell line K562 (33) was maintained in IMEM medium (Biofluids) containing 10% fetal bovine serum, 25 \( \mu \)g/ml gentamycin, and 1% antibiotic-antimycotic solution (Life Technologies, Inc.). In certain experiments, K562 cells were treated with 40 \( \mu \)M hemin for 48 h to induce erythroid differentiation before transfection. The mouse erythroleukemia cell line MEL (34) was maintained in Dulbecco’s modified Eagle’s medium (Biofluids) containing 5% fetal calf serum, 5% calf serum, and 1% antibiotic-antimycotic solution. Cells were grown in a humidified incubator at 37 °C in the presence of 5% \( \mathrm{CO}_2 \).

**Transient Transfections, Luciferase, and \( \beta \)-Galactosidase Assays**—Exponentially growing cells (5 \( \times \) 10\(^4\)) were collected by centrifugation at 240 \( \times \) g for 5 min at 4 °C, resuspended in 0.5 ml of IMEM containing 10% fetal bovine serum, 25 \( \mu \)g/ml gentamycin, and 1% antibiotic-antimycotic solution (Life Technologies, Inc.), and mixed with 3.5 ml of identical medium in each well of a 6-well plate. Plasmid DNA (4 \( \mu \)g in 150 \( \mu \)l of IMEM (for K562 cells) or IMEM (MEL cells)) was incubated with 16 \( \mu \)l of Superfect transfection reagent (Qiagen) for 15 min at room temperature and then added to cells. Cells were incubated for 40 h, harvested, and assayed for luciferase activity as described previously (10). Luciferase activity was normalized by the protein content of the lysate, determined by Bradford assay using \( \gamma \)-globulin as a standard. In certain experiments, the constitutively active \( \beta \)-galactosidase expression vector pCH110 (Amersham Pharmacia Biotech) (1 \( \mu \)g) was included in each transfection reaction, so that luciferase activity could be normalized for differences in transfection efficiency. \( \beta \)-Galactosidase activity was assayed with a luminescent substrate (Galacton Plus) according to the manufacturer’s instructions (TROPIX, Inc.). Since the expression of CBP increased the activity of the SV40 promoter, which contains \( \beta \)-galactosidase expression sequence from pCH110, the experiment shown in Fig. 10 was done without co-transfecting pCH110. Expression of E1A did not influence the activity of the SV40 promoter of pCH110. All transient transfection experiments except for Figs. 8 and 10 were done in the presence and absence of pCH110, and similar results were obtained. Expression vector pCH110 (Amersham Pharmacia Biotech) was kindly provided by Drs. Alex Vassilev and Yoshihiro Nakatani (National Institutes of Health).

**RT-PCR Analysis of Endogenous \( \gamma \)-Globin Gene Expression**—Exponentially growing K562 cells (12 \( \times \) 10\(^4\)) were transfected with a total of 72 \( \mu \)g of DNA in 288 \( \mu \)l of Superfect. The ratio of pCL1-E1A or pcDNA3 to EGFP was 5:1. Cells were incubated for 3 days, and equal numbers of EGFP-positive cells transfected with E1A or pcDNA3 were isolated by FACS. Trizol-extracted RNA was analyzed using the Access RT-PCR kit (Promega) according to the manufacturer’s instructions. Products were visualized by ethidium bromide staining of a 1.2% agarose gel.

**Expression and Purification of Recombinant Proteins**—A prokaryotic expression vector containing the cDNA for murine p300 was obtained from Dr. Timothy Emerson (Stanford University). Polymerase chain reaction (PCR) was used to ovexpressed in the BL21DE3LyS strain of Escherichia coli and purified on a column containing nickel-NTA resin equilibrated in buffer containing 8 M urea, as described previously (28). Protein samples were dialyzed overnight against 20 mM Tris, 0.1 mM EDTA, 5% glycerol, 50 mM NaCl, 5 M dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.5, at 4 °C with two changes of buffer. Protein concentrations were measured by Bradford assay using \( \gamma \)-globulin as a standard. Purified baculovirus expressed, flag epitope-tagged human p300 was kindly provided by Drs. Alex Vassilev and Yoshihiro Nakatani (National Institutes of Health).

**Solid-state Protein-Protein Interaction Assay**—Enzyme-linked immunosorbent assays were performed as described previously (28). Purified p300 or GST (100 ng) was immobilized in trilinate wells of a 96-well plate along with enzyme-linked immunosorbent-assay (ELISA) rat E1A/RIA number 3590. Wells were blocked by incubation with bovine serum albumin (1 mg) for 1 h. The immobilized protein was incubated with increasing amounts of purified p45 and then washed five times with 300 \( \mu \)l of wash buffer (phosphate-buffered saline containing 0.1% Tween 20). Anti-p45 antibody (28) (1/2000 dilution in wash buffer, 50 \( \mu \)l total) was added for 1 h, and then the plate was washed five times. After addition of horseradish peroxidase-conjugated goat anti-rabbit IgG (1/5000, Amersham) and 0.1% \( \mathrm{H}_2\mathrm{O}_2\) in 0.2% ABTS, the plate was washed five times, and the horseradish peroxidase substrate ABTS was added (0.2 mg/ml). Color development was allowed to proceed for 20 min, and absorbance measurements were made at 405 nm with an Eks800 universal microplate reader (BIO-TEK Instruments, Inc.), under conditions in which the absorbance was in the linear range. Incubations were done at room temperature with gentle agitation.
for transactivation may differ in cells of varying differentiation states. We tested whether HS2-mediated transactivation was also sensitive to E1A in hemin-treated K562 cells, which differentiate along the erythroid lineage. Similar to the results of Fig. 1, expression of E1A abolished HS2-mediated transactivation in induced K562 cells (data not shown).

MEL cells represent a later stage of erythroid differentiation than K562 cells and express β-globin rather than α- and γ-globins characteristic of K562 cells. We assessed the sensitivity of LCR-mediated transactivation to E1A in MEL cells to determine whether the sensitivity was dependent on the stage of erythroid differentiation. As shown in Fig. 2, transactivation of a β-globin promoter mediated by HS2 was strongly inhibited by E1A in MEL cells. In contrast to the K562 cell system, the inhibition was incomplete, suggesting that a component of the activity in MEL cells is resistant to inhibition by E1A. These results are consistent with a requirement of the E1A-sensitive factor for HS2-mediated transactivation at multiple stages of erythroid differentiation.

**Requirement of the E1A-sensitive Factor for Long-range Transactivation**—The transactivation property of the LCR is often studied with constructs containing the LCR positioned near (within 1–2 kb) a β-globin promoter linked to a reporter gene. However, within the endogenous β-globin locus, the LCR is 7–50 kb upstream of the β-globin promoters. Thus, it was important to determine whether the E1A sensitivity of LCR-mediated transactivation is also apparent over long distances. We asked whether transactivation of a γ-globin promoter by HS2-HS3 and by the mini-LCR positioned 5.1 kb from the promoter was sensitive to E1A in a transient transfection assay. Regardless of whether the LCR elements were positioned 20 base pairs or 5.1 kb from the promoter, E1A strongly inhibited transactivation (Fig. 3).

We also tested the influence of E1A on the activity of linearized templates in transient transfection assays to determine whether the requirement of the E1A-sensitive factor was unique to plasmids. Constructs containing HS2 20 base pairs or 5.1 kb from the promoter or the promoter alone were linearized downstream of the luciferase gene with NotI, and linear DNA was transfected into K562 cells. Expression of E1A completely inhibited the activity of both HS2-containing templates without affecting the activity of the construct containing the promoter alone (data not shown). Thus, short- and long-range transactivation mediated by the LCR are equally sensitive to inhibition by E1A with circular and linear templates.

The E1A-sensitive Factor Is Critical for Transactivation of a Chromosomal Reporter Gene and Endogenous γ-Globin Genes—The experiments described above used transient transfection assays, in which the DNA is nonreplicating and may not be assembled into organized chromatin. As the transactivation requirements may differ for templates in transient and stable assays, we asked whether HS2-mediated transactivation of an integrated reporter gene was sensitive to E1A. An expression vector encoding E1A or the blank vector and a vector encoding EGFP were transiently co-transfected into the K562 clonal cell line HS2(2.2)yLuc#20, which contains two copies of an integrated HS2(2.2)yLuc reporter gene. Cells positive for EGFP were isolated by FACS and assayed for luciferase activity. This procedure allows one to assess the influence of the transiently expressed E1A on the reporter gene present in all cells. Expression of E1A strongly reduced luciferase activity relative to the control vector (Fig. 4). Thus, the E1A-sensitive factor is required for strong LCR-mediated transactivation in stable transfection assays, in addition to transient assays as described above.

To determine the influence of E1A on endogenous γ-globin
gene expression, K562 cells were co-transfected with the control vector pcDNA3 or an E1A expression vector and a vector encoding EGFP. EGFP-positive cells were isolated by FACS, and RNA was analyzed by RT-PCR using primer pairs to detect human g-globin or HPRT transcripts. Expression of E1A strongly reduced the steady-state level of g-globin transcripts without influencing the level of HPRT transcripts (Fig. 5). The average decrease from two independent experiments was 3.2-fold. Since inhibition by E1A is apparent with the endogenous g-globin genes and is not unique to transfected LCR-containing constructs, the E1A-sensitive factor is likely to be important for the physiological regulation of the g-globin genes.

Requirement of NF-E2 but Not GATA-1 Sites of HS2 for E1A Sensitivity—As tandem NF-E2 sites and a GATA-1 site of HS2 are important for transactivation (8), and both NF-E2 and GATA-1 can physically interact with CBP/p300 (30, 37), we asked whether both sites are required for sensitivity to E1A. Importantly, distinct sequences of CBP/p300 are bound by NF-E2 and GATA-1, suggesting that a multivalent interaction by these factors may be required to recruit a CBP/p300-containing coactivator complex. As shown in Fig. 6A, deletion of the tandem NF-E2 sites of HS2 strongly reduced transactivation (99% decrease). The residual activity of the construct, which could be measured accurately, was increased 3.5-fold upon expression of E1A. E1A slightly increased the activity of the A-globin promoter alone (1.9-fold). Deletion of the GATA-1 site resulted in a 61% reduction in activity. Importantly, the activity of the GATA-1 mutant construct was strongly inhibited (87% decrease) by expression of E1A. Thus, the NF-E2 sites, but not the GATA-1 site, are required for E1A sensitivity as depicted in the model of Fig. 6B. We also tested whether the NF-E2 sites were necessary for E1A sensitivity in hemin-induced K562 cells. Similar to the uninduced cells, E1A did not inhibit the activity of the NF-E2 mutant construct in induced cells (data not shown).

Deletion of the NF-E2 sites of HS2 almost completely abolished transactivation by HS2, and therefore it is possible that the requirement of the NF-E2 sites for E1A sensitivity is indirect. Factors binding to the NF-E2 sites may be critical for forming a functional HS2 complex but might not directly mediate activation through the E1A-sensitive factor. To test whether the E1A-sensitive factor is required for transactivation by proteins binding to the tandem NF-E2 sites of HS2, we asked whether transactivation mediated by these sites alone was sensitive to E1A. The tandem NF-E2 sites from HS2 activated a minimal g-globin promoter 5.3-fold after transient transfection into K562 cells (Fig. 7). Expression of E1A strongly

![Fig. 3. Short- and long-range transactivation mediated by HS2-HS3 and HS1-HS4 are equally sensitive to inhibition by E1A. A, the reporter plasmids contained the human A-globin promoter linked to luciferase with or without HS2. Reporter plasmids were transiently transfected into MEL cells with or without expression plasmids encoding E1A (pCL1-E1A) or the blank vector pcDNA3. The b-galactosidase expression vector, pCH110, was included in all conditions. The graph on the left shows the absolute luciferase activities of the reporter constructs. The graph on the right shows the relative luciferase activities, where the luciferase activities of reporter constructs transfected without the blank expression vector (pcDNA3) or the E1A expression vector, were set at 100%. Luciferase activity was normalized by the protein concentration and the b-galactosidase activity of the lysate (mean ± S.E., n = 6).](image-url)
HS2(2.2) DNA-binding domain of GAL4 (GAL4-p45(1–90)) activated the expression of the transactivation domain of p45 fused to the between other cis elements within HS2. We then asked whether HS2. The construction maintained the natural spacing be-

inhibited transactivation, consistent with a direct involvement of the E1A-sensitive factor in transactivation mediated by the NF-E2 sites.

Since multiple factors can interact with the NF-E2 sites (15, 18–22), we wanted to determine whether transactivation mediated by NF-E2 was sensitive to E1A. Thus, we generated a mutant HS2 reporter construct (HS2(GAL4)yluc) with a single GAL4-binding site substituted for the tandem NF-E2 sites of HS2. The construction maintained the natural spacing between other cis elements within HS2. We then asked whether expression of the transactivation domain of p45 fused to the DNA-binding domain of GAL4 (GAL4-p45(1–90)) activated the HS2(GAL4)yluc reporter and whether activation was sensitive to E1A. Expression of GAL4-p45(1–90) activated the HS2(GAL4)yluc reporter 3.6-fold, and the activation was prevented by coexpression of E1A (Fig. 8A). E1A had no effect on the activity of the reporter alone. Expression of the GAL4 DNA-binding domain alone did not influence reporter activity (data not shown). As the expression of GAL4-p45(1–90) was driven by the CMV promoter, and this concentration of E1A does not inhibit the CMV promoter in K562 cells (data not shown), the inhibitory effect does not result from reduced expression of GAL-p45(1–90). Expression of GAL-p45(1–90) strongly activated (80-fold) a control promoter with five GAL4-binding sites linked to the adenovirus major late promoter TATA box (p55Tluic) (Fig. 8D). Similar to the HS2(GAL4)yluc reporter, E1A strongly inhibited transactivation. The GAL4-binding sites were required for transactivation by GAL4-p45(1–90), as expression of GAL4-p45(1–90) had no effect on the activity of the HS2ΔNF-E2)yluc reporter, which has a SalI site substituted for the tandem NF-E2-binding sites of HS2 and lacks GAL4-binding sites (Fig. 8C).

Involvement of CBP/p300 in LCR-mediated Transactivation—Although E1A inhibits CBP/p300, E1A can have multiple effects on cell function (42). Sequences within the amino terminus and the CR1 domain of E1A are important for CBP/p300 binding, whereas the CR1 and CR2 domains contain sequences necessary for retinoblastoma protein binding (36, 43). We tested E1A mutants defective in either CBP/p300 or retinoblas-
CBP to HS2 via a GAL4 DNA-binding domain at the site normally bound by NF-E2 strongly rescues the loss of activity resulting from deletion of the tandem NF-E2 sites. Since the NF-E2 sites, but not the GATA-1 site, of HS2 were required for E1A sensitivity, factors binding to the NF-E2 sites must be critical for recruitment of the E1A-sensitive factor. As mentioned above, a GST-p45 fusion protein has been reported to interact with GST fusions of CBP/p300 (30). However, we have not been able to coimmunoprecipitate endogenous NF-E2 and CBP/p300 using K562 nuclear extracts, which may reflect a highly regulated transient interaction between NF-E2 and CBP/p300 in cells. This could be analogous to the requirement for CREB to be phosphorylated by protein kinase A to bind CBP with high affinity (41). An alternative possibility is that the GST pull-down assays (30) may have detected a low affinity interaction not likely to occur in cells. Thus, we asked whether purified, full-length p45 binds to full-length p300 and whether the interaction is of high-affinity, which would further support a physiological role of CBP/p300 as a mediator of NF-E2 function. We overexpressed p45 in E. coli and used purified p45 in a quantitative, solid-state equilibrium binding assay to estimate its affinity for baculovirus expressed, purified p300. Incubation of increasing concentrations of p45 with immobilized p300 resulted in saturable binding with an estimated $K_D$ of

**Fig. 6.** Requirement of NF-E2 sites but not the GATA-1 site within HS2 for E1A sensitivity. A, the reporter plasmids contained the human $\beta$-globin promoter linked to luciferase with or without wild-type HS2, or NF-E2 or GATA-1-site mutants of HS2 ($\Delta$NF-E2 and $\Delta$GATA-1, respectively). The top graph shows the absolute luciferase activities of reporter constructs after transient transfection into K562 cells. Reporter plasmids were co-transfected with or without expression plasmids encoding E1A (pCL1-E1A) or the blank vector pcDNA3 (125 ng). The $\beta$-galactosidase expression vector, pCH110, was included in all conditions. Luciferase activity was normalized by the protein concentration and the $\beta$-galactosidase activity of the lysate (mean $\pm$ S.E., $n = 3$). The bottom graph shows normalized data, in which conditions with the pcDNA3 vector were set at 100%. B, model of NF-E2-mediated recruitment of a CBP/p300-containing coactivator complex. The transcription factor-binding sites within HS2 are indicated at the bottom. Two of these factors, NF-E2 and GATA-1, have been reported to interact with CBP/p300. The model assumes that a direct physical interaction between the p45 subunit of NF-E2 and CBP/p300 is critical for recruitment of the coactivator complex in K562 cells. In contrast, GATA-1 is not required to recruit the complex in these cells, but could potentially facilitate recruitment through interactions with the carboxyl-terminal domain of CBP/p300. The stoichiometry of NF-E2 binding to tandem sites within HS2 is unclear, and we have depicted a single NF-E2 heterodimer bound to the DNA.

**Fig. 7.** Transactivation mediated by the tandem NF-E2 sites of HS2 is inhibited by E1A. The reporter plasmids contained a minimal $\beta$-globin promoter linked to luciferase with and without the tandem NF-E2-binding sites from human HS2. Reporter plasmids were transiently transfected into K562 cells with or without expression plasmids encoding E1A (pCL1-E1A) or the blank vector pcDNA3. The $\beta$-galactosidase expression vector pCH110 was included in all conditions. The graph on the left shows the absolute luciferase activities of the reporter constructs. The graph on the right shows the relative luciferase activities, where the luciferase activities of reporter constructs transfected without the blank expression vector (pcDNA3) or the E1A expression vector (pCL1-E1A) were set at 100%. Luciferase activity was normalized by the protein concentration and the $\beta$-galactosidase activity of the lysate (mean $\pm$ S.E., $n = 3$).
Saturation resulted from the binding of a stoichiometric excess of p45 to the immobilized p300. This analysis assumes that 100% of the recombinant p45 is in a native conformation. Since p45 was purified in a denatured state and required renaturation, it is unlikely that all p45 molecules are native. If less than 100% of the p45 was competent for binding, the binding affinity would be even higher.

**DISCUSSION**

**An E1A-Sensitive Factor Is Critical for LCR-mediated Transactivation**—We have shown that the CBP/p300 inhibitor E1A strongly reduces LCR-mediated transactivation in transient and stable transfection assays and inhibits expression of endogenous γ-globin genes. Four lines of evidence support a functional relationship between the E1A sensitivity of LCR-mediated transactivation and CBP/p300 inhibition. First, recognition sites for a high-affinity CBP/p300-binding protein, NF-E2, were required for E1A sensitivity of LCR-mediated transactivation in K562 cells. Second, mutants of E1A defective in CBP/p300 binding (H3N, D2–36, and D38–67) had little effect on transactivation. In contrast, an E1A mutant defective in retinoblastoma protein binding (pm928) was an equally effective inhibitor of transactivation as wild-type E1A. Third, expression of CBP potentiated transactivation. Last, transactivation mediated by the activation domain of p45 was inhibited by E1A, and the NF-E2 requirement for HS2 enhancer activity could be bypassed by tethering CBP to HS2 via a GAL4 DNA-binding domain. The comparable requirement of the E1A-sensitive factor for short- and long-range transactivation and its importance for transactivation of a chromosomal reporter gene and endogenous γ-globin genes strongly suggests that the E1A-sensitive factor is a coactivator required for the enhancer function of the LCR.

The physiological importance of the E1A-sensitive factor is reinforced by the experiment of Fig. 5 and the results of Blobel et al. (37). Blobel and co-workers (37) showed that the conditional expression of E1A in MEL cells prevented endogenous γ-globin expression in the absence of E1A expression. This finding is consistent with the observation that E1A inhibits the transcription of endogenous γ-globin genes in transient and stable transfection assays. Moreover, the observation that E1A mutants defective in CBP/p300 binding only weakly inhibit HS2-mediated transactivation (Fig. 9) suggests that the E1A-sensitive factor is a coactivator required for the enhancer function of the LCR.

**Fig. 8.** Transactivation mediated by the activation domain of p45 is inhibited by E1A. The Gal4-p45(1–90) expression plasmid (1 μg) was transiently transfected into K562 cells with or without expression plasmids encoding E1A (pCL1-E1A) or the blank vector pcDNA3 (60 ng). A, fold activation of an HS2·gal reporter in which a single Gal4-binding site is substituted for the two NF-E2 sites of HS2. B, fold activation of the synthetic plasmid pGST1·luc containing five Gal4-binding sites upstream of the adenovirus major later promoter TATA box. C, fold activation of a NF-E2 site mutant of HS2 deleted for both NF-E2 sites. Luciferase activity was normalized by the protein concentration of the lysate (mean ± S.E., n = 3). Average absolute luciferase activities for reporters alone were 531 relative light units/μg for HS2·Gal4·luc, 4 relative light units/μg for pGST1·luc, and 214 relative light units/μg for HS2·(ΔNF-E2)·luc.

**Fig. 9.** E1A mutants defective in CBP/p300 binding only weakly inhibit HS2-mediated transactivation. A, diagram of wild-type and E1A mutant proteins. Conserved regions 1 and 2 are indicated by shaded boxes. Amino acids 1–289 refer to the E1A 13S gene product. B, K562 cells were transiently co-transfected with the luc reporter plasmid and either the blank vector pEF1α-neo or expression vectors encoding wild-type E1A (pEF1α-neoE1A) or mutants of E1A (pCL1-H3N, pEF1α-neoE1AΔ2–36, pEF1α-neoE1AΔ2–36, and pEF1α-neoE1Apm928). The β-galactosidase expression vector, pCH110, was included in all conditions. Luciferase activity was assayed in cell lysates and normalized by the protein concentration and the β-galactosidase activity of the lysate (mean ± S.E., n = 6). C, the transfection conditions were identical to B, except that the HS2·gal reporter plasmid was used.
NF-E2 strongly transactivates a reporter gene. K562 cells were transiently co-transfected with the βluc or HS2βluc reporter plasmids and either the blank vector pcDNA3 or an expression vector encoding CBP. Luciferase activity was assayed in cell lysates and normalized by the protein concentration of the lysate (mean ± S.E., n = 4).

![Figure 10](image1.png)

**FIG. 10.** Potentiation of HS2-mediated transactivation by CBP. MEL cells were transiently co-transfected with the βluc or HS2βluc reporter plasmids and either the blank vector pcDNA3 or an expression vector encoding CBP. The corrected values (mean ± S.E., n = 3) were plotted against the p45 concentration, and nonlinear regression analysis was used to estimate the *K*ₐ of the interaction.

**FIG. 12.** High affinity binding of p45 to p300. A, solid-state protein-protein interaction assay. p300 or GST were immobilized in the wells of a microtiter plate in triplicate. Vertical rows of the plate were incubated with increasing amounts of purified p45, followed by anti-p45 antibody and then a peroxidase-conjugated secondary antibody. p5 binding was measured by adding a peroxidase substrate. B, quantitative analysis. The absorbance values at 405 nm were corrected by subtracting the background absorbance from the corresponding wells containing immobilized GST. The corrected values (mean ± S.E., n = 3) were plotted against the p45 concentration.

β-globin gene expression. However, in the MEL cell system, β-globin gene expression occurs upon induction of erythroid differentiation with dimethyl sulfoxide. Thus, the inhibition by E1A could have resulted from perturbation of multiple regulatory steps necessary for differentiation. Our results in K562 cells provide direct evidence that the E1A-sensitive factor is required to maintain steady-state levels of endogenous γ-globin mRNA in a system that does not require differentiation to activate β-globin genes.

To determine whether the E1A-sensitive factor was recruited to the LCR through protein-protein interactions with LCR-binding proteins, we defined the cis-acting elements required for E1A sensitivity. We tested the hypothesis that a multivalent interaction between NF-E2, GATA-1, and CBP/p300 is required to efficiently recruit the complex. However, only the NF-E2 sites were required for E1A sensitivity, inconsistent with a critical role for GATA-1 in CBP/p300 recruitment to HS2 for activation of the γ-globin promoter in K562 cells (Fig. 6B).

It is formally possible that the NF-E2 sites are required to form a functional HS2 complex and therefore the requirement of the sites for recruitment of the E1A-sensitive factor may be indirect. This is unlikely, as the inhibition of transactivation by E1A is nearly complete, mimicking the effect of deleting the NF-E2 sites. No other deletions of cis-acting elements within HS2 result in such strong inhibition of transactivation. Furthermore, the results of Fig. 8 showing that transactivation mediated by the activation domain of p45 is abolished by E1A supports the hypothesis that NF-E2 is directly required to recruit or utilize the E1A-sensitive factor.

Although the potentiation of HS2-mediated transactivation by expression of CBP was consistently greater than the effect of deleting the NF-E2 sites, inconsistent with a critical role for GATA-1 in CBP/p300 recruitment to HS2 for activation of the γ-globin promoter in K562 cells (Fig. 6B), the stimulation was only moderate. Furthermore, only moderate stimulation of HS2 luc was observed in K562 cells (data not shown). Two considerations are relevant to this point. First, MEL and K562 cells already express CBP/p300. Therefore, CBP/p300 may not be limiting in these systems. Second, CBP/p300 is likely to function in a large heteromic complex, and the transient expression of a single component may not efficiently generate functional complexes. The strong stimulation of transactivation through the LCR by
The coactivator function of CBP/p300 is apparent in transient transfection assays in multiple systems (52). Thus, if the coactivator function requires histone acetylation, a fraction of the transiently transfected DNA templates must be organized into chromatin. As multiple copies of the transiently transfected DNA templates are present in a cell, and the chromatin organization of these templates is likely to be heterogeneous, it is difficult to determine whether a low percentage of the templates contain similar chromatin structures. Importantly, the E1A-sensitive factor is required for LCR-mediated transactivation in stable transfection assays and for expression of endogenous γ-globin genes, where templates are likely to have a more homogeneous chromatin structure than in transient transfection assays.

We have proposed that the ability of the LCR to confer high-level transcription to the β-globin genes over long distances on a chromosome requires the recruitment of chromatin remodeling enzymes, which modulate the chromatin structure of the β-globin promoters (3, 38, 46). The strong synergism among the hypersensitive sites, which is necessary for long-range transactivation (38), may be explained by the recruitment of distinct classes of coactivators that function collectively to catalyze a chromatin structure transition of the promoters at the appropriate developmental stage. The results described herein suggest that CBP/p300 is one such coactivator critical for long-range transactivation by the LCR. As CBP/p300 can exist as a complex with other HATs such as PCAF, additional factors may be important for the coactivator function. Our previous work (28) implicated WW domain proteins as other factors required for NF-E2-mediated transactivation of the β-globin genes in CB3 cells. Although the endogenous WW domain interactors have not been identified, we hypothesized that the ubiquitin ligase WWP1 may be a functionally relevant interactor, based on its expression in fetal liver and high affinity for the transactivation domain of NF-E2. The two enzymes, a HAT and a ubiquitin ligase, may be part of a larger complex of interacting components necessary for LCR function.

It is unknown whether HATs are requisite coactivators for long-range transactivation. In this regard, a recent report by Krumm et al. (53) provided evidence for the involvement of PCAF and p300 in long-range transactivation of a simple, synthetic promoter. Expression of PCAF or p300 fused to the GAL4 DNA-binding domain stimulated transactivation when GAL4 sites were placed 3.1 kb downstream of the promoter. At least two mechanisms may explain how regulatory complexes stimulate transactivation over long distances on chromosomes. The complex may mediate the unfolding of chromatin of an entire chromosomal domain, resulting in enhanced accessibility of cis-acting sequences throughout the domain. Alternatively, the complex may have a local action on the chromatin structure surrounding a promoter. Since loss of the LCR in its normal chromosomal context does not abrogate the general DNase I sensitivity of the β-globin locus (54), a potential indicator of unfolded chromatin, it is unlikely that chromatin modifying enzymes recruited by the LCR mediate a broad unfolding of the entire β-globin domain. Rather, the LCR may exert local effects on the chromatin structure of the β-globin promoters, analogous to the restricted acetylation of histones on the human interferon-β promoter (55) and a plasmid-based HIS3 promoter in Saccharomyces cerevisiae (56).

In summary, our studies implicating the E1A-sensitive factor represent the first demonstration of a coactivator required for LCR function. It will be of considerable interest to ask whether the conditional expression of E1A, CBP/p300, and WWP1 influence the acetylation and ubiquitination state of histones within the β-globin locus or of other factors required for transcription of the β-globin genes.

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REFERENCES
1. Grosveld, F., van Assendelft, G. B., Greaves, D. R., and Kollias, G. (1987) Cell 51, 975–985
2. Forrester, W. C., Takegawa, S., Papayanopoulou, T., Stamatakyanopoulou, G., and Groudine, M. (1987) Nucleic Acids Res. 15, 10159–10177
3. Bresnick, E. H. (1997) Chemtracts Biochem. Mol. Biol. 10, 1601–1609
4. Talbot, D., Philippsen, S., Fraser, P., and Grosveld, F. (1996) EMBO J. 15, 2169–2177
5. Philippsen, S., Talbot, D., Fraser, P., and Grosveld, F. (1996) EMBO J. 15, 2169–2176
6. Talbot, D., and Grosveld, F. (1996) EMBO J. 13, 1391–1398
7. Liu, D., Chang, J. C., Moi, P., Liu, W., Kan, Y. W., and Curtin, P. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3899–3903
8. Caterina, J. J., Ciavatta, D. J., Donze, D., Behringer, R. R., and Townes, T. M. (1994) Nucleic Acids Res. 22, 1006–1011
9. Elnitski, L., Miller, W., and Hardison, R. (1997) J. Biol. Chem. 272, 369–378
10. Lam, L. T., and Bresnick, E. H. (1996) J. Biol. Chem. 271, 32421–32429
11. Ney, P. A., Sorrentino, B. P., McDonagh, K. T., and Nienhuis, A. W. (1990) Genes Dev. 4, 993–1000
12. Moon, A. M., and Ley, T. J. (1991) Blood 77, 2272–2284
13. Chang, J. C., Liu, D., and Kan, Y. W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3107–3110
14. Gong, Q., and Dean, A. (1993) Mol. Cell. Biol. 13, 911–917
15. Andrews, N. C., Erdjument-Bromage, H., Davidson, M. B., Tempst, P., and Orkin, S. H. (1993) Nature 362, 722–728
16. Ney, P. A., Andrews, N. C., Jane, S. M., Saler, B., Parucher, M. E., Weremowicz, S., Morton, C. C., Goff, S. C., Orkin, S. H., and Nienhuis, A. W. (1993) Mol. Cell. Biol. 13, 5604–5612
17. Andrews, N. C., Kotkow, K. J., Ney, P. A., Erdjument-Bromage, H., Tempst, P., and Orkin, S. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11488–11492
18. Chan, J. Y., Han, X. L., and Kan, Y. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11371–11375
19. Caterina, J. J., Denze, D., Sun, C. W., Ciavatta, D. J., and Townes, T. M. (1994) Nucleic Acids Res. 22, 2383–2390
20. Moi, P., Chan, K., Asunis, I., Cao, A., and Kan, Y. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9926–9930
21. Kim, M., Liu, Z., and Zang, F. (1997) Curr. Opin. Cell Biol. 9, 240–246
22. Igarashi, K., Hoshino, H., Muto, A., Suwabe, N., Nishikawa, S., Nakauchi, H., and Yamamoto, M. (1998) J. Biol. Chem. 273, 11783–11790
23. Lo, S. J., Rowan, B., and Ben-David, Y. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8398–8402
24. Kotkow, K. J., and Orkin, S. H. (1995) Mol. Cell. Biol. 15, 4640–4647
25. Shivdasani, R. A., Rosenblatt, M. F., Zucker-Franklin, D., Jackson, C. W., Hunt, P., Sartis, C. J., and Orkin, S. H. (1995) Cell 81, 695–704
26. Bean, T. L., and Ney, P. A. (1997) Nucleic Acids Res. 25, 2559–2551
27. Amrolia, P. J., Ramamurthy, L., Saluja, D., Tanese, N., Jane, S. M., and Cunningham, J. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10051–10056
28. Mosser, E. A., Kasanov, J. D., Forsberg, E. C., Kay, E. B., Ney, P. A., and Bresnick, E. H. (1998) Biochemistry 37, 13686–13695
29. Garver, N. R., Garva, R., Ernkevich, K., Sudd, M., and Chen, C. K. J. (1997) J. Biol. Chem. 272, 24105–24108
30. Cheng, X., Reginato, M. J., Andrew, N. C., and Lazar, M. A. (1997) Mol. Cell. Biol. 17, 1407–1416
31. Pirezi, G., McConnell, S. J., Uveges, A. J., Carter, J. M., Sparks, A. B., Kay,
β-Globin LCR Coactivator

B. K., and Fowlkes, D. M. (1997) J. Biol. Chem. 272, 1411–1416

32. Webster, J. C., Jewell, C. M., Bodwell, J. E., Munck, A., Sar, M., and Cidlowski, J. A. (1997) J. Biol. Chem. 272, 9287–9293

33. Dean, A., Erard, F., Schneider, A. P., and Schechter, A. N. (1981) Science 212, 459–461

34. Marks, P. A., Sheffrey, M., and Rifkind, R. A. (1985) Prog. Clin. Biol. Res. 191, 183–203

35. Ericsson, J., and Edwards, P. A. (1998) J. Biol. Chem. 273, 17865–17870

36. Blobel, G. A., Nakajima, T., Eckner, R., Montminy, M., and Orkin, S. H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2061–2066

37. Chrivia, J. C., Kwok, R. S., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) Nature 365, 855–859

40. Fry, C. J., Slansky, J. E., and Farnham, P. J. (1997) Mol. Cell. Biol. 17, 1966–1976

41. Flint, J., and Tran, V. K., and Goodman, R. H. (1997) Recent Prog. Horm. Res. 52, 103–119

46. Forsberg, E. C., Lam, L. T., Yang, X. J., Nakatani, Y., and Bresnick, E. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 95, 8756–8760

50. Zhang, W., and Bieker, J. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5984–5989

51. Imhof, A., Yang, X. J., Ogryzko, V. V., Nakatani, Y., Wolfe, A. P., and Ge, H. (1997) Curr. Biol. 7, 689–692

52. Goldman, P. S., Tran, V. K., and Goodman, R. H. (1997) Mol. Cell. Biol. 3, 447–455

53. Krumm, A., Madisen, L., Yang, X. J., Goodman, R., Nakatani, Y., and Groudine, M. (1998) Mol. Cell 2, 447–455

55. Parekh, B. S., and Maniatis, T. (1999) Mol. Cell 3, 125–129

56. Kuo, M. H., Zhou, J., Jambeck, P., Churchill, M. E., and Allis, C. D. (1998) Genes Dev. 12, 627–639

57. Hung, H. L., Lau, J., Kim, A. Y., Weiss, M. J., and Blobel, G. A. (1999) Mol. Cell Biol. 19, 3496–3505