

A Conserved Motif Present in a Class of Helix-Loop-Helix Proteins Activates Transcription by Direct Recruitment of the SAGA Complex

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Summary

The class I helix-loop-helix (HLH) proteins, which include E2A, HEB, and E2-2, have been shown to be required for lineage-specific gene expression during T and B lymphocyte development. Additionally, the E2A proteins function to regulate V(D)J recombination, possibly by allowing access of variable region segments to the recombination machinery. The mechanisms by which E2A regulates transcription and recombination, however, are largely unknown. Here, we identify a novel motif, LDFS, present in the vertebrate class I HLH proteins as well as in a yeast HLH protein that is essential for transactivation. We provide both genetic and biochemical evidence that the highly conserved LDFS motif stimulates transcription by direct recruitment of the SAGA histone acetyltransferase complex.

Introduction

The helix-loop-helix family of transcriptional regulators mediates a number of important developmental events in eukaryotes, including hematopoiesis, myogenesis, neurogenesis, and sex determination (Cline, 1989; Weintraub et al., 1991; Murre and Baltimore, 1992; Murre et al., 1994; Bain and Murre, 1998). These proteins share a DNA binding and dimerization motif that contains a basic region adjacent to two amphipathic alpha helices separated by a flexible loop structure and is known as the basic helix-loop-helix (bHLH) (Murre et al., 1989). The bHLH domain has been shown to bind DNA sequence elements known as E boxes. The E box, which has the core consensus CANNTG, is found in a variety of cell type-specific gene promoter and enhancer elements (Murre and Baltimore, 1992). Mutational analysis has demonstrated that the E box sites are critical for regulating lineage-specific gene expression (Murre and Baltimore, 1992).

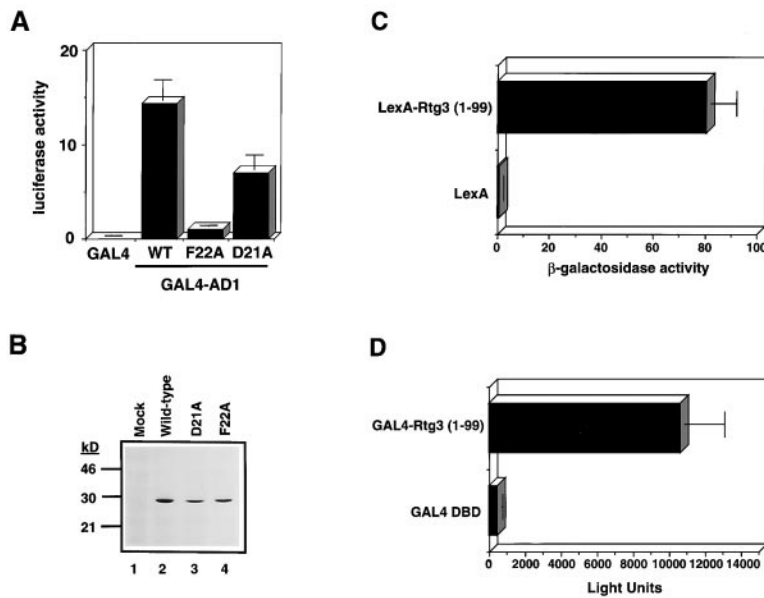
The E2A gene, which encodes both the E12 and E47

bHLH proteins, is indispensable for proper B lymphocyte development (Bain et al., 1994; Zhuang et al., 1994). Mice containing a targeted disruption of the E2A gene have no detectable B cells in the peripheral lymphoid organs (Bain et al., 1994; Zhuang et al., 1994). Developmental arrest occurs at an early stage since no immunoglobulin (Ig) rearrangements can be detected in the bone marrow and fetal liver of E2A (–/–) mice (Bain et al., 1994; Zhuang et al., 1994). The E2A proteins are required for the transcriptional regulation of a large number of B lineage-specific genes, including *Rag-1*, *EBF*, Ig heavy chain, and $\lambda 5$ (Schlissel et al., 1991; Bain et al., 1994; Choi et al., 1996; Sigvardsson et al., 1997; Kee and Murre, 1998). The E2A gene products have also been implicated in V(D)J recombination. Ectopic expression of E47 in a pre-T cell line leads to increased levels of DJ rearrangement (Schlissel et al., 1991). Furthermore, in the absence of E2A, certain TCR $\gamma\delta$ V(D)J rearrangements are severely impaired (Bain et al., 1999). How E2A proteins regulate V(D)J recombination, however, has not yet been elucidated. E2A protein binding sites are present in both Ig enhancer elements as well as in the promoter regions of Ig and TCR V regions and have been shown to regulate germline transcription through these DNA segments (Redondo et al., 1990, 1991; Takeda et al., 1990; Sleckman et al., 1996). That E2A controls accessibility to the recombination machinery was demonstrated in E2A-deficient thymocytes in which the levels of double-stranded DNA breaks at the recombination signal sequences were dramatically affected (Bain et al., 1999). Taken together, these data suggested that one plausible role for E2A proteins in lymphocyte development is to regulate V(D)J recombination by controlling accessibility of the Ig and TCR variable, diversity, and joining segments to the recombination machinery.

Two activation domains, termed AD1 and AD2/LH, have been identified in the amino-terminal half of E2A (Aronheim et al., 1993; Quong et al., 1993; Massari et al., 1996). These domains are conserved in and restricted to a subset of the HLH proteins, which includes E2A, E2-2, and HEB. Both AD1 and AD2 have the ability to activate transcription in yeast and mammalian cells (Aronheim et al., 1993; Quong et al., 1993; Massari et al., 1996). Although AD1 and AD2 share no sequence homology, both contain a potential α helix that is important for function (Quong et al., 1993; Massari et al., 1996).

When tethered to DNA, eukaryotic transcriptional activation domains must recruit the general transcription machinery, which includes RNA polymerase II, the general transcription factors (GTFs), and cofactors in order to stimulate gene transcription (reviewed in Zawel and Reinberg, 1995). Although studies have shown that an activator can make direct contact with the GTFs, many activators are thought to communicate with the transcription machinery via interactions with cofactors known as adaptors or coactivators. Among the first coactivators identified were those associated with the TBP-containing complex, TFIID, which are known as TBP-associated factors or TAFs (Dynlacht et al., 1991). The TAFs were identified on the basis of their requirement for

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(D) Rtg3p(1-99) activates transcription in mammalian cells. The cells were harvested 48 hr posttransfection and assayed for luciferase activity. Data shown represent the average values from a typical experiment that was performed in triplicate. Error bars indicate standard deviations.

One protein was identified, called Rtg3p, which contained a region of homology to AD1 (Figures 1A and 1B). Intriguingly, Rtg3p, like E2A, is a member of the helix-loop-helix family of transcription factors (Jia et al., 1997). The region of similarity to AD1 was located at the extreme amino terminus of Rtg3p, a position similar to the location of AD1 in the class I HLH proteins (Figure 1A). Sequence alignment of this region of Rtg3p with the vertebrate E proteins revealed a significant homology within the AD1 helix (Figure 1B). Rtg3p displays a 31% identity/53% similarity to the E2A AD1 over a 36-amino acid segment (Figures 1A and 1B). Most striking is the presence of an absolutely conserved block of residues, LDFS, which is present in each of the HLH proteins presented here (Figure 1B).

Previous studies have demonstrated that the region of AD1 containing the LDFS motif can adopt an α -helical structure that is critical for its activity in both yeast and mammalian cells (Massari et al., 1996). Therefore, we analyzed this region of Rtg3p for potential secondary structure using both the Chou-Fasman and Robson-Garnier algorithms (Chou and Fasman, 1974; Garnier et al., 1978). Both algorithms predict a helical structure present between amino acids 5 and 36 of Rtg3p (data not shown). Interestingly, when the homologous amino-terminal regions of E2A and Rtg3p are plotted on a helical wheel, the residues conserved between E2A and Rtg3p form two groups present on opposite faces of the helix (not shown). Molecular modeling of the conserved region of AD1 further reveals that the highly conserved LDFS residues lie on opposing faces of the helix (Figure 1C).

LDFS Motif Is Required for Transactivation

In order to determine whether the LDFS motif is required for transcriptional activation, we generated mutations within the AD1 helical region that were designed to eliminate these conserved residues. An alanine was substituted for the conserved aspartic acid in LDFS at position

21 (D21A) of the human E2A AD1 domain, and the conserved phenylalanine in LDFS was replaced by an alanine at amino acid position 22 (F22A). Next, we introduced either the wild-type or mutant GAL4-AD1 proteins into HeLa cells along with a luciferase reporter gene driven by five GAL4-binding sites (Figure 2A). Mutation of the aspartic acid had some effect on AD1 activity, with levels dropping to 50% of the wild-type value (Figure 2A). The F22A mutant, however, was severely crippled in its transactivation capability, showing a 15-fold decrease in activity (Figure 2A). Western blot analysis demonstrated that both the wild-type and mutant AD1 proteins were expressed at comparable levels in HeLa cells (Figure 2B). The activities of the D21A and F22A AD1 mutants were also determined in *Saccharomyces cerevisiae*. Whereas the D21A mutation had little effect, the F22A mutant showed a 4-fold reduction in activity as compared to the wild-type AD1 domain (data not shown). These data demonstrate that the LDFS motif is required for AD1 activity in both yeast and mammalian cells.

The Rtg3p AD1-like Domain Activates Transcription and Requires the LDFS Motif

The data described above indicate that the LDFS motif is required for AD1-mediated transactivation. To determine whether the Rtg3p amino-terminal region containing the LDFS motif is also capable of activating transcription, the first 99 amino acids were fused in-frame to the LexA DNA-binding domain (DBD) and expressed in a yeast strain harboring a *LacZ* reporter plasmid driven by three LexA-binding sites. Plasmid-bearing yeast were then assayed for β -galactosidase activity (Figure 2C). As expected, LexA-Rtg3p(1-99) was capable of activating transcription of the *LexAop-LacZ* reporter gene (Figure 2C). Although LexA-Rtg3p(1-99) was a significantly weaker activator than LexA-AD1, it showed activity at least 100-fold higher than that of LexA alone (Figure 2C and data not shown).

Figure 2. The LDFS Motif Is Required for AD1-Mediated Transactivation

(A) Transient transfection analysis of GAL4-AD1 mutants in HeLa S3 cells. Shown are average values of a typical experiment performed in duplicate. Luciferase activity as measured in light units has been normalized to β -galactosidase activity. Error bars represent standard deviations.

(B) Western blot analysis showing levels of the GAL4-AD1 derivatives in HeLa S3 cells. Mock, cells transfected without expression plasmid. The GAL4 fusion proteins were detected using a GAL4 monoclonal antibody as described (Massari et al., 1996).

(C) Rtg3p(1-99) activates transcription in yeast. Plasmids expressing either LexA-Rtg3p(1-99) or the LexA DNA-binding domain were introduced into the yeast strain PSY316, which contained the *LexAop-LacZ* reporter plasmid JK103. Cells were grown to mid-log phase and assayed for β -galactosidase activity. Values shown indicate the average of four independent transformants. β -galactosidase activity is measured in units.

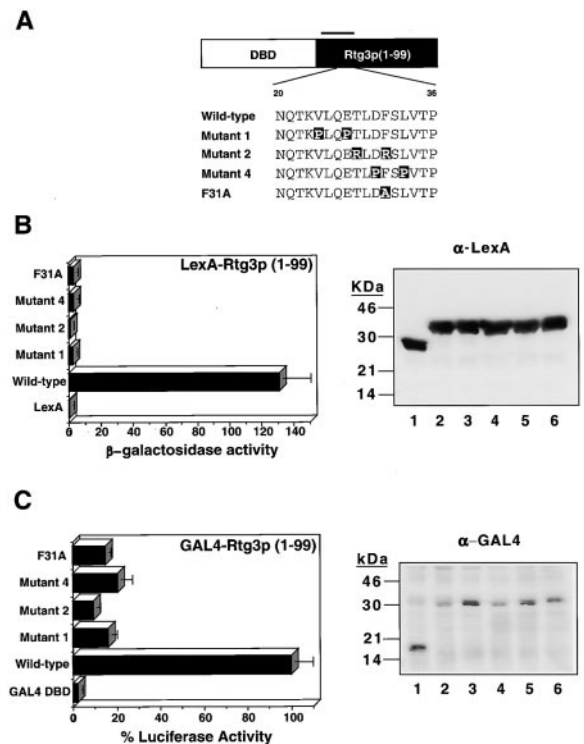


Figure 3. The Integrity of the Putative Helical Region of Rtg3p(1-99) Is Essential for Its Activity

(A) Summary of the Rtg3p mutants used in this study. Shown is a schematic depiction of the Rtg3p(1-99) protein fused to either the GAL4 or LexA DNA-binding domain (DBD). The black bar indicates the position of the predicted helix present in the amino-terminal region of Rtg3p. Amino acid substitutions introduced into the Rtg3p mutants are highlighted by blackened boxes.

(B) Activity of the LexA-Rtg3p(1-99) derivatives in the yeast strain PSY316. Yeast were cotransformed with the indicated LexA-Rtg3p expression vector and the *LacZ* reporter plasmid, JK103, and assayed for β -galactosidase activity. To the right of the histogram is a Western blot showing the levels of the LexA-Rtg3p fusions present in whole-cell extracts derived from the same yeast transformants used for the β -galactosidase assay. Lane 1, LexA; lanes 2-6, LexA-Rtg3p(1-99) derivatives. Lane 2, WT; lane 3, mutant 1; lane 4, mutant 2; lane 5, mutant 4; lane 6, mutant F31A.

(C) Activity of the various GAL4-Rtg3p(1-99) mutant proteins relative to GAL4-Rtg3p WT in HeLa S3 cells. Shown in the right panel is a Western blot of whole-cell lysates of HeLa S3 cells transfected with the various GAL4-Rtg3p(1-99) expression plasmids. Lane 1, GAL4 DBD; lanes 2-6, GAL4-Rtg3p(1-99) derivatives. Lane 2, WT; lane 3, mutant 1; lane 4, mutant 2; lane 5, mutant 4; lane 6, mutant F31A.

To determine if the Rtg3p AD1-like region could function as an activator in mammalian cells, the Rtg3p(1-99) segment was fused in-frame with the GAL4 DNA-binding domain, and the resulting expression vector was introduced by transient transfection into HeLa cells along with a luciferase reporter gene under the control of five GAL4-binding sites (Figure 2D). Activation of the luciferase reporter by GAL4-Rtg3p(1-99) was consistently observed, although it was substantially weaker than that of GAL4-AD1 (Figure 2D and data not shown). These data demonstrate that the Rtg3p AD1-like domain shares similar functional properties with the E2A AD1 transactivation motif in both yeast and mammalian cells.

To assess whether the LDFS motif present in Rtg3p

Table 1. Toxicity of AD1 Overexpression in Yeast Correlates with Transcriptional Activity

GAL4-AD1 Fusion ^a	Mutation ^a	Transactivation ^b	Slow Growth ^c
Wild type	—	+	+
Mutant 1	E15P, D18P	—	—
Mutant 2	L19R, F22R	—	—
Mutant 3	S58G, W59Y	+	+
Mutant 4	D21P, M24P	—	—
Mutant 5	S48A	+	+
Mutant 6	S67A, S68A	+	+
GAL4-E2-2 AD1	—	+	+
GAL4-HEB AD1	—	+	+

^aThe GAL4-E2A AD1 and GAL4-E2-2 AD1 2 μ expression plasmids have been described previously (Massari et al., 1996). Mutants 1, 2, and 4 target the potential α helix of AD1.

^bTransactivation is defined here as the ability to activate transcription of an integrated *GAL1-lacZ* reporter gene present in the yeast strain yWAM2 (Wang and Reed, 1993) or strain Y190 (Durfee et al., 1993) for E2-2 and HEB. Reporter gene activation was assayed by a qualitative X-gal filter lift assay. Blue colony color is indicated as a plus and white as a minus. These data are consistent with quantitative β -galactosidase assays performed previously (Massari et al., 1996).

^cTransformants that grew very slowly on selective media as compared to empty vector controls are indicated by a plus. Normal growth is denoted by a minus. These tiny colonies were barely visible after 4 days at 30°C.

contributed to its ability to activate transcription, a series of amino acid substitutions were generated within and surrounding the LDFS motif (Figure 3A). Both the wild-type and mutant forms of LexA-Rtg3p(1-99) were introduced into yeast in order to determine their transcriptional activity (Figure 3B). Significantly, mutations that targeted the LDFS motif exhibited a dramatically reduced activity (Figure 3B). For example, substitution of the conserved phenylalanine residue for alanine at amino acid position 31 of Rtg3p resulted in a 40-fold loss of activity as compared to wild type (Figure 3B).

When the same Rtg3p mutants were tested as GAL4 DBD fusions in mammalian cells, their activities were strikingly similar to those seen in yeast (Figure 3C). The transactivation potential of the proline substitution mutants (mutants 1 and 4) and the F31A mutant were reduced by greater than 80% (Figure 3C). The arginine substitutions within the putative helical region of Rtg3p had the most dramatic effects on activity, reducing transactivation function by 90% in HeLa cells and by approximately 99% in yeast (Figures 3B and 3C). Western blot analysis of the wild-type and mutant Rtg3p fusion proteins in both yeast and HeLa cells revealed that the fusions were expressed at comparable levels (Figures 3B and 3C). These data suggest that, like AD1, the region of Rtg3p containing the LDFS motif is critical for its ability to activate transcription.

Toxicity of GAL4-Rtg3p and GAL4-AD1 in Yeast Requires the Presence of Ada2 and Gcn5

During the course of our transactivation studies, we noticed that overexpression of GAL4-AD1 in yeast was growth inhibitory (Table 1). Yeast transformed with high-copy plasmids expressing GAL4-AD1 but not the GAL4 DNA-binding domain (DBD) alone grew slowly and ap-

peared as tiny colonies after incubation for many days at 30°C (Table 1). The toxic phenotype was also conferred by overexpression of either the GAL4-E2-2 or GAL4-HEB AD1 fusion proteins (Table 1). Interestingly, there was a correlation between transactivation capability and toxicity of the various GAL4-AD1 derivatives (Table 1). For example, mutations in the LDFS motif that severely compromised activity in yeast were not toxic when expressed as GAL4 fusions (Table 1).

The acidic activation domains of VP16, Bel-1, and p53 have also been shown to be toxic when overexpressed as GAL4 fusions in yeast (Berger et al., 1992; Blair et al., 1994; Candau et al., 1997). As with the AD1 domain, mutations within VP16 that reduce transactivation in yeast also relieve toxicity (Berger et al., 1992). These data suggest that like VP16, the toxic effects of AD1 overexpression may be due to transcriptional squelching (Berger et al., 1992). Mutations in the *ADA/GCN5* genes, which encode subunits of the SAGA and Ada HAT complexes, relieve the toxic effects of overexpression of GAL4-VP16 (Berger et al., 1992; Pina et al., 1993; Horiuchi et al., 1995, 1997; Grant et al., 1997). Therefore, we tested whether yeast strains deleted for either the *ADA2* or *GCN5* genes would be resistant to GAL4-AD1-mediated toxicity. High-copy plasmids driving expression of GAL4-AD1, GAL4-AD1 mutant 2, or GAL4 DBD alone were transformed into the yeast strain PSY316 or into isogenic strains harboring a deletion of either the *ADA2* ($\Delta ada2$) or *GCN5* ($\Delta gcn5$) gene (Figure 4A). Neither the GAL4 DBD nor a transcriptionally inactive derivative of GAL4-AD1 was toxic when overexpressed in these strains (Figure 4A). As expected, GAL4-AD1 induced a slow growth phenotype in the PSY316 strain, as indicated by the presence of tiny colonies (Figure 4A). Surprisingly, however, the $\Delta ada2$ and $\Delta gcn5$ strains overexpressing GAL4-AD1 grew normally (Figure 4A). An isogenic $\Delta ada2$ strain was also completely resistant to GAL4-AD1 overexpression (data not shown). The resistance to toxicity was not due to a lack of expression of GAL4-AD1, as immunoblot analysis confirmed the presence of the AD1 fusion proteins in the $\Delta ada2$ and $\Delta gcn5$ strains (data not shown).

Because of the remarkable functional similarities of the E2A AD1 domain with the conserved region of Rtg3p, we tested whether overexpression of GAL4-Rtg3p(1-99) in yeast would be growth inhibitory. The GAL4 DBD, GAL4-Rtg3p(1-99) wild-type, and the transcriptionally compromised mutant, GAL4-Rtg3p mutant 2, were transformed separately into either PSY316 wt or $\Delta ada2$ mutant yeast. Transformants expressing GAL4 DBD alone or GAL4-Rtg3p mutant 2 grew normally (Figure 4B). However, wild-type yeast harboring the GAL4-Rtg3p(1-99) plasmid formed tiny, slow growing colonies on the selective media (Figure 4B). This phenotype is indistinguishable from that of wild-type yeast overexpressing GAL4-AD1. In contrast, expression of GAL4-Rtg3p(1-99) in the $\Delta ada2$ strain did not cause a slow growth phenotype (Figure 4B).

The Activity of the LDFS Motif Is Dependent upon Ada2p and Gcn5p

Previous studies indicated that the E2A proteins are required for the induction of lymphocyte-specific gene

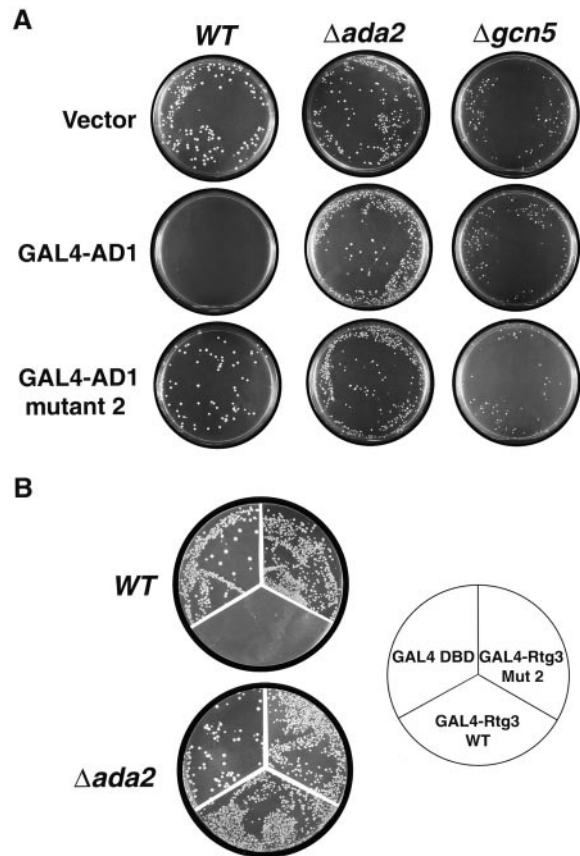


Figure 4. Phenotype of Yeast Overexpressing GAL4-AD1 or GAL4-Rtg3p(1-99)

(A) Overexpression of GAL4-AD1 in *ada2* and *gcn5* mutants is not toxic. High-copy plasmids encoding GAL4 DNA binding-domain alone (vector), GAL4-AD1 wt, or GAL4-AD1 mutant 2 were transformed into the indicated strains and allowed to grow on synthetic complete media lacking histidine and containing 2% dextrose for 4 days at 30°C before being photographed.

(B) Overexpression of GAL4-Rtg3(1-99) is toxic in yeast. The yeast strain PSY316 (WT) and an isogenic mutant strain PSY $\Delta ada2$ were transformed with 2 μ -based plasmids constitutively expressing GAL4 DBD (pMA424), GAL4-Rtg3p(1-99) wild type, or GAL4-Rtg3p(1-99) mutant 2. Transformants were grown for 3 days at 30°C before being photographed.

expression as well as for regulation of V(D)J recombination (Schlissel et al., 1991; Bain et al., 1994; Choi et al., 1996; Sigvardsson et al., 1997; Kee and Murre, 1998; Bain et al., 1999). This raised the possibility that E2A gene products may function to control accessibility of the variable region segments to the recombination-activating (RAG) proteins. Thus, it is conceivable that the E2A proteins allow access to Ig and TCR loci by recruitment of protein(s), such as HATs, that are capable of modifying chromatin structure. Consistent with such a model, the AD1 overexpression studies in yeast suggested to us that an activation domain present in E2A may function through a HAT complex. To determine whether the AD1 domain requires the Ada or SAGA HAT complexes for activity, we analyzed the ability of the E2A AD1 domain to activate transcription in $\Delta ada2$ and $\Delta gcn5$ strains. Deletion of either of these genes renders

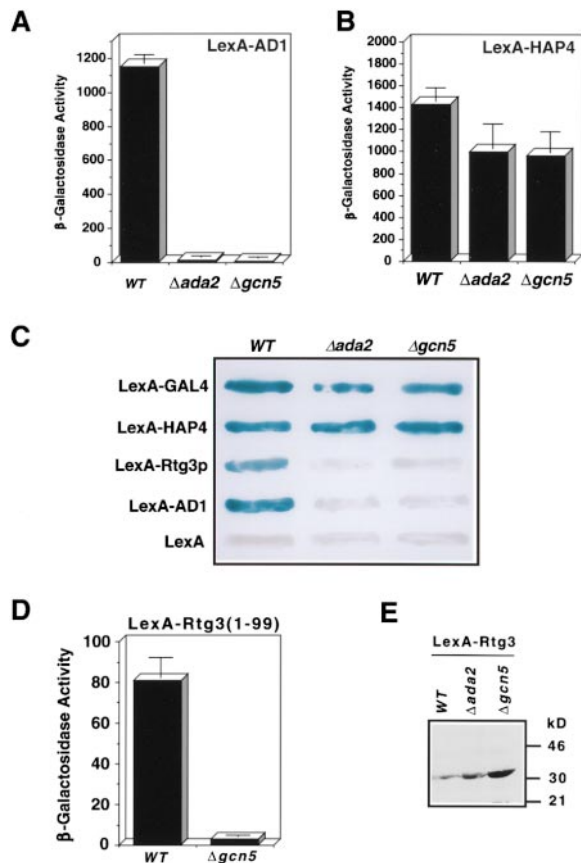


Figure 5. AD1 Activates Transcription through an Ada/Gcn5-Dependent Pathway

(A) The LexA-AD1 expression vector was transformed into each of the indicated yeast strains along with the *LexAop-LacZ* reporter plasmid, JK103. Transformants were subsequently measured for β-galactosidase activity using a quantitative liquid assay.

(B) As a positive control, β-galactosidase activity was measured from the indicated strains carrying a LexA-HAP4 expression vector. Each value shown represents the average of three independent transformants from a typical assay. Each assay was performed at least three times, yielding comparable results. Error bars represent standard deviations. β-galactosidase activity is given in units.

(C) X-gal plate assay. Transformants harboring the *LacZ* reporter plasmid, JK103, and the indicated LexA fusion expression vector were initially selected on synthetic medium lacking uracil and histidine and then streaked onto X-gal indicator plates lacking uracil and histidine. The plates were incubated at 30°C for 20 hr before being photographed.

(D) Quantitative β-galactosidase assay measuring the transcriptional activity of LexA-Rtg3p(1-99) in the yeast strains PSY316 (WT) and PSYΔada2. Shown are average values of four independent transformants with error bars representing standard deviations.

(E) Western blot assay showing the levels of the LexA-Rtg3p(1-99) fusion protein in the indicated yeast strains.

the Ada and SAGA complexes devoid of HAT activity (Grant et al., 1997, 1998a). Both wild-type and mutant yeast strains were cotransformed with a LexA-AD1 expression plasmid and a *LacZ* reporter gene driven by LexA-binding sites. Individual transformants were subsequently assayed for β-galactosidase activity (Figure 5A). Strikingly, the transactivation capability of the LexA-AD1 protein was reduced over 100-fold in the Δada2 and Δgcn5 strains (Figure 5A). As a control, the transcriptional activity of LexA-HAP4 was assessed in both

the wild-type and Δada2 and Δgcn5 mutant strains (Figure 5B). The HAP4 activation domain has been previously shown to activate through an Ada/Gcn5-independent mechanism (Berger et al., 1992). As expected, the activation function of HAP4 was not affected in the Δada2 and Δgcn5 backgrounds (Figure 5B).

To determine whether the AD1-like region of Rtg3p similarly requires the Ada or SAGA complexes for function, we tested a LexA-Rtg3p(1-99) fusion for activity in the PSY316 wt, Δada2, and Δgcn5 strains. Wild-type and mutant yeast strains carrying a LexA-Rtg3p(1-99) expression plasmid and a *LexA-LacZ* reporter were patched onto X-gal plates to assess β-galactosidase gene activity (Figure 5C). Similar to the LexA-AD1, LexA-HAP4, and LexA-GAL4 activators, LexA-Rtg3p(1-99) was able to activate the *LacZ* reporter gene in the PSY316 strain, albeit more weakly (Figure 5C). As expected, both LexA-HAP4 and LexA-GAL4 were capable of activating transcription in the mutant strains as well since they have been shown to function through an Ada/Gcn5-independent pathway (Berger et al., 1992; Pina et al., 1993). There was, however, a dramatic reduction in LexA-Rtg3p(1-99) activity in the Δada2 and Δgcn5 mutants, as these strains failed to turn blue on X-gal indicator media (Figure 5C). Quantitative β-galactosidase assays demonstrated that transactivation capability of LexA-Rtg3p(1-99) was reduced 30-fold in the Δgcn5 background (Figure 5D).

One explanation for the inability of the LexA-AD1 and LexA-Rtg3p(1-99) fusions to activate in the Δada2/gcn5 mutant strains could be due to reduced expression or stability of the activators. Western blot analysis, however, confirmed that both LexA-AD1 and LexA-Rtg3p(1-99) were expressed at comparable levels in both wild-type and Δada2/Δgcn5 strains (Figure 5E and data not shown). In summary, both the AD1 and Rtg3p AD1-like domains absolutely require Ada2p and Gcn5p and, therefore, functional Ada or SAGA complexes to activate transcription in budding yeast.

AD1 Directly Interacts with the SAGA Complex In Vitro and Requires the LDFS Motif

The genetic data presented above clearly demonstrate that AD1 requires Ada or SAGA complexes for function in yeast. In order to determine if these complexes are being directly recruited by AD1, we performed a series of glutathione S-transferase (GST) pulldown assays. Native SAGA complexes were purified from yeast extracts (Grant et al., 1997). GST fusion proteins bearing wild-type AD1 or mutant derivatives were first loaded onto beads and then incubated with purified SAGA complexes (Utley et al., 1998; Grant et al., 1998b). Next, both the supernatant and the washed bead fractions were assayed for their ability to acetylate a nucleosomal substrate and analyzed by SDS-PAGE electrophoresis and fluorography. Indeed, GST-AD1 was able to interact with the purified SAGA complexes, as indicated by the histone H3 acetylase activity associated with the bead fraction (Figure 6A). These pulldown experiments were carried out in parallel with three GST-AD1 mutants: mutant 1, mutant 2, and mutant 4 (Figure 6A). Each of these mutant AD1 proteins contains a disruption of the conserved helix and were shown to be defective for activation in both yeast and mammalian cells (Massari et al.,

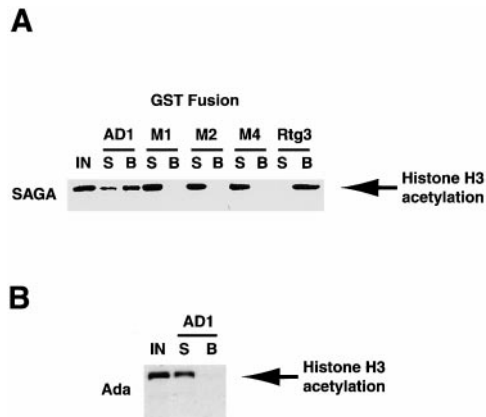


Figure 6. AD1 and Rtg3p(1-99) Directly Interact with the SAGA HAT Complex In Vitro

(A) A GST pull-down assay was performed with purified SAGA complexes and GST-AD1 (AD1), and the following GST-AD1 mutants: mutant 1 (M1), mutant 2 (M2), mutant 4 (M4), and GST-Rtg3p(1-99) (Rtg3). After the binding reaction was completed, both the supernatant (S) and the bead (B) fractions were assayed for HAT activity using a nucleosomal substrate. Histone H3 acetylation (arrow), which indicates the presence of SAGA HAT activity, was revealed by fluorography. IN represents 100% of SAGA HAT activity used as input.

(B) AD1 does not interact with the Ada complex. The GST pull-down assay was performed as described above.

1996). All three mutants showed a dramatic reduction in their ability to bind SAGA as compared to the wild-type GST-AD1 fusion (Figure 6A). Significantly, mutants 2 and 4, both of which contain a disruption of the LDFS motif, did not exhibit detectable binding to SAGA (Figure 6A).

To determine whether a GST-Rtg3p(1-99) fusion protein could interact with the SAGA complex, a GST-Rtg3p(1-99) fusion protein was incubated with purified SAGA complexes and assayed for its ability to acetylate nucleosomes (Figure 6A). The GST-Rtg3p(1-99) fusion bound strongly to the SAGA complex, as all the HAT activity was associated with the bead fraction (Figure 6A). In summary, these data demonstrate that the interaction between AD1 and the SAGA complex is specific and requires the integrity of the LDFS motif.

Our genetic studies suggest that the LDFS motif may also interact with the related HAT complex, Ada. To determine if Ada was also capable of interacting with the LDFS motif, we performed pull-down assays with purified Ada complexes and GST-AD1 (Figure 6B). In contrast to the results seen with SAGA, Ada was not capable of interacting with the LDFS motif (Figure 6B).

Discussion

The genetic and biochemical experiments presented here demonstrate that the direct recruitment of the nuclear HAT complex, SAGA, by a highly conserved motif present in a class of HLH proteins, is necessary to activate transcription. These data strongly suggest that E2A, HEB, and E2-2 regulate transcription by directing chromatin modifying activity to target genes. Additionally, the findings described here may have implications

for the regulation of DNA rearrangements that occur during lymphocyte development.

A Novel, Highly Conserved Motif Restricted to a Subset of HLH Proteins Is Required for Transactivation and Recruitment of the SAGA Complex

E2A, HEB, and E2-2 gene products are closely related in their expression patterns and biochemical properties. They also share significant sequence homology in regions outside of their HLH domain. Most striking is their sequence homology in the AD1 and AD2 transcriptional activation domains. The studies described here reveal that another HLH protein, Rtg3p, shares significant homology with the class I HLH-restricted AD1 domain. Rtg3p functions as a transcriptional activator and is required for the expression of the *CIT2* gene, which encodes an isoform of citrate synthase (Jia et al., 1997; Rothermel et al., 1997). Rtg3p also contains the LDFS motif in its amino-terminal domain. The LDFS motif is present within a region of AD1 that has been shown by circular dichroism analysis to be helical (Massari et al., 1996). The mutational analysis of Rtg3p is consistent with the idea that a helical secondary structure is a conserved and essential feature of this particular transactivation domain. Additionally, we show that substitutions within the LDFS motif dramatically reduce the transactivation ability of Rtg3p.

Does recruitment of SAGA by Rtg3p play a role in *CIT2* gene expression? A recent study has described changes in global gene expression patterns in yeast strains lacking components of the SAGA complex (Holstege et al., 1998). In *Gcn5* null mutant strains, *CIT2* gene expression was reduced to approximately 50% of wild-type levels (www.wi.mit.edu/young/expression.html). Consistent with the findings presented here, these data indicated that a functional SAGA complex is required for proper *CIT2* gene regulation.

The absolute conservation of the LDFS motif suggests that it may form a surface that mediates protein-protein interactions. The SAGA complex directly interacts with the amino-terminal activation domains of both E2A and Rtg3p in a manner that is dependent upon the integrity of the helix and the LDFS motif. Furthermore, both the molecular modeling and helical wheel analysis reveal that these conserved amino acids form groups on opposite faces of the proposed helix. We suggest that these conserved residues directly interact with a subunit(s) present in the SAGA complex in a manner that requires contact from both sides of the helix. This raises the question: which component(s) of SAGA contact the LDFS motif? To address this issue, we have performed a series of pull-down assays with GST-AD1 and subset of proteins found in the SAGA complex, including Ada1, Spt3, Spt7, Spt8 and Spt20, TAF_{II} 17/20, TAF_{II} 25, TAF_{II} 60, TAF_{II} 68, TAF_{II} 90, and Tra1. None of these SAGA components were individually capable of interacting with the LDFS motif (C. M. and M. E. M., unpublished data). The Ada complex, like SAGA, contains the Ada2, Ada3, and *Gcn5* proteins. Since the AD1 domain does not bind to purified Ada complexes, it seems unlikely that the LDFS motif is interacting with the Ada2, Ada3, or *Gcn5* components present within SAGA. Future studies will be directed at understanding the LDFS-SAGA interaction in greater detail. In particular, it will be interesting to determine whether opposing faces of the helix

interact with one particular component of SAGA or with distinct polypeptides within the complex.

The Role of the LDFS Motif in E2A-Mediated Transactivation

Previous studies have shown that the nuclear HAT p300 is capable of enhancing E2A-directed transcriptional activation (Eckner et al., 1996; Qiu et al., 1998). Transient transfection experiments performed in mammalian cells have demonstrated that p300 potentiates E box-dependent reporter gene activation by E47 (Eckner et al., 1996). More recently, p300 has been shown to enhance the ability of both AD1 and AD2 to activate transcription in transfection assays (Qiu et al., 1998). These studies, however, did not demonstrate a direct interaction with p300 and E2A in vitro, opening the possibility that their association is indirect. Here, we demonstrate that the AD1 transactivation domain can directly interact with a distinct nuclear HAT complex, called SAGA, that has been highly conserved throughout evolution. Taken together, these studies suggest that the E proteins may stimulate transcription through the selective recruitment of different HAT activities to target genes.

The amino-terminal transactivation domains present in E2A may also play an important role in the development of two distinct subsets of human leukemia. In pro-B acute lymphoblastic leukemia (ALL), a t(17;19) chromosomal translocation results in the fusion of the *E2A* gene to a gene encoding a novel bZIP family transcription factor called HLF (Inaba et al., 1992). In 25% of pre-B ALLs, a t(1;19) translocation fuses *E2A* to a homeobox gene called *Pbx1* (Kamps et al., 1991). Both chromosomal translocations result in the expression of a chimeric protein that is composed of the amino-terminal transactivation domains of E2A linked to a heterologous DNA-binding domain (Kamps et al., 1991; Inaba et al., 1992). It is likely that the development of lymphomas may result from the inappropriate expression of target genes normally regulated by Pbx1 or HLF (Hunger et al., 1992; Dederer et al., 1993; Yoshihara et al., 1995). In support of this, E2A-Pbx1 but not Pbx1 has been shown to be a potent transactivator (van Dijk et al., 1993; Lu et al., 1994). Deletion studies have demonstrated that the AD1 domain is required for both E2A-Pbx1 and E2A-HLF-mediated transformation of NIH 3T3 cells (Monica et al., 1994; Yoshihara et al., 1995). The integrity of the AD1 domain is also necessary for the antiapoptotic effects of E2A-HLF (Inukai et al., 1998). Based on the data presented here, it is likely that both the E2A-Pbx1 and E2A-HLF chimeric oncoproteins require recruitment of nuclear HATs in order to exert their transforming activities in vivo.

The Potential Role for SAGA in V(D)J Recombination

It is intriguing that the E2A proteins have been implicated in both Ig and TCR V(D)J recombination. Overexpression of E47 in a pre-T cell line leads to the induction of IgH DJ rearrangements (Schlissel et al., 1991). Rearrangements to particular TCR γ and δ V regions are significantly reduced in E2A-deficient thymocytes, presumably due to an inability to efficiently cleave the DNA at the recombination signal sequence (Bain et al., 1999). These data identified the E2A proteins as transcription

factors that have the ability to influence V region gene usage during the recombination process. Furthermore, regulation of ordered V(D)J rearrangement is sensitive to the dosage of E2A, as mice heterozygous for E2A show significant alterations in rearrangement levels (Bain et al., 1999). Recently, we have obtained direct evidence that the E2A proteins regulate Ig kappa VJ recombination as well (B. Romanow and C. M., unpublished data). E2A has also been implicated in the control of Ig isotype switching (Goldfarb et al., 1996).

There are various other ways in which E2A might regulate the accessibility of the recombination signal sequences (RSSs) to the recombination machinery. A number of studies have suggested that transcriptional enhancers play important roles in modulating the accessibility of the gene segments to the recombination machinery. Interestingly, E2A protein-binding sites have been identified in the enhancer elements of each of the Ig enhancers, as well as in the TCR β and δ enhancers (Redondo et al., 1990, 1991; Takeda et al., 1990; Sleckman et al., 1996). Targeted deletion of the Ig enhancers leads to an impairment of both light and heavy chain gene rearrangement (Sleckman et al., 1996). These data have raised the question: how do E2A proteins control DNA rearrangement? We would like to propose that the LDFS motif present in E2A allows the recruitment of a mammalian HAT complex to target genes. Modification of nucleosomes by HAT activity could open up chromatin and allow accessibility to recombination factors. Indeed, *hADA2* and *hGCN5* are expressed in B cells (M. E. M. and C. M., unpublished data), suggesting that hGCN5 HAT complexes are available for recruitment by DNA-bound E2A homodimers. Interestingly, hGCN5 also interacts with Ku and the DNA-dependent protein kinase DNA-PK, which are involved in V(D)J recombination (Blunt et al., 1995; Finnie et al., 1995; Barlev et al., 1998). It will be interesting to determine whether dominant-negative forms of hGCN5 have the ability to block DNA rearrangement mediated by the E2A proteins.

Conclusion

The data described here identify a novel motif, LDFS, which directs histone acetyltransferase activity to nucleosomes of target genes regulated by a set of HLH proteins. Both the LDFS motif and its molecular target, the SAGA complex, are strikingly conserved throughout eukaryotic evolution and may function to regulate transcription and gene rearrangement.

Experimental Procedures

Plasmid Construction

Details on the construction of plasmids used in this study are available upon request or by visiting <http://www.molecule.org/cgi/content/full/4/1/63/DC1>.

Yeast Strains and Media

The yeast strains PSY316 (*ade2-101 Δhis3-200 leu2-3,112 lys2 ura3-53*) and the isogenic derivatives PSY $\Delta ada2$ and PSY $\Delta gcn5$ have all been described (Berger et al., 1992; Candau et al., 1996). Yeast transformations were done according to a modification of the lithium acetate method (Durfee et al., 1993). Yeast media were prepared as described (Ausubel et al., 1991).

β -Galactosidase Assays

Qualitative β -galactosidase assays were performed as follows: in brief, yeast transformants were transferred to nitrocellulose, permeabilized in liquid nitrogen for 20 s, and incubated on Whatmann 3 mm paper soaked with a 1 mg/mL solution of x-gal. Filters were allowed to develop for 1 hr at 30°C before the results were recorded. For the experiment presented in Figure 4C, yeast transformants were patched on to X-gal indicator plates as described (Ausubel et al., 1991). Quantitative β -galactosidase assays were performed as described (Ausubel et al., 1991).

Western Blotting

Yeast transformants expressing the appropriate LexA fusion were grown in synthetic media lacking histidine to an OD₆₀₀ of 0.4–0.6. Approximately 1.5 mL of mid-log culture was harvested by centrifugation at 12,000 \times g for 2 min. Cell pellets were immediately resuspended in 50 μ l 2 \times SDS sample buffer (Ausubel et al., 1991) containing 10% 2-mercaptoethanol and frozen on dry ice. Samples were then boiled for 5 min and allowed to cool. Protein concentration of each sample was determined by the Lowery method (Harlow and Lane, 1988). Thirty micrograms of each sample was fractionated on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to Immobilon membrane (Millipore). LexA proteins were detected with a rabbit polyclonal LexA antibody (kindly provided by Roger Brent) and a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody using electrochemiluminescence as described by the manufacturer (Amersham). HeLa S3 cells were lysed in 2 \times SDS sample buffer, boiled 10 min, and centrifuged for 5 min to remove insoluble material. HeLa S3 cell extract (35 μ g) was used for Western blotting assays using the method described above.

Transient Transfection Assays

Transient transfection of HeLa S3 cells was performed using the Superfect reagent as described previously (Massari et al., 1998). HeLa cells were transiently transfected with the 250 ng of the appropriate expression plasmids and 1 μ g of 5 \times GAL4 LUC reporter plasmid. Twenty-five nanograms of a CMV- β -gal was cotransfected as a control for transfection efficiency. The total amount of DNA used in each sample was adjusted to 5 μ g by adding the pBluescript II plasmid. For Western blot analysis of GAL4-AD1 and GAL4-Rtg3p(1–99) fusions, 10 μ g of expression vector was transfected into 10⁶ HeLa S3 cells using Superfect reagent according to the manufacturer's instructions (Qiagen). Cells were harvested 48 hr posttransfection and processed as described above for immunodection.

Purification of HAT Complexes and GST Pulldown Assays

Bacterial strain BL21(DE3) was transformed with the appropriate GST-AD1 or GST-Rtg3p(1–99) expression plasmid and grown to an OD₆₀₀ of 0.6. Cultures were subsequently induced for 2 hr with 0.4 mM IPTG, and cells were harvested by centrifugation and lysed by sonication six times in 30 s bursts. GST fusions were purified from the clarified bacterial lysates using glutathione-agarose beads as described previously (Ausubel et al., 1991). Purified proteins were dialyzed against the following buffer: 100 mM NaCl, 50 mM HEPES (pH 7.5), 10% glycerol, 0.1% Tween-20, 0.5 mM DTT, 0.5 mM PMSF, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin A. GST fusion protein concentration was estimated by Coomassie brilliant blue staining of samples run on an SDS-polyacrylamide gel. Yeast whole-cell extracts and isolation of HAT complexes were performed as described (Grant et al., 1997), except that the order of the columns was modified. The SAGA complex was purified over Ni²⁺-NTA agarose (Qiagen), followed by Mono-Q HR 5/5 (Pharmacia), Mono-S HR 5/5 (Pharmacia), histone agarose (Sigma), and Superose 6 HR 10/30 (Pharmacia) columns. The GST pulldown assay was performed as described (Grant et al., 1998b; Utley et al., 1998).

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