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The Human Cut Homeodomain Protein Represses Transcription from the c-*myc* Promoter

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Studies of the c-*myc* promoter have shown that efficient transcription initiation at the P2 start site as well as the block to elongation of transcription require the presence of the ME1a1 protein binding site upstream of the P2 TATA box. Following fractionation by size exclusion chromatography, three protein-ME1a1 DNA complexes, a, b, and c, were detected by electrophoretic mobility shift assay. A cDNA encoding a protein present in complex c was isolated by screening of an expression library with an ME1a1 DNA probe. This cDNA was found to encode the human homolog of the *Drosophila* Cut homeodomain protein. The bacterially expressed human Cut (hu-Cut) protein bound to the ME1a1 site, and antibodies against hu-Cut inhibited the ME1a1 binding activity c in nuclear extracts. In cotransfection experiments, the hu-Cut protein repressed transcription from the c-*myc* promoter, and this repression was shown to be dependent on the presence of the ME1a1 site. Using a reporter construct with a heterologous promoter, we found that c-*myc* exon 1 sequences were also necessary, in addition to the ME1a1 site, for repression by Cut. Taken together, these results suggest that the human homolog of the *Drosophila* Cut homeodomain protein is involved in regulation of the c-*myc* gene.

The control of c-*myc* expression has been shown to be critical for both cellular proliferation and differentiation (10, 18, 30, 31, 41). The c-*myc* gene belongs to the family of immediate-early response genes whose expression is activated by a variety of mitogenic stimuli in quiescent fibroblasts and lymphocytes (1). Inhibition of c-*myc* expression in proliferating cells lead to growth arrest (9, 12, 29, 46), suggesting that c-*myc* is required for continuous cell proliferation. Induction of c-*myc* expression in quiescent cells was also found to be essential for the transition from G_0 to G_1. However, in contrast to expression of other immediate-early response genes, c-*myc* expression is not restricted to this brief G_0-to-G_1 transition period but rather remains constant through the cell cycle in proliferating cells (21, 37, 45). Upon induction of cellular differentiation in a variety of cell lines, c-*myc* expression was rapidly down-regulated (15, 17, 25, 38, 42, 47). The importance of c-*myc* down-regulation during differentiation was further evidenced from the effect of introducing sense or antisense c-*myc* sequences into cells. The presence of antisense RNA or DNA, which reduced c-*myc* RNA levels, triggered the differentiation of HL60 and MEL cells, whereas constitutive c-*myc* expression from a transfected plasmid blocked cellular differentiation (11, 14, 23, 24, 34, 35, 50).

Transcription of the c-*myc* gene is initiated from two major start sites termed P1 and P2, which are located 164 bp apart in the mouse. P2 is the predominant initiation site, giving rise to 75 to 90% of c-*myc* mRNAs (31, 41). Expression of c-*myc* is controlled by intricate regulatory mechanisms. Several positive and negative *cis*-acting elements have been identified both upstream and downstream of the transcription initiation sites, and presumably these *cis*-acting elements coordinate c-*myc* expression in response to external stimuli. Regulation has been shown to occur at multiple levels, including both transcription initiation and elongation, mRNA stability, translation, and protein stability (41).

Several *cis*-acting regulatory elements have been identified between the P1 and P2 initiation sites. The ME1a1 protein binding site was defined by DNase footprinting analysis as a 25-bp region situated just upstream of the P2 TATA box (3). From deletion analysis, the ME1a1 site was shown to be important for efficient initiation at the P2 start site (3, 8, 32). Deletion or mutation of this site strongly decreased the level of P2-initiated transcripts (8, 32). Aside from its effect on transcription initiation, the ME1a1 site was also shown to be involved in the regulation of transcription elongation. Thus, increased readthrough transcription was observed following deletion of the ME1a1 site from the c-*myc* promoter (32). In addition, using a construct in which c-*myc* exon 1 sequences were placed downstream of a heterologous promoter, we showed that transcription elongation was greatly reduced following insertion of the ME1a1 site upstream of the TATA box (16). Moreover, mutated versions of the ME1a1 sites were used to establish a correlation between in vitro binding to cellular factors and the ability to confer block to transcription elongation in vivo, strongly implicating sequence-specific transcription factors in the control of transcription elongation (16).

In light of the importance of the ME1a1 site in the regulation of both transcription initiation and elongation, we set out to characterize the cellular factors that interact with this site. In this report, we demonstrate that three cellular factors bind to ME1a1. By screening a cDNA expression library with an ME1a1 DNA probe, we isolated a cDNA encoding the human homolog of the *Drosophila* Cut protein. We show that the human Cut (hu-Cut) protein binds to the ME1a1 binding site and represses transcription from the c-*myc* promoter.

MATERIALS AND METHODS

Preparation of nuclear extracts and size exclusion chromatography. HeLa cell nuclear extracts were prepared by the procedure of Dignam et al. (13). Extracts were fractionated on a Waters Protein Pak Glass 300 SW size exclusion column,

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using a Waters model 625 high-pressure liquid chromatograph (HPLC). Usually, 1 mg of crude nuclear extract was applied to the column and run at a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected.

λgt11 library screening. The human placenta λgt11 library was a generous gift from Morag Park. Screening was performed basically as described by Singh et al. (39). Briefly, 2×10^4 PFU was plated on 150-mm-diameter plates and incubated at 37°C for 3 h. The plates were overlaid with isopropylthiogalactopyranoside (IPTG)-saturated nitrocellulose filters and incubated for 4 h. The filters were then removed, a second IPTG-saturated nitrocellulose filter was overlaid on the plate, and the plate was incubated for another 4 h. The filters were then transferred to BLOTTO (50 mM Tris [pH 7.5], 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% powdered milk) and incubated for 1 h. They were transferred to binding buffer (25 mM NaCl, 10 mM Tris [pH 7.5], 1 mM $MgCl_2$, 5 mM EDTA [pH 8.0], 1 mM dithiothreitol) and incubated for 12 h. The filters were then hybridized for 4 h with concatemerized copies of the ME1a1 binding site (16) (2×10^6 cpm/ml) and poly(dI-dC)-poly(dI-dC) (5 μg/ml; Pharmacia). They were washed four times for 8 min each time with binding buffer, patted dry, and exposed for 12 to 24 h on Kodak X-AR film with intensifying screens at −70°C. By this approach, one cDNA, called 76.1 was isolated. It contained sequences from nucleotide 3061 to 5374 according to the numbering of the CCAAT displacement protein (CDP) cDNA (33). To obtain a longer cDNA, the library was rescreened with cDNA 76.1 as a probe. A second cDNA, called 76.2, that was isolated is composed of sequences from nucleotides 1605 to 5374. This cDNA contained the coding regions for all three Cut repeats, the homeodomain, and the carboxy-terminal region.

Plasmid construction. All *myc* constructs have been described previously: pMBgCAT (49), Δ46 (3), and MEC and MEC-ME1a1 (16). Constructs Gal4-E1b-CAT and GAL4-VP16 have been described elsewhere (27, 38b). The pSG-Cut expression vector was generated by cloning cDNA 76.2 in the pSG424 vector (38b), digested with *Bgl*II and *Bam*HI, which removed the Gal4 DNA binding domain. As a control, the *Bgl*II-*Bam*HI-digested pSG424 vector was religated, generating the pSG vector. The ME1a1-CAT construct was made by inserting six ME1a1 oligonucleotides into the *Xba*I site of the Gal4-E1b-CAT construct. E1-CAT and ME1a1-E1-CAT were constructed by inserting the blunt-ended *Hin*dIII-*Bgl*II fragment of c-*myc* exon 1 in the *Sma*I site of the Gal4-E1b-CAT and ME1a1-CAT constructs, respectively. The hu-Cut bacterial expression vector GST (glutathione *S*-transferase)-Cut was constructed by cloning cDNA 76.1 into the *Eco*RI site of the bacterial expression vector pGEX-2T (Pharmacia).

Bacterial expression and purification of recombinant protein. *Escherichia coli* DH5 bacterial cells containing the GST-Cut expression vector were grown to an optical density of 0.5, IPTG was added to a final concentration of 1 mM, and cells were grown for 90 min and harvested by centrifugation. Bacterial cell lysates were prepared by sonication, and recombinant protein was purified with glutathione-Sepharose 4B beads (Pharmacia) which were extensively washed with phosphate-buffered saline. The recombinant protein was eluted with 25 mM glutathione (pH 8.0). A Bradford assay (Bio-Rad) was performed to determine the protein concentration, using bovine serum albumin as control.

Generation of hu-Cut antibodies. To generate polyclonal antibodies against hu-Cut, rabbits were injected with 500 μg of purified bacterial fusion protein in Freund's complete adjuvant. The animals were boosted twice with 250 μg of protein, and serum was collected 10 days after the last boost. The polyclonal antibodies were purified by affinity chromatography. Because antibodies directed against GST may be produced, the serum was passed through two GST affinity columns to remove them. The flowthrough depleted of GST antibodies was then applied to a GST-Cut affinity column to isolate antibodies against hu-Cut. Monoclonal antibodies were generated by Molecular Immunogenetics (San Andreas, Calif.), using the bacterially expressed fusion protein. They were purified on a protein A affinity column (Pharmacia).

EMSA. Electrophoretic mobility shift assays (EMSA) were performed with oligonucleotides encoding the ME1a1 binding site (16) and either 50 ng of purified bacterially expressed fusion protein or 3 μl of fractionated HeLa cell nuclear extracts. To deplete the extracts of hu-Cut, 50 ng of purified polyclonal antibodies, 150 ng of monoclonal antibodies, or 100 ng of preimmune antibodies was added to 30 μl of fractionated extracts and incubated at room temperature for 45 min. Protein G-Sepharose (Pharmacia) was then included for 30 min, and the immune complex was removed by centrifugation. Binding reactions were performed in 25 mM NaCl–10 mM Tris (pH 7.5)–1 mM $MgCl_2$–5 mM EDTA (pH 8.0)–5% glycerol–1 mM dithiothreitol, in a final volume of 20 μl, with 1 μg of poly(dI-dC) and 100 ng of specific competitor, where specified. Specific competitors were oligonucleotides encoding either wild-type, mutant 1, or mutant 2 ME1a1 binding sites (16). Samples were incubated at room temperature for 5 min, 20,000 cpm (100 pg) of end-labeled probe was added, and the samples were incubated for 15 min. The samples were then loaded on a 5% polyacrylamide gel (30:1) and electrophoresed at 8 V/cm for 3 h in 50 mM Tris–0.38 M glycine–1 mM EDTA (pH 8.5). The gel was dried and visualized by autoradiography.

CAT assays. 293 cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum. Plasmid DNA was introduced by the calcium phosphate precipitation technique (48). Typically, 5 μg of reporter construct and 6 μg of pSG or pSG-Cut were added to 3×10^5 cells in 100-mm-diameter plates. Where specified, 5 μg of Gal4-VP16 expression vector was transfected along with 5 μg of either pSG or pSG-Cut for a total of 10 μg of expression vectors. CMV-β-gal (5 μg) was added to all transfections as a control for transfection efficiency. The cells were harvested 48 h after transfection. Extracts were prepared and chloramphenicol acetyltransferase (CAT) assays performed as described previously (19). The CAT assays were quantitated on a PhosphoImager (Fuji).

RESULTS

Multiple cellular factors bind to ME1a1. The ME1a1 binding site has been shown to play an important role in the regulation of both transcription initiation and elongation within the c-*myc* gene. As a first step toward the purification of ME1a1-binding proteins, a HeLa crude nuclear extract was fractionated on an HPLC size exclusion column, and the fractions were assayed by EMSA for binding to the ME1a1 DNA sequence. As a probe, we used double-stranded oligonucleotides encoding the 25-bp region previously shown to be protected in DNase footprinting assay (3). Three protein-DNA complexes, termed a, b, and c, were detected upon fractionation of the crude extract (Fig. 1). Complex a corresponds to the unique protein-DNA complex previously detected in crude HeLa nuclear extracts (16). The factor responsible for complex a eluted in a broad peak and was detected in fractions 2 to 7. Complex b was present in fractions 6 to 8, whereas complex c was detected in fractions 2 and 3, corresponding to molecular masses of 75 to 85 kDa and 180 to 200 kDa, respectively. Thus, in fractionated extracts, at least three proteins were found to

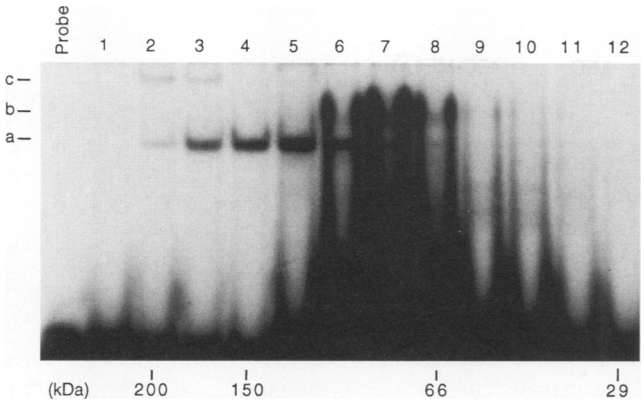

FIG. 1. Fractionation of HeLa nuclear extract by size exclusion chromatography. HeLa nuclear extract was fractionated on a Protein Pak Glass 300 SW size exclusion column, and 0.5-ml fractions were collected. EMSA were performed with the ME1a1 oligonucleotide (16). The void volume was not collected; therefore, fraction 1 corresponds to the first proteins eluting from the column. Fraction numbers are indicated above the lanes. The three protein-DNA complexes, a, b, and c, are indicated. The relative positions of several molecular weight markers are indicated at the bottom.

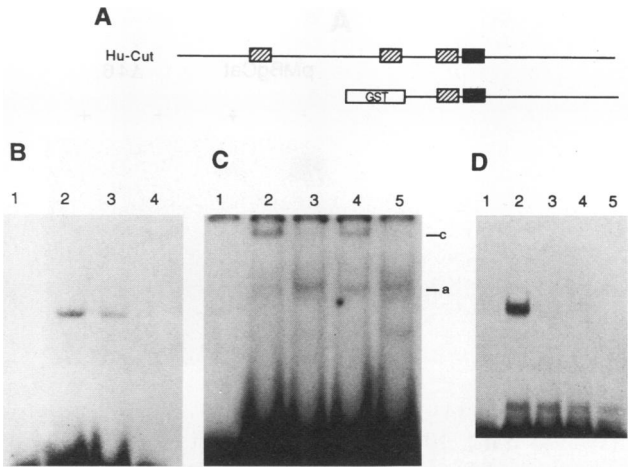

FIG. 2. Binding of hu-Cut to ME1a1. (A) Schematic representation of the hu-Cut protein as well as the GST/Cut fusion protein expressed in bacteria. The Cut repeats (hatched boxes) and the homeodomain (black box) are depicted. (B) EMSA using purified bacterially expressed fusion protein and the ME1a1 binding site. Lanes: 1, probe alone; 2, 50 ng of fusion protein; 3, 50 ng of fusion protein plus 1 μg of poly(dI-dC); 4, 50 ng of fusion protein plus 100 ng of unlabeled ME1a1 oligonucleotides. (C) EMSA using fraction 2 of the size exclusion chromatography depleted of hu-Cut with monoclonal and polyclonal antibodies. Lanes: 1, probe alone; 2, no antibodies; 3, incubated with purified monoclonal antibodies; 4; incubated with purified antibodies from the prebleed; 5, incubated with purified polyclonal antibodies. Complexes a and c are indicated. (D) EMSA using purified bacterially expressed fusion protein and the ME1a1 binding site. Lanes: 1, probe alone; 2, 50 ng of fusion protein; 3 to 5, 50 ng of fusion protein plus 100 ng of unlabeled specific competitor DNAs (wild-type, mutant 1, and mutant 2 ME1a1 oligonucleotides, respectively).

form a complex with the ME1a1 binding site, suggesting that potentially three factors can bind to this site in the cell.

A cDNA encoding the human homolog of the *Drosophila* Cut homeodomain protein is isolated by screening an expression library with the ME1a1 site. To isolate cDNAs encoding proteins that bind to the ME1a1 site, a human placenta λgt11 cDNA expression library was screened with multimerized copies of the ME1a1 site. From 1 million plaques screened and after three rounds of purification, one positive clone was isolated. DNA sequencing of the cDNA and a search of GenBank DNA sequences demonstrated that the isolated cDNA was 100% homologous to the cDNA encoding CDP (33), the human homolog of the *Drosophila* Cut homeodomain protein. The human and *Drosophila* Cut proteins exhibit similar structural organizations, with a region of about 60 amino acids predicted to form an amphipathic coiled-coil structure, three 73-amino-acid regions called Cut repeats, and a Cut-type homeodomain (33). These two proteins are 50% identical (68% highly conserved) within these domains, but the rest of the proteins appear to be quite divergent (33). Two other Cut-related proteins, Clox and Cux, have recently been cloned from dog and mouse cells. These proteins are similarly related to *Drosophila* Cut (2a, 45a).

A bacterially expressed GST/hu-Cut fusion protein binds to the ME1a1 site. To confirm that it binds to the ME1a1 site, a portion of the hu-Cut protein was expressed in bacteria as a GST fusion protein. The recombinant protein, schematically illustrated in Fig. 2A, contained Cut repeat 3, the homeodomain, and the carboxy terminus fused to GST. EMSA were performed with purified GST/Cut fusion proteins and labeled ME1a1 oligonucleotides as a probe. We detected one protein-DNA complex which was not competed for with a 1,000-fold excess of poly(dI-dC) but was completely eliminated by a 100-fold excess of unlabeled ME1a1 (Fig. 2B, lanes 2 to 4). Therefore, a bacterially expressed hu-Cut protein can specifically bind to the ME1a1 DNA sequence.

Antibodies against hu-Cut prevent the formation of complex c. To test whether hu-Cut was one of the ME1a1-binding proteins detected in fractionated nuclear extracts, polyclonal and monoclonal antibodies were generated against the bacterially expressed fusion protein. In Western blots (immunoblots) with HeLa crude nuclear extracts, both purified antibodies specifically recognized a protein of the expected 200-kDa molecular mass (data not shown). When EMSA were performed with fraction 2 from size exclusion chromatography, protein-DNA complexes a and c were detected (Fig. 2C, lane 2). Prior incubation of fraction 2 with either monoclonal or polyclonal anti hu-Cut antibodies specifically eliminated complex c (Fig. 2C, lanes 3 and 5). This was not due to a general inhibitory effect, since the formation of complex a was not reduced in the presence of anti-hu-Cut antibodies. The addition of preimmune serum had no effect on either complex a or complex c (Fig. 2C, lane 4). We conclude that the hu-Cut protein is involved in the formation of complex c in fractionated nuclear extracts.

The hu-Cut protein represses transcription from the c-*myc* promoter in vivo. To investigate the effect of the hu-Cut protein on c-*myc* expression in vivo, transient cotransfection experiments were performed in 293 cells by using the vector pSGCut as an effector plasmid (Fig. 3D) and two c-*myc*/CAT constructs as reporter constructs. Plasmid pMBgCAT contains c-*myc* exon 1 sequences plus 1,141 bp of 5′ flanking sequences. Plasmid Δ46 derives from pMBgCAT and contains a 16-bp deletion overlapping the ME1a1 site (Fig. 3D). Cotransfection of the hu-Cut expression vector resulted in a two- to threefold reduction in CAT activity from the wild-type c-*myc*/CAT construct. Repression was dependent on the ME1a1 site, since hu-Cut had no effect on Δ46 (Fig. 3A and C). However, the deletion in Δ46 itself reduced CAT activity. This result con-

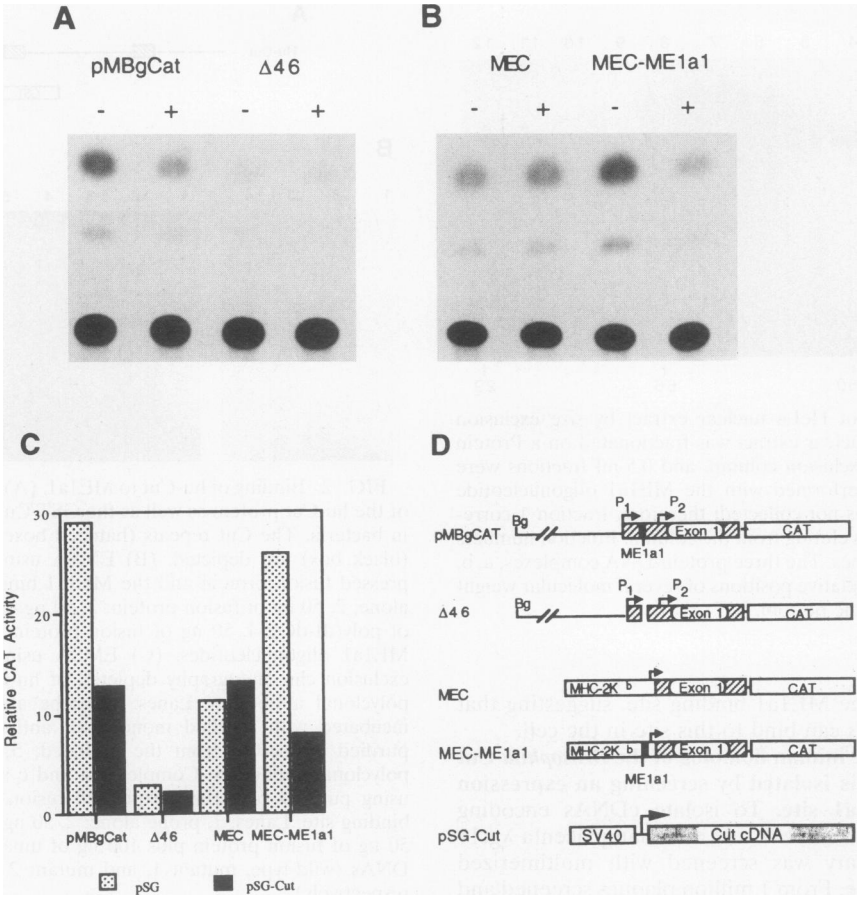

FIG. 3. Cotransfection experiments with c-*myc* promoter constructs and hu-Cut. (A) CAT assay from cotransfection of pMBgCAT and Δ46 with either pSG (−) or pSG-Cut (+). (B) CAT assay from cotransfection of MEC and MEC-ME1a1 with either pSG (−) or pSG-Cut (+). (C) Graphic representation of relative CAT activity for the various c-*myc* promoter constructs. The results are averages of two independent transfections. CAT activities were corrected for transfection efficiencies by β-galactosidase assays. (D) Schematic representation of the constructs used for transfection.

firms the importance of the ME1a1 site for transcription initiation and raises the possibility that hu-Cut had no effect on Δ46 because of its low transcription level. Since expression was much lower with Δ46, the absence of repression by hu-Cut may have resulted from the low transcription level. To overcome this problem, we inserted the ME1a1 site into a heterologous promoter construct (Fig. 3D) in which the *H-2K*^{b} major histocompatibility class gene promoter was linked to the c-*myc* first exon. Cotransfection of hu-Cut had no effect on expression of the original MEC plasmid; however, a threefold repression in CAT activity was observed with the MEC-ME1a1 construct (Fig. 3B and C). Taken together, these results indicate that hu-Cut can repress transcription from the c-*myc* promoter in vivo and that the ME1a1 site is required for repression.

The reporter constructs tested in Fig. 3 all contained exon 1 sequences between the initiation site and the CAT coding sequences. To test whether the ME1a1 site was sufficient to repress transcription, we inserted six ME1a1 sites in the $(Gal4)_5$-E1b CAT construct, which contains the minimal E1b promoter plus five binding sites for the Gal4 DNA binding domain. Transcription was activated following cotransfection of a vector expressing Gal4/VP16. hu-Cut had no effect on basal transcription (Fig. 4, lane 1). In addition, hu-Cut did not affect Gal4/VP16-activated transcription (Fig. 4, lane 2 versus lane 3). We then tested whether repression could be restored by the addition of c-*myc* exon 1 sequences. In the presence of both the ME1a1 site and the first exon, hu-Cut repressed CAT activity by three- to fourfold (Fig. 4, lanes 5 and 6). However, no repression was observed when ME1a1 binding sites were removed from the reporter construct, leaving only c-*myc* exon 1 sequences from +138 to +516 relative to the P1 start site (Fig. 4, lane 7 versus lane 8). These results demonstrate that the ME1a1 site is necessary but not sufficient for repression by hu-Cut and that other sequences within the first exon are also required.

Mutations that reduce the block to elongation of transcription do not affect binding by hu-Cut. We previously established a correlation between the block to elongation of transcription and binding to ME1a1 (16). In these studies, binding experiments were performed with whole nuclear extract and therefore detected only binding of complex a. We therefore verified whether mutations in ME1a1 which reduced the block to elongation of transcription also reduced binding by hu-Cut. As shown in Fig. 2D, binding of a GST/hu-Cut fusion protein to an ME1a1 probe is competed for equally efficiently by both mutated oligonucleotides (Fig. 2D, lane 2 versus lanes 3 and 4). These results suggest that binding of hu-Cut to ME1a1 is not involved in the regulation of transcription elongation.

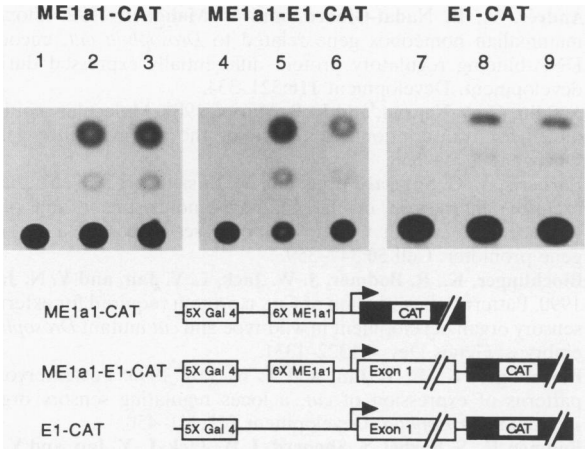

FIG. 4. Cotransfections with minimal promoter constructs. Cotransfections were performed with either ME1a1-CAT, ME1a1-E1-CAT, or E1-CAT as the reporter construct and either 10 μg of pSG-Cut (lanes 1, 4, and 7), 5 μg of Gal4-VP16 plus 5 μg of pSG (lanes 2, 5, and 8), or 5 μg of Gal4-VP16 plus 5 μg of pSG-Cut (lanes 3, 6, and 9). The reporter constructs are schematically represented at the bottom.

DISCUSSION

The ME1a1 protein binding site is a 25-bp region of the c-*myc* promoter that plays an important role in the regulation of c-*myc* expression. ME1a1 was shown to be necessary for efficient transcription initiation at the P2 start site as well as for the control of transcription elongation. In this report, fractionation studies indicated that three different transcription factors could interact with the ME1a1 binding site. This 25-bp region therefore comprises three, possibly overlapping, recognition sites for transcription factors that are likely to compete for binding. By screening a cDNA expression library with an ME1a1 DNA probe, we isolated a cDNA encoding the human homolog of the *Drosophila* Cut homeodomain protein (33). Several lines of evidence suggest that the human Cut homeodomain protein binds to the ME1a1 sequence and is involved in the control of c-*myc* expression. First, in EMSA, bacterially expressed recombinant human Cut proteins bound to the ME1a1 sequence. Second, incubation of nuclear extracts with either monoclonal or polyclonal anti-hu-Cut antibodies resulted in the specific depletion of the ME1a1 binding activity c. The same antibodies did not inhibit binding activity a, and serum from nonimmunized animals did not affect either binding activity. Third, in transient cotransfection assays, the human Cut protein repressed expression from an intact c-*myc* promoter and from a heterologous promoter in which one copy of the ME1a1 binding site had been inserted, indicating that repression by hu-Cut was dependent on the presence of the ME1a1 binding site.

Cotransfection experiments using heterologous promoter constructs indicated that in addition to the ME1a1 site, c-*myc* exon 1 sequences were necessary for the repression by hu-Cut. We do not currently know the basis for this requirement; however, several hypotheses can be envisaged. It is possible that hu-Cut repressed expression at the level of transcription elongation when c-*myc* exon 1 sequences were inserted between the transcription start site and the CAT coding sequences. We did not obtain any evidence of modulation at the level of transcription elongation (data not shown), but a block to elongation of transcription is not detected, at least in our hands, in run-on assays using nuclei prepared from transiently transfected cells. Formal testing of this hypothesis will therefore await the establishment of stably transfected cells expressing hu-Cut under the control of an inducible promoter. Another set of results, however, make this hypothesis rather unlikely, since mutations in ME1a1 that were previously shown to reduce the block to elongation of transcription did not reduce binding by hu-Cut. Taken together, these results suggest that hu-Cut represses c-*myc* at the level of transcription initiation and that, in addition to ME1a1, at least one other *cis*-acting regulatory element located in c-*myc* exon 1 is required for this modulation. We favor the hypothesis that binding of hu-Cut to the ME1a1 site is facilitated by protein-protein interactions with other sequence-specific factors, possibly even hu-Cut itself, that interact with other sites within c-*myc* exon 1.

Recently, two independent groups have isolated the cDNAs for zinc finger proteins that bind to ME1a1. These proteins were designated MAZ (*myc*-associated zinc) (8) and ZF87 (36). It remains to be determined whether these two isolates represent the same zinc finger protein and whether either one or both of these are responsible for the formation of ME1a1 complexes a and b. ZF87 was cloned as a protein that binds to the ME1a2 site, upstream of ME1a1; however, it can also bind to ME1a1. The factor responsible for complex a also can bind to both sites (16a). Taken together, these results suggest that ZF87 is involved in the formation of complex a.

Several sequences have been demonstrated to serve as binding sites for mammalian Cut proteins. These include the FP sequence (GCTTTTCAGTTGACCAATGATTATTAGC CAATTTCTGATAAAAGAAAAGGAAACCGATTGC), the C3S sequence (AAAAGAAGCTTATCGATACCGT) (22), and the ME1a1 sequence (GGAAAAAGAAGGGAGGGG AGGGATCC) for the human CDP/hu-Cut protein and the βe2 subelement (GATCTGTGAGCTGTGGAATGTAAGG GAGATC) for the canine Clox (Cut-like homeobox) protein. We note that the FP, ME1a1, and C3S sequences share an AAAAGAA motif and that an AAGGGAG motif is present in both ME1a1 and the βe2 subelement. Whether these motifs are sufficient for binding by Cut remains to be determined. In addition, we and others have recently found that Cut repeats 1, 2, and 3 function as specific DNA binding domains (22, 45a). The hu-Cut protein may therefore contain up to four specific DNA binding domains: the three Cut repeats and the Cut homeodomain. Future work will address which of these DNA binding domains is involved in targeting hu-Cut to the c-*myc* promoter.

Studies in *Drosophila melanogaster* found that Cut was necessary for proper cell type specification in several tissues, including the external sense organs, the Malpighian tubules, muscles, and the tracheal system (5–7, 28). Thus, in various *cut* mutants, these structures did not develop, and when tested, ectopic expression of Cut did not induce cell differentiation but changed the cell type specificity of differentiating cells. Since no target genes have been identified in *D. melanogaster*, it is not known whether Cut acts as a positive or negative regulator of gene expression in specifying cell identity. The recent isolation of three mammalian Cut-related genes, the CDP/hu-Cut protein in humans, Clox in dogs, and Cux in mice, may provide us with some clues about the biochemical function of Cut (references 2a, 33, and 45a and this report). CDP has been shown to bind to upstream regulatory sequences of several genes, including genes encoding the cytochrome β heavy chain (gp91-phox) (40), human γ-globulin (43, 44), and the sea urchin histone H2B (4). Expression of these genes coincides with down-regulation of CDP binding activity upon cellular differentiation, suggesting that CDP may repress their expres-

sion in undifferentiated cells. In transient transfection experiments, Clox and Cux have been shown to repress transcriptional activity of the β major histocompatibility complex enhancer and of the *Ncam* promoter, respectively (2a, 45a). In the present study, we demonstrated that hu-Cut could repress expression from the c-*myc* promoter. Taken together, these results suggest that Cut acts as a negative regulator of gene transcription. We cannot, however, exclude the possibility that Cut also acts as a positive regulator of gene expression in certain situations.

The study of negative transcriptional regulators in recent years has uncovered multiple mechanisms by which repression can be exerted. Some repressors act by interacting with specific DNA binding sites overlapping those of transcriptional activators, thereby competing for DNA site occupancy and preventing the action of activators on the targeted promoters. Other repressors interact directly with an activator, thereby inhibiting its function, a mechanism referred to as quenching. Alternatively, binding of the repressor may directly repress transcription, a mechanism termed active repression. Binding of CDP to several regulator sequences has been shown to prevent the binding of CF1, a transcriptional activator. Thus, it has been proposed that CDP/hu-Cut represses gene expression as a result of competitive binding (2a, 33). In cotransfection experiments with the c-*myc* promoter, it is conceivable that hu-Cut also functions by preventing the interaction of a transactivator with the ME1a1 site. Indeed, the low level of expression observed with a construct that has a 16-bp deletion within the ME1a1 sequence suggests that ME1a1 is also a binding site for a transcriptional transactivator (mutation Δ46) (reference 32 and this study). On the other hand, we also observed repression by Cut when the ME1a1 site was inserted within the *H-2K*^{b} major histocompatibility complex promoter and the GAL4-E1b minimal promoter. The fact that repression was effected when the ME1a1 binding site was moved to two different heterologous promoters argues against competition with an activator as the mechanism for repression but instead suggests that Cut acts as an active repressor. In accordance with this hypothesis, the carboxy-terminal region of hu-Cut contains an alanine-rich domain. Similar alanine-rich sequences have been identified within the repressor domain of the Krüppel, even-skipped, and Engrailed homeodomain proteins (20, 26). Future experiments will determine whether the carboxy-terminal region of hu-Cut is involved in repression.

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