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A SWI/SNF–Related Chromatin Remodeling Complex, E-RC1, Is Required for Tissue-Specific Transcriptional Regulation by EKLF In Vitro

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Summary

Erythroid Krüppel-like factor (EKLF) is necessary for stage-specific expression of the human β-globin gene. We show that EKLF requires a SWI/SNF–related chromatin remodeling complex, *E*KLF coactivator-*r*emodeling *c*omplex *1* (E-RC1), to generate a DNase I hypersensitive, transcriptionally active β-globin promoter on chromatin templates in vitro. E-RC1 contains BRG1, BAF170, BAF155, and INI1 (BAF47) homologs of yeast SWI/SNF subunits, as well as a subunit unique to higher eukaryotes, BAF57, which is critical for chromatin remodeling and transcription with EKLF. E-RC1 displays functional selectivity toward transcription factors, since it cannot activate expression of chromatin-assembled HIV-1 templates with the E box–binding protein TFE-3. Thus, a member of the SWI/SNF family acts directly in transcriptional activation and may regulate subsets of genes by selectively interacting with specific DNA-binding proteins.

Introduction

Cell type–specific transcriptional activators and repressors play a critical role in controlling gene expression that ultimately results in tissue differentiation and specialization. In the cell, transcription factors must work within and around the confines of chromatin. The basic unit of chromatin is the nucleosome, 146 base pairs of DNA wrapped almost two turns around a histone octamer (Luger et al., 1997). The structure of chromatin itself provides one important level of gene regulation, as demonstrated by the observation that transcriptionally active genes are characterized by an open chromatin structure, whereas inactive genes are packaged in a highly condensed chromatin configuration (Weintraub and Groudine, 1976).

Several chromatin remodeling complexes have recently been described. These include SWI/SNF, RSC, NURF, CHRAC, ACF, and FACT (reviewed in Pazin and Kadonaga, 1997; Armstrong and Emerson, 1998; Cairns, 1998; Kadonaga, 1998). With the exception of FACT, all the complexes contain an ATPase activity required for nucleosome disruption or remodeling. Thus far, only ACF and NURF have been shown to function in concert with an activator to stimulate transcription from a chromatin-assembled template in vitro (Ito et al., 1997; Mizuguchi et al., 1997).

Among the chromatin remodeling complexes, yeast SWI/SNF was the first to be identified. Genetic studies revealed that SWI/SNF is required for the transcription of a subset of genes including *HO, SUC2, ADH1, ADH2, GAL1*, and *GAL10*. Suppressors of SWI/SNF mutations were identified as histones, indicating that the SWI/SNF complex functions to counteract the repressive effects of chromatin on transcription (reviewed in Winston and Carlson, 1992). Indeed, both yeast and human SWI/SNF complexes function in vitro to disrupt chromatin structure and facilitate factor binding (Côté et al., 1994; Imbalzano et al., 1994; Kwon et al., 1994). Multiple mammalian SWI/SNF complexes (BAF complexes) have been identified and shown to be diverse in subunit composition (Wang et al., 1996a, 1996b, 1998). The mammalian SWI/SNF (BAF) complexes contain either BRG1 or human brahma (hbrm). These ATPases are homologous to the yeast protein SWI2/SNF2 (Khavari et al., 1993; Muchardt and Yaniv, 1993). BAF155 (BRG1-associated factor, 155 kDa) and BAF170 are both homologs of yeast SWI3. Interestingly, subunits unique to SWI/SNF–like complexes of higher eukaryotes are now being identified. For example, BAF57 is found in mammalian complexes (Wang et al., 1998), and its counterpart, BAP111, was identified in the *Drosophila* SWI/SNF–related complex (Papoulas et al., 1998). These subunits both contain an HMG-like domain, and no such subunits are found in the yeast complexes.

To examine how chromatin structure is modified to allow transcription of a tissue-specific, developmentally regulated gene, we have chosen the human β-globin promoter as a model system. The β-globin locus is well characterized, consisting of an upstream locus control region (LCR) followed by five genes (5′-ε, Gγ, Aγ, δ, β-3′) arranged in the order of their developmental expression. The open chromatin configuration of the β-globin locus in expressing erythroid cells is DNase I sensitive with active promoters showing hypersensitivity (Groudine et al., 1983). DNA-binding sites for erythroid-restricted transcription factors are concentrated in the LCR and individual gene promoters and enhancers. These protein-binding motifs include sites for the erythroid factors GATA-1, NF-E2, and EKLF (reviewed in Baron, 1997). Of these factors, EKLF is a key regulator of β-globin expression. Transgenic mice that lack EKLF die of anemia at the developmental stage when the adult form of β-globin is normally expressed (Nuez et al., 1995; Perkins et al., 1995). In these mice, the β-globin promoter remains in a closed chromatin configuration (no hypersensitivity), yet expression of early globin genes is normal, indicating that EKLF is a true stage-specific regulator of the adult β-globin gene (Wijgerde et al., 1996).

EKLF expression is restricted to the normal sites of hematopoiesis. It contains a proline-rich activation domain at the amino terminus and three TFIIIA-like zinc fingers within the carboxyl terminus (Miller and Bieker,

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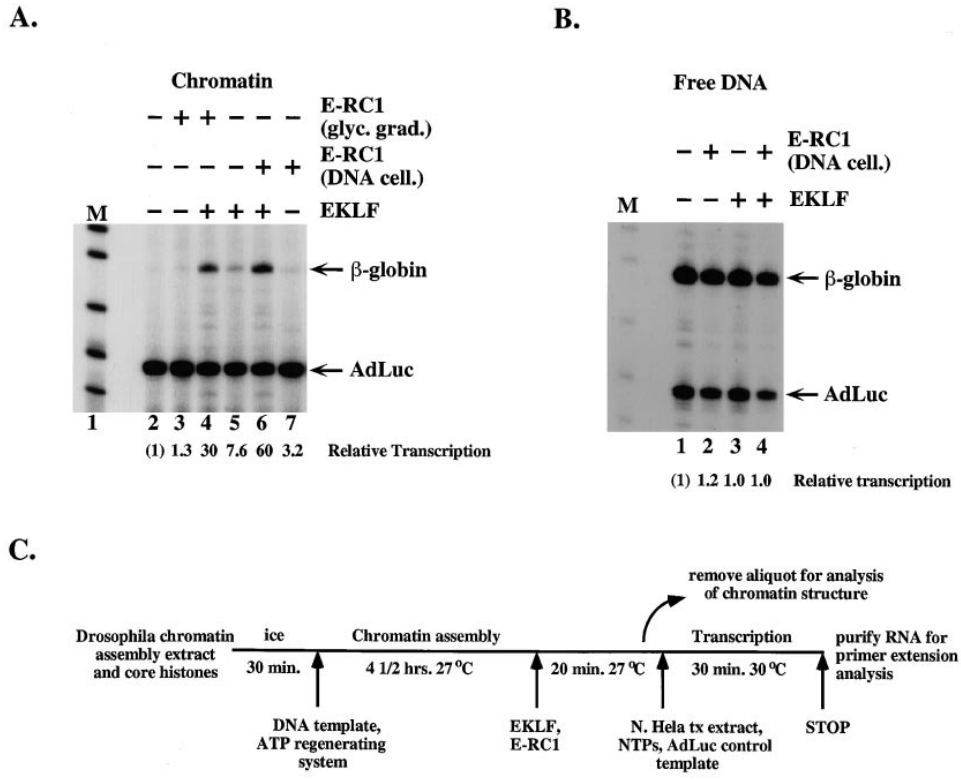

Figure 1. Transcriptional Activation of Chromatin-Assembled β-Globin Template Requires Both EKLF and a Coactivator, E-RC1

(A) Transcription of chromatin-reconstituted β-globin template. Glycerol gradient fraction or DNA–cellulose fraction containing the EKLF coactivator E-RC1 and recombinant purified EKLF protein were added to 1 μg chromatin as indicated. Relative fold activation is shown below each lane. Quantitation was performed using a Molecular Dynamics phosphorimager. Fold transcription activation was determined by subtracting a background value for each lane from both β-globin and AdLuc signals. The control AdLuc signal for lane 2 was set at one and the relative AdLuc values were divided into the appropriate β-globin signal. Repressed β-globin transcription (lane 2) was then set at one. Primer extension products are marked by arrows. M, MspI-digested pBR322 as a molecular weight marker.

(B) EKLF and the coactivator E-RC1 have no effect on transcription of free DNA. β-globin template (250 ng) was incubated with 2.5 μl DNA–cellulose fraction containing E-RC1 and/or 9.3 pmol EKLF, as indicated, and transcribed as described for the chromatin templates in Experimental Procedures. Relative fold transcriptional activation is shown under each lane. M, MspI-digested pBR322 as a molecular weight marker.

(C) Experimental flowchart. See Experimental Procedures for detailed protocols.

1993). EKLF was the founding member of the Krüppel-like factor family, named as such because the zinc fingers possess a high degree of similarity to the *Drosophila* gap gene *Krüppel*. Other members of this new family include LKLF (lung Krüppel-like factor), found predominantly in lung and spleen (Anderson et al., 1995); BKLF (basic Krüppel-like factor), highly expressed in the mouse embryonic midbrain (Crossley et al., 1996); and GKLF (gut-enriched Krüppel-like factor), an epithelial-specific factor associated with growth arrest (Shields et al., 1996). EKLF regulates the human β-globin promoter through the −90 CACCC sequence. Naturally occurring mutations within this CACCC box result in β-thalassemias in humans (Orkin et al., 1982; Kulozik et al., 1991), caused by reduced levels of β-globin expression due to decreased DNA binding and transcriptional activation by EKLF (Feng et al., 1994). Thus, EKLF is unquestionably critical for stage-specific expression of the β-globin gene (Donze et al., 1995).

To understand the mechanism of action of this key erythroid regulator, we have reproduced EKLF-mediated transcriptional activation of the β-globin promoter using an in vitro chromatin assembly system. We show that EKLF requires a coactivator to form a transcriptionally competent β-globin promoter by structurally remodeling repressed chromatin in an ATP-dependent manner. We identify the coactivator E-RC1 as a member of the mammalian SWI/SNF family of chromatin remodeling complexes. Although E-RC1 has general chromatin disruption activity, it is unable to activate transcription in combination with another DNA-binding protein, TFE-3, on the chromatin-assembled human immunodeficiency virus-1 (HIV-1) promoter. Thus, E-RC1 displays selectivity toward EKLF and demonstrates potential functional diversity among members of the mammalian SWI/SNF family.

Results

Transcription from the Chromatin-Reconstituted Human β-Globin Promoter Depends upon EKLF and a Coactivator, E-RC1

Plasmids containing the intact β-globin promoter from −385 to +18, linked to the *CAT* gene, were assembled

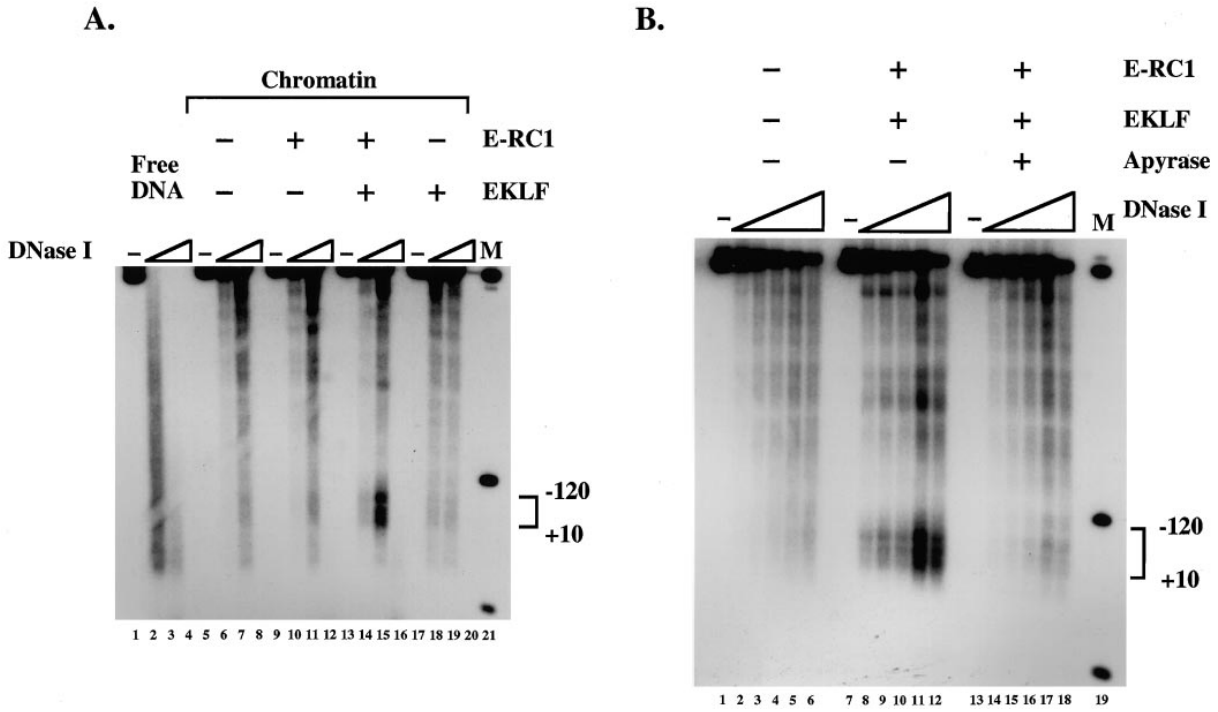

Figure 2. The Chromatin Structure of the β-Globin Promoter Is Disrupted Prior to Transcription and This Structural Change Is ATP Dependent

(A) Hypersensitive site formation occurs only in the presence of both EKLF and E-RC1. After assembly, chromatin was incubated with DNA–cellulose E-RC1 fraction and/or EKLF as indicated. The chromatin reactions were then split, and one-half was transcribed as a control (data not shown). Remaining chromatin was divided into three tubes (100 ng chromatin per tube) and digested with DNase I for 1 min at 27° C. Triangles indicate increasing amounts of DNase I. Free DNA was digested with 0, 0.0015, 0.0045, or 0.0135 U DNase I (3.1 U/μg). Chromatin reactions were digested with 0, 0.3, 0.9, or 2.7 U DNase I. Following purification, DNA was digested with NcoI. The indirect end-labeling procedure is described in Experimental Procedures. Brackets indicate the promoter region from −120 to +10. M, β-globin plasmid digestions used as molecular weight markers. Bands represent digests of NcoI (4.7 kb), NcoI/BsaAI (841 bp), and NcoI/EcoRI (301 bp).

(B) The DNase I hypersensitive site is abolished in the presence of apyrase. After assembly, 1 μg chromatin was incubated with either buffer or 0.2 U apyrase as indicated at 27°C for 10 min. Reactions were then incubated with DNA–cellulose E-RC1 fraction and/or EKLF as indicated. Chromatin was divided into six tubes with 150 ng chromatin per tube and digested with 0, 0.9, 1.2, 1.5, 1.8, or 2.1 U DNase I. Triangles indicate increasing amounts of enzyme. Brackets show −120 to +10 of the promoter region. M, molecular weight markers as described in Figure legend 2A.

into chromatin using *Drosophila* embryonic extracts (Bulger and Kadonaga, 1994). As expected, chromatin-assembled templates are transcriptionally repressed (Figure 1A, lane 2). We initially examined whether binding of the erythroid-specific transcriptional activator EKLF to the −90 CACCC box in the human β-globin promoter could generate an open chromatin structure and allow transcription to occur. These experiments indicated that EKLF alone could not efficiently activate β-globin expression (lane 5), which led us to search for a possible coactivator of EKLF function. Chromatographic fractionation of protein extracts from mouse erythroleukemia (MEL) cells, which represent the definitive erythroid lineage and express high levels of endogenous β-globin genes, resulted in the purification of an activity, *E*KLF coactivator-*r*emodeling *c*omplex *1* (please see Figure 4C for an SDS-PAGE analysis of purified E-RC1). This complex functioned with EKLF to activate chromatin-assembled β-globin promoters (lanes 4 and 6), yet was insufficient by itself (lanes 3 and 7). Additionally, neither EKLF nor E-RC1 could individually activate transcription when bound to the DNA template prior to chromatin formation (data not shown).

The reconstituted chromatin templates used in these experiments contain evenly spaced nucleosomes, as determined by micrococcal nuclease analysis, and the bulk chromatin structure is unaffected by the addition of EKLF and E-RC1 (data not shown). EKLF and E-RC1 are added after nucleosome assembly is complete (as diagrammed in Figure 1C), suggesting that an active chromatin remodeling process is occurring. Moreover, EKLF and E-RC1 are unable to stimulate transcription of free DNA (Figure 1B), indicating that they function specifically through chromatin structure.

Chromatin Structural Remodeling of the β-Globin Promoter Requires Both EKLF and E-RC1

To examine the nucleosomal structure of in vitro–assembled β-globin promoters, indirect end-labeling analysis of DNase I digested chromatin was performed. Importantly, these digestions were carried out prior to transcription and thus reflect potentially active, rather than transcribing, promoters. A DNase I hypersensitive region from approximately −120 to +10 in the β-globin promoter was observed in the presence of both EKLF and E-RC1 (Figure 2A, lane 15). This pattern resembles

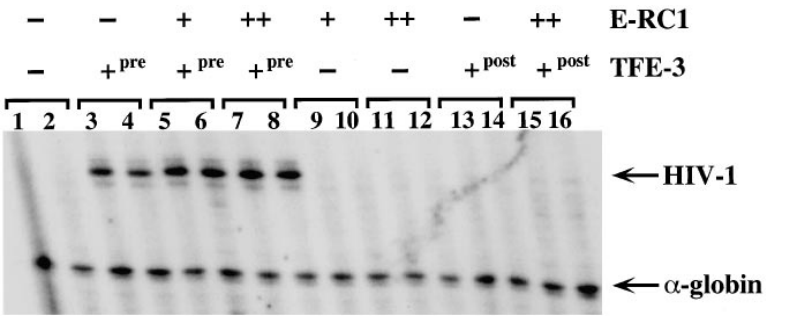

Figure 3. E-RC1 Cannot Function with TFE-3 to Activate Transcription of Chromatin-Assembled HIV-1 Templates

Transcriptional activation of chromatin-reconstituted HIV-1 template. The plasmid template was assembled into chromatin in the presence (lanes 3–8) or absence (lanes 1, 2, 9–16) of purified TFE-3. Following assembly, the DNA–cellulose fraction of E-RC1 (2.5 μl, lanes 5 and 6 and 9 and 10; or 5 μl, lanes 7 and 8, 11 and 12, 15 and 16) and TFE-3 (lanes 13–16) were added to 100 ng of HIV-1 template and incubated for 10 min at room temperature. Transcription proceeded following the addition of a nuclear HeLa extract and incubation at 30°C for 30 min. Primer extension products of the HIV-1 promoter and the α-globin internal control are indicated by arrows.

the hypersensitive structure of the active β-globin promoter in vivo which maps from 100–150 bp 5′ of the cap site (Groudine et al., 1983). Neither EKLF (lanes 17–19) nor E-RC1 (lanes 10–12) were sufficient alone to generate an open chromatin configuration. Since most chromatin remodeling events are energy-dependent, we asked whether hypersensitive site formation in the β-globin promoter was dependent on ATP. Chromatin templates were incubated with the ATP-hydrolyzing reagent apyrase prior to addition of EKLF and E-RC1. Indeed, formation of the open β-globin promoter structure by these proteins requires ATP (Figure 2B, compare lanes 8–12 to 14–18).

E-RC1 Displays Functional Selectivity for EKLF

To analyze the specificity of E-RC1 as a coactivator of EKLF, we examined whether it could function with another protein, TFE-3, to facilitate transcription from the HIV-1 promoter. TFE-3 is a member of the helix-loop-helix family of DNA-binding proteins and interacts with an E box motif at −177 within the HIV-1 enhancer. Recombinant TFE-3 and other HIV-1 enhancer–binding proteins have been shown to activate efficiently transcription of a chromatin-assembled HIV-1 template in vitro (Sheridan et al., 1995). As shown in Figure 3, prebinding of TFE-3 alone to HIV-1 DNA followed by chromatin assembly with *Drosophila* embryo extracts generates a structure that is active for transcription in vitro (lanes 3 and 4) relative to chromatin-repressed templates lacking prebound TFE-3 (lanes 1 and 2). Addition of E-RC1 to TFE-3-bound HIV-1 chromatin failed to appreciably increase transcription (lanes 5–8). When E-RC1 was analyzed for its ability to facilitate TFE-3 binding to assembled chromatin (postbinding), as β-globin activation by EKLF is normally assayed, no transcription occurred with (lanes 15 and 16) or without E-RC1 (lanes 13 and 14). Thus, E-RC1 displays functional selectivity for EKLF, as it is not a coactivator of TFE-3. These results indicate that E-RC1 does not function generally to facilitate the interaction of DNA-binding proteins with chromatin templates to enable transcriptional activation. Instead, E-RC1 shows selectivity in the factors and genes that it regulates. Although it is unlikely that E-RC1 is a coactivator only of EKLF, the data suggest that its function may be restricted to a specific class of transcriptional activators.

The EKLF Coactivator E-RC1 Is Identified as a Member of the Mammalian SWI/SNF Family of Chromatin Remodeling Complexes

E-RC1 was purified according to the scheme shown in Figure 4A. By glycerol gradient sedimentation, E-RC1 was identified as a large 18.7S multiprotein complex that cofractionates with known mammalian SWI/SNF subunits. When fractions across the 20%–35% glycerol gradient were assayed, transcriptional activity peaked in fractions 4 and 5 (Figure 4B, lanes 7 and 8), as did DNase I hypersensitive site formation (data not shown). An SDS-PAGE analysis shows the purity and protein subunit composition of these fractions (Figure 4C). Western blot analyses using antibodies to mammalian SWI/SNF subunits were conducted on the same glycerol gradient fractions (Figure 4D). The subunits BAF155, BAF57, and BRG1 all comigrate with the transcriptional activity in fractions 4 and 5. Moreover, BAF170 and INI1 (BAF47) proteins also peak in these fractions (data not shown). Interestingly, BRG1 protein is found in a second peak (fractions 7 and 8) that does not support β-globin transcription, suggesting that other BRG1-containing complexes with different functions may exist. E-RC1 cofractionated with the core SWI/SNF subunits BAF57, BAF155, and BRG1 through each of the three column steps (data not shown) and may represent a major SWI/SNF activity in MEL cells. A small amount of RNA polymerase II was present in the DNA–cellulose fraction, as detected by Western blot analysis using an antibody to the CTD. However, after the glycerol gradient step, RNA polymerase II was only detectable in fraction 8 and, thus, did not comigrate with the EKLF-dependent E-RC1 coactivator (data not shown). All antibodies to mammalian SWI/SNF subunits tested so far have comigrated with this transcriptional activity. Although E-RC1 was originally purified from erythroid cells, it may not be tissue-restricted. A partially purified E-RC1 fraction from HeLa cells also displays EKLF coactivator activity.

To determine whether purified E-RC1 displays classical chromatin remodeling activity, a mononucleosomal DNase I footprinting assay was performed (Figure 5A). A rotationally positioned nucleosome was assembled by salt dialysis onto a DNA fragment containing a single copy of the sea urchin 5S nucleosome-positioning sequence. When this positioned nucleosome was digested with DNase I, a 10 bp repeat ladder was produced (lane 3). In the presence of ATP, E-RC1 was able to remodel

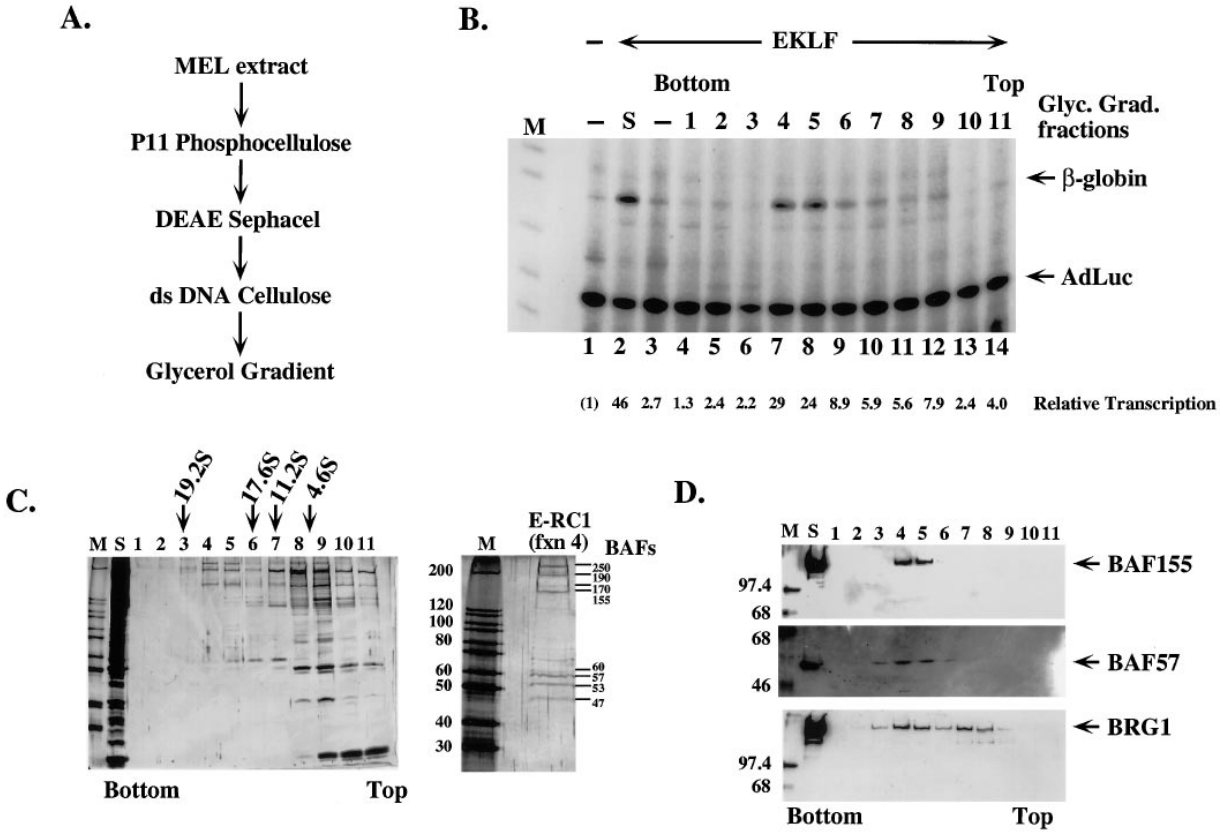

Figure 4. The EKLF Coactivator E-RC1 Is Identified as a Mammalian SWI/SNF-Related Complex

(A) Diagram of coactivator purification. See Experimental Procedures for details.

(B) Transcriptional activation of chromatin reconstituted β-globin template with EKLF and glycerol gradient fractions of E-RC1. A 13 ml gradient was divided into 11 fractions from 1 (bottom) to 11 (top). Fifty microliters of each fraction and EKLF protein was added to 1 μg chromatin. Reactions were then split, and half was DNase I digested and analyzed for hypersensitivity by indirect end-labeling analysis (data not shown). The remaining chromatin was transcribed. M, MspI-digested pBR322 as a molecular weight marker. Relative fold transcriptional activation is given under each lane.

(C) SDS-PAGE analysis of E-RC1 glycerol gradient fractions. Ten microliters of each fraction was analyzed by 7.5% SDS-PAGE and visualized by silver staining. S, DNA–cellulose fraction that was the starting material for glycerol gradients. M, 10 kDa molecular weight markers (GIBCO). Sedimentation values for standards are shown: thyroglobulin (19.2S), apoferritin (17.6S), β-amylase (11.2S), and bovine serum albumin (4.6S). The S value for the active complex was calculated from three separate glycerol gradients; the average was 18.7S.

(D) Western blot analysis of glycerol gradient fractions reveals that E-RC1-mediated transcriptional activity comigrates with SWI/SNF subunits. Ten microliters of each glycerol gradient fraction was electrophoresed on a 7.5% SDS-PAGE and the subunit composition determined by Western blot analysis. S, 90 mM DNA–cellulose fraction that was the starting material for the gradient. M, ECL markers, sizes indicated.

and disrupt the nucleosome to produce a DNase I digestion pattern intermediate between that of a nucleosome and free DNA (lanes 7 and 8). In the absence of ATP, no nucleosome disruption by E-RC1 was observed (lanes 4 and 5). This DNase I pattern is consistent with that generated by other SWI/SNF–related complexes purified from both yeast (Côté et al., 1994) and HeLa cells (Kwon et al., 1994).

To confirm the role of one of the SWI/SNF subunits in transcription, immunoneutralizations with an antibody to the HMG domain–containing BAF57 SWI/SNF subunit were conducted. As shown in Figure 5B, anti-BAF57 antibody specifically interfered with transcriptional activation, neutralizing 73% of β-globin promoter activity but not that of a control template (AdLuc) (compare lanes 3 and 4). As controls, preimmune sera and anti-Rsc6 had no effect (compare lane 4 with 5 and 6). Thus, a key SWI/SNF subunit of E-RC1 plays a functional role in EKLF-dependent β-globin transcription.

Immunoprecipitations from E-RC1 chromatographic fractions using antibodies to BAF155 and BAF57 were carried out to further confirm that E-RC1 is a member of the mammalian SWI/SNF family. SWI/SNF complexes cannot be eluted intact from antibody beads, but they have been shown to retain activity while on the beads after thorough washing. For example, mammalian SWI/SNF complexes on beads are able to disrupt mononucleosomes and facilitate factor binding (Wang et al., 1996a). As shown in Figure 5C, both BAF57 and BAF155 immunoprecipitates were active for DNase I hypersensitive site formation in the presence of EKLF (lanes 5 and 17, respectively). A mock immunoprecipitate shows limited activity (lane 7). From this data, we conclude that mammalian SWI/SNF immunoprecipitates from E-RC1

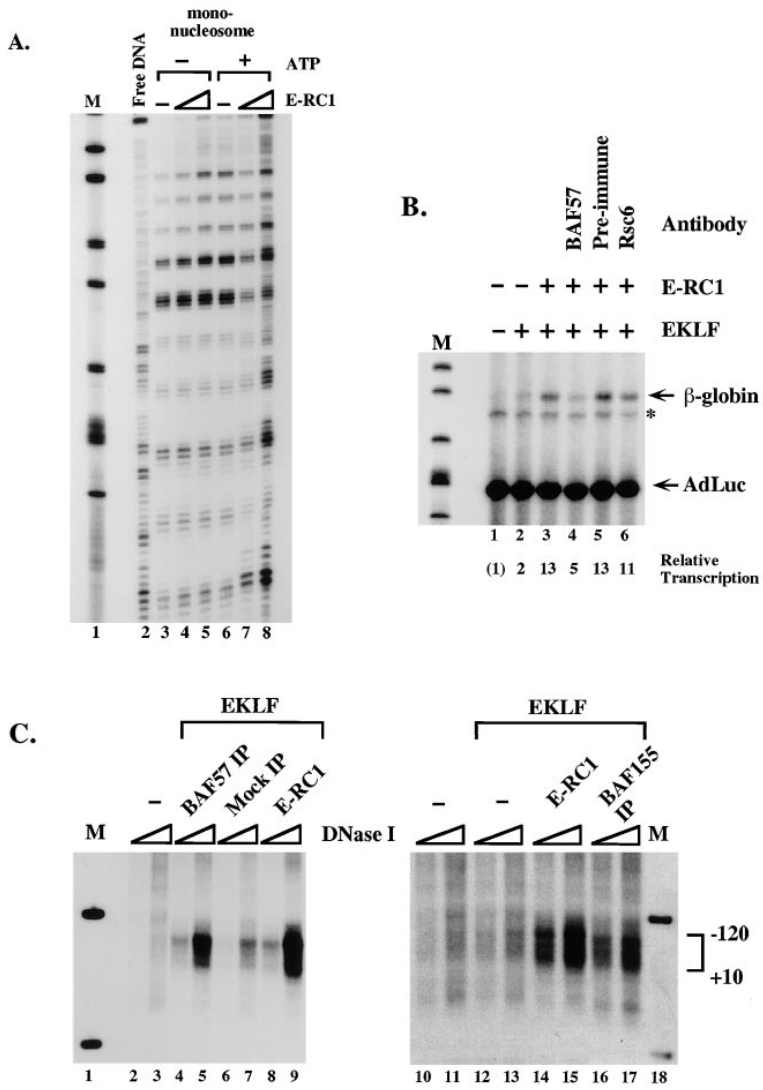

Figure 5. Characterization of Purified E-RC1 and Confirmation of the Role of SWI/SNF Subunits in β-Globin Transcription

(A) Purified E-RC1 possesses classical chromatin remodeling activity. DNase I footprinting of 5S DNA reconstituted by salt dialysis into a mononucleosome. Following salt dialysis and sucrose gradient purification, mononucleosomes were incubated with E-RC1 in either the presence or absence of 1 mM ATP for 20 min at 27°C. Free DNA was digested with 0.005 U DNase I and mononucleosomes with 0.15 U DNase I per reaction. Digestions took place at 27°C for 1 min. Triangles indicate increasing amounts of E-RC1 (glycerol gradient fraction 4) of 1 μl or 2.5 μl. M, MspI-digested pBR322 as a molecular weight marker.

(B) Immunoneutralizations confirm that BAF57 is an integral component of E-RC1. DNA–cellulose E-RC1 fraction was incubated with anti-sera for 30 min on ice prior to addition to 0.5 μg chromatin template. Relative fold transcriptional activation is shown under each lane. Asterisk indicates nonspecific band. M, MspI-digested pBR322 as a molecular weight marker.

(C) Immunoprecipitations with anti-BAF57 and BAF155 antibodies pull down a complex sufficient to form a DNase I hypersensitive site with EKLF in the β-globin promoter. E-RC1 (DEAE fraction) was used as a source for either BAF155 or BAF57 immunoprecipitations. The complexes bound to protein A beads were washed extensively as described in Experimental Procedures and then added with EKLF to 0.5 μg chromatin template.

fractions are able to function with the erythroid protein EKLF to alter the chromatin structure of the β-globin promoter. Thus, E-RC1 is a true member of the mammalian SWI/SNF group.

By Western blot analysis, we were able to compare BAF57 levels in E-RC1 preparations to a titration of bacterially expressed BAF57 of a known concentration (data not shown). One subunit of BAF57 is present in SWI/SNF purified from Jurkat T cells that express an HA-tagged BAF57 (Wang et al., 1998). For the purpose of these calculations, we are assuming the same is true of E-RC1. When the DNA–cellulose fraction is used as a source of E-RC1, one complex is present for every template—or, approximately, 1 E-RC1 per 27 nucleosomes. Glycerol gradient–purified E-RC1 is used at a ratio of 1 complex for every 49 nucleosomes, with comparable results for both transcription (Figure 1A) and hypersensitive site formation (data not shown).

To determine whether or not other members of the SWI/SNF (BAF) group of chromatin remodeling complexes could replace E-RC1, we obtained SWI/SNF purified from Jurkat T cells, expressing an HA-tagged BAF57 polypeptide (Wang et al., 1998). The activity of this complex was measured by a DNase I footprinting analysis of a positioned mononucleosome, as in Figure 5A. This particular SWI/SNF complex cannot substitute for E-RC1 in either EKLF-dependent β-globin transcriptional activation or in DNase I hypersensitive site formation in the β-globin promoter when used at ratios of up to 1 complex per 40 nucleosomes (data not shown). When E-RC1 and T cell–derived SWI/SNF are compared on an SDS-PAGE, their subunit compositions appear almost identical, with some differences in minor bands (Figure 6). With the exception of the few nonspecific bands in the Jurkat SWI/SNF preparation (see Figure 5a; Wang et al., 1998), all of the major mammalian SWI/SNF subunits comigrate with the major polypeptides in purified E-RC1. Although E-RC1 and the T cell–derived SWI/SNF appear to be functionally distinct, it is important to note that

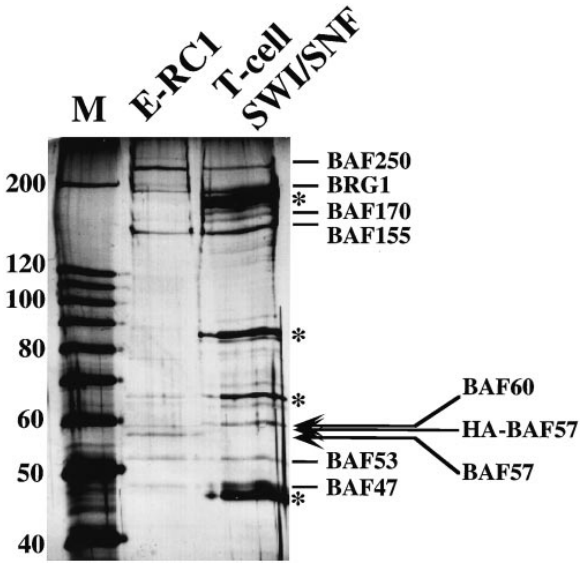

Figure 6. SDS-PAGE Analysis Comparing Purified E-RC1 with Jurkat T Cell SWI/SNF

Equal amounts of each complex were electrophoresed on a 7.5% SDS-PAGE and visualized by silver staining. M, 10 kDa protein molecular weight markers (GIBCO) with sizes indicated. BAF subunits are identified at the right. Nonspecific bands resulting from HA-immunopurification of T cell SWI/SNF are indicated by asterisks.

other SWI/SNF family members may share the same characteristics as E-RC1, and the ability to activate transcription by EKLF may be a general property of SWI/SNF complexes.

E-RC1-Dependent β-Globin Promoter Activation Requires an Intact −90 EKLF-Binding Site and EKLF Activation Domain

Many β-thalessemias are caused by mutations within the β-globin locus. One such point mutation occurs within the −90 EKLF-binding site of the β-globin promoter at −87 (C to G) (Orkin et al., 1982), which effectively eliminates EKLF binding and transactivation (Feng et al., 1994). The reduction of β-globin expression seen in β-thalessemia patients has been reproduced in our in vitro system using chromatin-reconstituted templates. As shown in Figure 7A, the chromatin-assembled mutant β-globin promoter is not competent for transcriptional activation by EKLF and E-RC1 (compare lanes 5 and 6 with 13 and 14). However, both wild-type and mutant promoters transcribe with equal efficiency as free DNA (Figure 7B). A DNase I footprint analysis confirms that EKLF is unable to bind the mutant site (Figure 7C, compare lanes 2 and 3 with 5 and 6). Therefore, EKLF must bind to its site at −90 to function as an activator of a chromatin-assembled promoter, and its regulatory effects are more pronounced in a chromatin environment than as free DNA.

EKLF contains an N-terminal proline-rich activation region, with the minimal activation domain extending from amino acids 20 to 124. This domain is essential for transactivation in transient transfections (Bieker and Southwood, 1995). Two deletion mutants, Δ20–60 and Δ60–196, that remove portions of this core domain are unable to activate transcription in either transient transfections (Chen and Bieker, 1996) or in our in vitro system (Figure 8A, compare lane 3 with 4 and 5). Both protein mutants are able to bind DNA with equal or greater efficiency than wild-type protein in a DNase I footprinting analysis of free DNA (Figure 8B). Thus, our E-RC1-dependent in vitro chromatin transcription assay reflects genuine transactivation by EKLF.

Although the activation domain of EKLF is required for transcription of a chromatin-assembled template (Figure 8A), it is not needed to generate an open promoter conformation. As shown in Figure 8C, both protein deletion mutants are capable of forming a DNase I hypersensitive region (lanes 7–12) that is indistinguishable from that generated by wild-type protein (lanes 4–6). As observed with intact EKLF, the deletion mutants are dependent upon the presence of E-RC1 for hypersensitive site formation, as they cannot function alone (data not shown). Thus, the activation domain of EKLF must function at a step subsequent to the generation of an open chromatin structure at the promoter. The β-thalassemia −87 point mutant promoter, which is not bound by EKLF, was also assayed for hypersensitive site formation. These studies indicate that the mutant β-globin promoter is transcriptionally inert as a result of a closed chromatin conformation that is shown by a reduction in DNase I hypersensitivity (Figure 8C, lanes 16–18). From these data, we conclude that EKLF binding, in combination with E-RC1, is necessary for an open chromatin configuration within the β-globin promoter.

Discussion

We have reproduced stage-specific activation of a chromatin-assembled human β-globin promoter in vitro. We find that chromatin remodeling and transcriptional activity are dependent upon both the erythroid Krüppel-like factor (EKLF) and a SWI/SNF complex, E-RC1. This demonstrates that a member of the mammalian SWI/SNF (BAF) family of complexes can regulate transcription of a chromatin template in vitro. Furthermore, our analyses provide insight into the specific mechanisms by which the critical erythroid factor EKLF functions within chromatin.

Possible Mechanism of Action of SWI/SNF–Related E-RC1

Our studies reveal that EKLF is dependent upon E-RC1 for the formation of an open, poised promoter. Several potential mechanisms of action exist. EKLF may first bind its site within the promoter and identify nearby nucleosomes as a target for E-RC1 activity. Yeast SWI/SNF has been shown to act transiently to disrupt a nucleosome marked by GAL4–AH binding (Owen-Hughes et al., 1996). E-RC1 may also function to promote EKLF nucleosomal interaction, since yeast SWI/SNF enhances nucleosomal binding of several transcription factors, including GAL4 derivatives (Côté et al., 1994; Kwon et

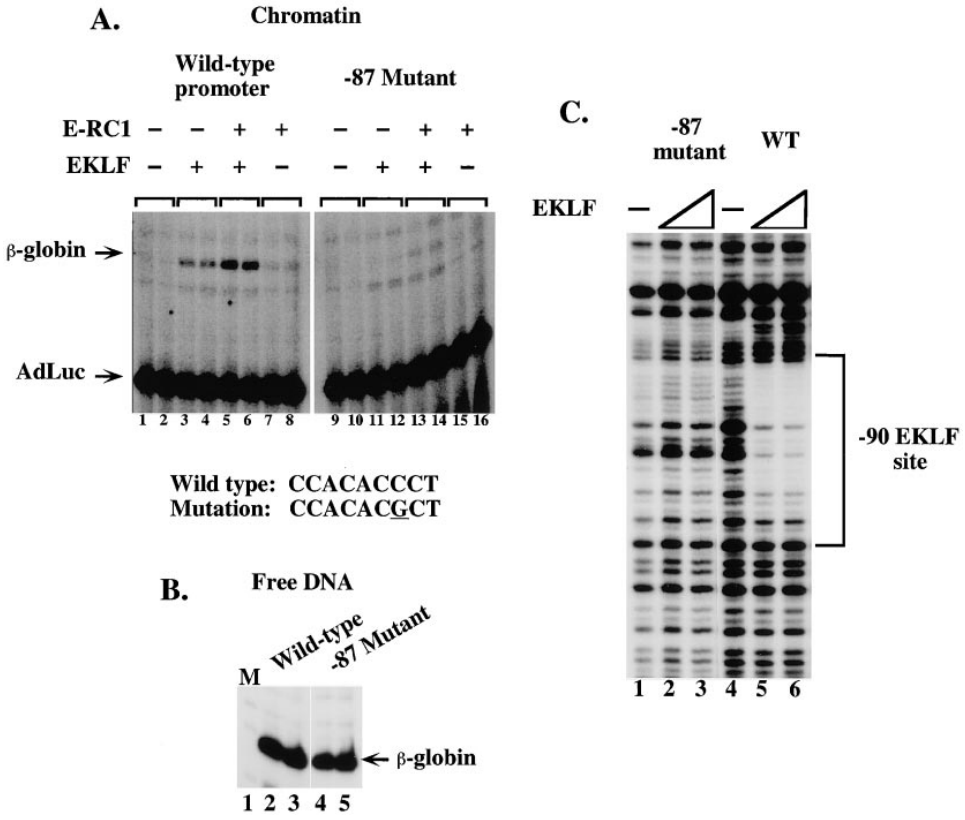

Figure 7. A β-Globin Promoter Containing a Naturally Occurring β-Thalassemia Point Mutation in the −90 EKLF Site Is Not Transcriptionally Activated by EKLF and E-RC1

(A) Transcription of chromatin-assembled β-globin templates. One microgram template, containing either wild-type or mutant promoter, was incubated with E-RC1 (DEAE fraction) and/or EKLF protein as indicated. In this case, E-RC1 and EKLF protein were incubated with the template throughout chromatin assembly; the results are not affected as a result of the change in the time of factor addition. The sequence of the wild-type and mutant EKLF sites are shown.

(B) The EKLF mutant β-thalessemia promoter is readily transcribed as free DNA. One microgram template was carried through a mock chromatin-assembly protocol, with buffers substituting for the chromatin-assembly extract and histones. M, MspI-digested pBR322 as a molecular weight marker.

(C) DNase I footprint analysis of wild type and mutant β-globin promoters. DNA fragments spanning −170 to −3, from either the wild-type or −87 point mutant promoter, were generated by PCR and footprinted with 7.5 pmol or 15 pmol EKLF as indicated by triangles. The EKLF DNA-binding site is marked by brackets.

al., 1994), TATA-binding protein (TBP) (Imbalzano et al., 1994), Sp1, USF, and NF-KB (Utley et al., 1997).

Studies both in vitro and in vivo suggest that SWI/SNF functions to assist transcription factors that have difficulty accessing their binding sites. In vitro, multimerized factor binding sites enhanced nucleosomal interaction by a transcriptional activator and lowered the stimulatory effect of SWI/SNF (Utley et al., 1997). In vivo, GAL4 is dependent upon SWI/SNF to function at low-, but not high-, affinity GAL4 sites (Burns and Peterson, 1997). In the case of the human β-globin promoter, the initial DNase I resistant structure indicated that a majority of templates are likely to have a nucleosome located at the −90 EKLF site. E-RC1 may enhance EKLF binding to its nucleosomal site in addition to promoting nucleosome perturbation. Many transcription factors can be prebound to templates prior to chromatin formation. In this way, they presumably bypass the requirement of any complexes that would facilitate their binding to a nucleosomal site. However, EKLF cannot activate transcription or remodel chromatin in the absence of E-RC1 even when prebound to the template (data not shown), suggesting that E-RC1 is functioning at a step subsequent to factor binding.

Further studies are necessary to address whether or not EKLF directly interacts with E-RC1 to recruit the complex to the β-globin promoter. A preliminary analysis of EKLF deletion mutants indicates that the C-terminal zinc fingers are a potential site for interaction (data not shown). SWI/SNF increases transcriptional activation by the glucocorticoid receptor and the estrogen receptor when assayed in yeast, and SWI3 is coimmunoprecipitated with a 150-amino-acid fragment centered on the zinc finger region of the glucocorticoid receptor (Yoshinaga et al., 1992).

Transcriptional Activation of the β-Globin Promoter

Once the promoter resides in an open chromatin configuration, transcriptional activation can occur. Although the EKLF activation domain does not play a role in hypersensitive site formation, it is critical at this stage. Our

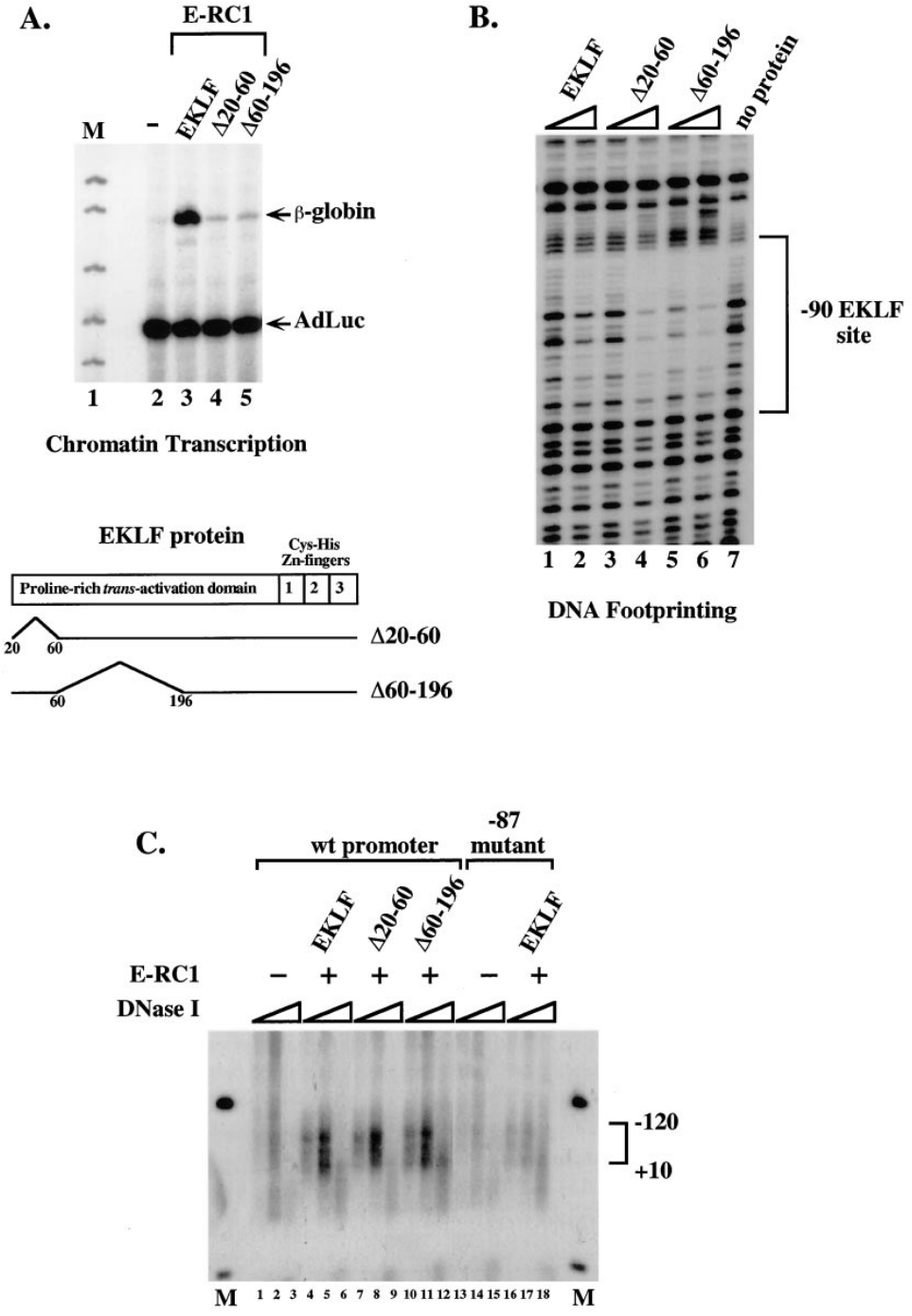

Figure 8. The Proline-Rich Activation Domain of EKLF Is Necessary for Transcriptional Activation

(A) Transcription of chromatin-assembled β-globin template is only observed with a complete EKLF activation domain. Chromatin was incubated with DNA–cellulose E-RC1 fraction and either wild-type or mutant EKLF protein as indicated. M, MspI-digested pBR322 as a molecular weight marker. EKLF protein domains are shown below.

(B) DNA footprint of β-globin promoter with EKLF mutants lacking the activation domain. PCR-generated fragment from −170 to −3 of the β-globin promoter was incubated with 3 pmol protein or 7.5 pmol protein as indicated by triangles. The EKLF site is shown by brackets.

(C) DNase I hypersensitive site formation within the β-globin promoter is dependent on an intact −90 CACCC element and is independent of an EKLF activation domain. Indirect end-labeling of DNase I digested chromatin. Chromatin templates containing either the wild-type promoter (lanes 1–12) or the −87 point mutant (lanes 13–21) were incubated with DNA–cellulose E-RC1 fraction and wild-type or mutant EKLF protein as shown. Reactions were then split, and half was transcribed as a control (data not shown). The remaining chromatin was divided into three tubes with 150 ng chromatin per tube and digested with 1.5, 2, or 3 U of DNase I. Triangles indicate increasing amounts of DNase I. Brackets show the −120 to +10 region of the promoter. M, digested β-globin plasmid as a size marker.

results are in agreement with previous studies that indicated that the proline-rich activation domain is required for erythroid cell–specific activation of the β-globin gene (Bieker and Southwood, 1995). The proline-rich domain of the transcriptional activator CTF1 has been shown to bind TFIIB and facilitate its recruitment to a TBP–DNA complex (Kim and Roeder, 1994). Likewise, the activation domain of EKLF may target a component of the RNA polymerase II machinery. Our findings are consistent with studies of GAL4–VP16 (Pazin et al., 1994) and the thyroid hormone receptor (Wong et al., 1997) that maintained that disruption of the chromatin structure of a promoter may occur in the absence of an activation domain but is not, in itself, sufficient to allow transcription. Gene activation in the context of chromatin has recently been reviewed (Armstrong and Emerson, 1998; Cairns, 1998; Kadonaga, 1998).

Functional Selectivity of Chromatin Remodeling Complexes

A fascinating issue that will no doubt be the focus of much study in the future is, how prevalent is functional selectivity among chromatin remodeling complexes? Mammalian SWI/SNF family members are more diverse and heterogeneous than the yeast version, suggesting that different complexes may play distinct roles in the cell (Wang et al., 1996a, 1996b). E-RC1 shows selectivity by facilitating EKLF-dependent β-globin transcription but not TFE-3-dependent HIV-1 expression, even though it remodels nucleosomes nonspecifically (Figure 5A). Other members of the SWI/SNF family may also share this property and the ability to function with EKLF. Selectivity of transcription factor interaction may, in fact, explain why yeast SWI/SNF regulates only a subset of genes in vivo (reviewed in Winston and Carlson, 1992).

Multiple chromatin modifying activities are also present in the *Drosophila* assembly extracts used in our experiments. These include NURF (Tsukiyama and Wu, 1995), ACF (Ito et al., 1997), and CHRAC (Varga-Weisz et al., 1997). Both ACF and NURF have been shown to potentiate transcriptional activation in vitro (Ito et al., 1997; Mizuguchi et al., 1997). We cannot rule out the possibility that these or other complexes present in the *Drosophila* extracts are contributing to the transcription we observe. However, since purified E-RC1 is absolutely required for transcriptional activation and chromatin disruption, these complexes are not sufficient to function with EKLF. By contrast, another hematopoietic factor, NF-E2, does not require an exogenous SWI/SNF chromatin remodeling activity but can cooperate with those complexes present in *Drosophila* extracts to reconfigure the LCR/HS2 enhancer (Armstrong and Emerson, 1996). We believe that the observation that EKLF, TFE-3, and NF-E2 appear to function with distinct chromatin modifying activities is relevant. EKLF demonstrates a preference for a SWI/SNF remodeling complex, and this type of specificity may play a role in many instances of highly regulated transcription. The erythroid-specific EKLF is the founding member of a family of Krüppel-like factors with similar zinc-finger domains. It will be exciting to pursue whether these family members also show a functional requirement for E-RC1.

Implications for β-Globin Gene Locus Regulation

The β-globin locus is an ideal model system for the exploration of the interdependence of tissue- and stage-specific expression with precise chromatin structures. Our identification of an EKLF coactivator as a member of the SWI/SNF (BAF) family of chromatin remodeling complexes provides further evidence for that relationship. In addition to its role in the β-globin promoter, EKLF is necessary for the formation of hypersensitive site 3 (HS3) within the locus control region (LCR) (Wijgerde, et al. 1996). Further efforts are underway to elucidate the role of E-RC1 in EKLF-mediated chromatin remodeling of the LCR and LCR promoter communication within the β-globin gene locus.

Experimental Procedures

Plasmid Constructions

βCAT plasmid was constructed as described (Jane et al., 1992). Full-length EKLF was cloned into the NdeI and BamHI sites of the pET-14b bacterial expression vector (Novagen). EKLFΔ20–60 was generated after ApaI restriction to remove the amino terminus. EKLFΔ60–196 was made by digestion with ApaI and SacII and religated.

Protein Purification

Histidine-tagged wild-type and mutant EKLF proteins were expressed in *E. coli* BL21(DE3)pLysS or BL21(DE3) cells. All proteins were purified under denaturing conditions on Ni^{2+}-NTA resin (Qiagen) and then renatured by step dialysis.

MEL (mouse erythroleukemia) cells were grown and the protein extracts prepared as previously described (Armstrong and Emerson, 1996). All purification steps were performed at 4°C. For E-RC1 purification, approximately 120 mg protein were loaded onto phosphocellulose P-11 resin (Whatman), 8–10 ml bed volume, equilibrated in 0.1 M KCl P-11 buffer (20 mM HEPES [pH 7.9], 20% glycerol, 0.2 mM EDTA, 2 mM DTT). The column was washed with 0.1 M KCl P-11 buffer. Step elutions of 0.3 M KCl, 0.6 M KCl, and 1.0 M KCl were collected. E-RC1 eluted in the 0.6 M KCl peak, which was dialyzed into dialysis buffer (20 mM HEPES [pH 7.9], 50 mM KCl, 0.2 mM EDTA, 10% glycerol, 2 mM DTT). E-RC1 was then loaded onto a DEAE-sephacel (Pharmacia) column equilibrated in dialysis buffer. After washing, E-RC1 was eluted with dialysis buffer containing 0.25 M KCl and dialyzed into dialysis buffer. The 0.25 M DEAE fraction was loaded on a dsDNA–cellulose (Sigma) column equilibrated in DNA–cellulose buffer (20 mM HEPES [pH 7.9], 5 mM $MgCl_2$, 10% glycerol, 0.1% Brij-35, 1 mM DTT) containing 25 mM ammonium sulfate. After extensive washing, E-RC1 was eluted with DNA–cellulose buffer containing 90 mM ammonium sulfate and dialyzed into DNA–cellulose dialysis buffer (20 mM HEPES [pH 7.9], 100 mM KCl, 3 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 0.1% NP-40, 0.5 mM DTT). The 90 mM DNA–cellulose fraction was applied to a 13 ml 20%–35% glycerol gradient, approximately 320 μg protein per gradient, and centrifuged in an SW41 rotor at 29,000 rpm for 24 hr. Glycerol gradients were prepared in DNA–cellulose dialysis buffer except that NP-40 was omitted. Gradients were divided into 11 fractions that were dialyzed separately into dialysis buffer.

Chromatin Assembly and Transcription Reactions

Chromatin was reconstituted as described (Armstrong and Emerson, 1996). Following assembly, the chromatin template (1 μg in 100 μl) was incubated with wild-type or mutant EKLF protein (37 pmol each) and E-RC1 fractions, as described in the figure legends, for 20 min at 27°C. The reactions were then split for either transcription or structural analysis. To allow transcription, 25 μl nuclear HeLa extract (typically about 8 mg/ml), prepared as described (Dignam et al., 1983), was added to 0.5 μg of chromatin and incubated on ice for 10 min. Transcription mix was added (20 mM HEPES [pH 7.9], 50 mM KCl, 5 mM $MgCl_2$, 0.2 mg/ml BSA, 0.5 mM [each] ATP,

CTP, UTP, GTP, 0.7 μg/ml Adeno-luciferase control template [Ad-Luc], 1 mM DTT) in a final volume of 150 μl. Transcription proceeded at 30°C for 30 min and was stopped by the addition of 250 μl of transcription stop buffer (1% SDS, 20 mM EDTA). The purified RNA product was analyzed by primer extension analysis.

DNase I Digestion and Indirect End-Labeling Analysis

Following incubation of the chromatin with transcription factors, 100–150 ng chromatin was digested with DNase I for 1 min at 27°C. Purified DNA was digested with NcoI and analyzed by Southern blot hybridization (Gene Screen Plus) with a random prime-labeled 301 bp EcoRI/NcoI fragment from the βCAT plasmid.

Mononucleosome Disruption Assays

Sequence-positioned mononucleosomes (5S) were reconstituted by salt dialysis and subject to sucrose gradient sedimentation as described (Workman and Kingston, 1992), using purified *Drosophila* core histones at a 1:1 histone:DNA ratio. DNase I footprinting analysis was carried out as described in the legend to Figure 5A.

DNase Footprinting

A 167 bp fragment from −170 to −3 of the human β-globin promoter was generated by PCR (5′ primer: AAGTCCAACTCCTAAGCCAG and 3′ primer: GCAATAGATGGCTCTGCCCT) using a labeled 5′ primer and gel purified. Footprinting reactions were carried out in a 25 μl volume in dialysis buffer containing 5 mM $MgCl_2$, 5 μl $ZnSO_4$, 50 μg/ml bovine serum albumin, and 4 ng/μl poly dIdC. EKLF protein was added in the amounts listed in the figure legends and incubated with approximately 10,000 cpm of probe for 25 min at room temperature. DNase I was added (5 ng), and cleavage proceeded for 1 min at 25°C. Reactions were stopped by the addition of 5× nuclease stop buffer to 1× concentration. DNA was electrophoresed on a 6% acrylamide-urea gel.

Western Blot Analysis, Immunoneutralizations, and Immunoprecipitations

Glycerol gradient fractions were electrophoresed on a 7.5% SDS-PAGE gel and blotted onto Hybond-C membrane (Amersham). Western blot analyses were carried out using a 1:1000 dilution of primary antisera (Anti-BAF155, BAF57, BAF170, INI1[BAF47], and BRG1). Westerns were developed using the Pierce SuperSignal ECL reagents. Immunoneutralizations were accomplished by the incubation of 5 μl DNA–cellulose E-RC1 fraction with 2.5 μl antisera or preimmune sera on ice for 30 min prior to addition of the chromatin template. Immunoprecipitations were performed by diluting the E-RC1 fraction 5-fold (from 100 μl to 500 μl) in 0.15 M KCl buffer D (20 mM HEPES [pH 7.9], 0.25 mM EDTA, 20% glycerol, 0.1% Tween 20). Antisera was added (2.5 μl) and the fraction was incubated overnight, rotating slowly at 4°C. Protein A beads were blocked by incubation with 1 mg/ml bovine serum albumin for 1 hr, rotating at 4°C. Beads were washed once in 0.1 M KCl buffer D, and 15 μl was added to each reaction. After 2 hr incubation rotating at 4°C, the beads were spun down at 10,000 rpm for 30 s. The beads were washed two times each with 0.5 M KCl buffer D, 0.1 M KCl buffer D containing 0.2 M guanidine HCl, and 0.1 M KCl buffer D. During the last wash, the beads were split into two tubes. One-half of the beads were analyzed by SDS-PAGE and SWI/SNF–related complexes visualized by silver stain (data not shown); the other 7.5 μl was added with EKLF to the chromatin templates. DNase I hypersensitive site analysis proceeded as previously described.

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