COUP-TF (chicken ovalbumin upstream promoter transcription factor)-interacting protein 1 (CTIP1) is a sequence-specific DNA binding protein

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Chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting proteins 1 and 2 [CTIP1/Evi9/B cell leukaemia (Bcl) 11a and CTIP2/Bcl11b respectively] are highly related C_2H_2 zinc finger proteins that are abundantly expressed in brain and the immune system, and are associated with immune system malignancies. A selection procedure was employed to isolate high-affinity DNA binding sites for CTIP1. The core binding site on DNA identified in these studies, 5′-GGCCGG-3′ (upper strand), is highly related to the canonical GC box and was bound by a CTIP1 oligomeric complex(es) *in vitro*. Furthermore, both CTIP1 and CTIP2 repressed transcription of a reporter gene harbouring a multimerized CTIP binding site, and this repression was neither reversed by trichostatin A (an inhibitor of known class I and II histone deacetylases) nor stimulated by co-transfection of a COUP-TF family member. These results demonstrate that CTIP1 is a sequence-specific DNA binding protein and a *bona fide* transcriptional repressor that is capable of functioning independently of COUP-TF family members. These findings may be relevant to the physiological and/or pathological action(s) of CTIPs in cells that do not express COUP-TF family members, such as cells of the haematopoietic and immune systems.

Key words: Bcl11b, Evi9/Bcl11a, leukaemia, oligomerization, transcriptional repression.

INTRODUCTION

C_2H_2 zinc finger proteins comprise the largest family of transcription factors in eukaryotes [1], and play important roles in development in both animals [2–14] and plants [15–17]. The complete sequence of the *Caenorhabditis elegans* genome revealed that 157 open reading frames encode C_2H_2 zinc finger proteins, whereas some 40 open reading frames in the *Saccharomyces cerevisiae* genome encode such proteins [18]. The *Drosophila* genome encodes over 400 C_2H_2 zinc finger proteins, while nearly 900 are present in the human genome [1], many of which function as important transcriptional regulators, such as the Ikaros family of proteins implicated in lymphocyte commitment and differentiation [19].

C_2H_2 zinc finger proteins are typically modular proteins composed of C_2H_2 zinc binding motifs, which generally confer sequence-specific DNA binding activity, and transcriptional regulatory domains [20]. Structurally, the C_2H_2 zinc finger motif is comprised of a β-hairpin followed by an α-helix that folds around a single zinc ion [21]. Sequence-specific recognition of DNA is mediated primarily by interactions between the variable amino acids within and around the α-helix and nucleotides within the major groove of DNA [21]. Several studies have attempted to establish a recognition code for the DNA binding specificity of C_2H_2 zinc finger proteins (reviewed in [20,22]). Although preferences for certain amino acid side chains to contact specific bases have been identified, the structural details are complex, and a simple recognition code has not been elucidated [20,22].

We previously identified two novel and related C_2H_2 zinc finger proteins, chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting proteins 1 and 2 [CTIP1/Evi9/B cell leukaemia (Bcl) 11a and CTIP2/Bcl11b respectively] [23]. CTIPs mediate transcriptional repression when tethered to a promoter by interaction with a DNA binding protein, such as ARP1, a member of COUP-TF subfamily of orphan nuclear receptors ([23]; D. Avram, unpublished work). Transcriptional repression mediated by CTIP1 [23] and CTIP2 (D. Avram, unpublished work) was found to be insensitive to reversal by trichostatin A (TSA), suggesting that mechanisms other than recruitment of TSA-sensitive class I and II histone deacetylases to the template may underlie CTIP-mediated repression.

Dysregulated expression of either CTIP1 or CTIP2 has been implicated in proliferative diseases in mammals. For example, the mouse CTIP1/Evi9 locus was identified as a site of retroviral integration in BXH2 murine myeloid leukaemia [24], and the human genomic locus of CTIP1, chromosome 2p13 [25], has been found to be translocated [t(2;14)(p13;q32.3)] in human lymphomas and B-cell chronic lymphocytic leukaemias [26]. Moreover, the *CTIP1* gene is located within a chromosomal region that is frequently rearranged in other human neoplasias [27]. The human genomic locus of CTIP2, chromosome 14q32, is associated with a translocation [t(15;14)(q35;q32)] that appears to result in acute T lymphoblastic leukaemia [28]. These findings suggest that aberrant expression of CTIPs in haematopoietically derived cells results in transformation of the cells and contributes to the generation of a malignant phenotype. However, the molecular basis for the transforming activity of aberrantly expressed CTIPs is presently unknown.

CTIP1 and CTIP2 share similarity over large blocks of amino acids [23]. Among these regions of similarity there are two,

Abbreviations used: Bcl, B cell leukaemia; CAT, chloramphenicol acetyltransferase; COUP-TF, chicken ovalbumin upstream promoter transcription factor; CTIP, COUP-TF-interacting protein; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; HEK, human embryonic kidney; NP40, Nonidet P40; RE, response element; *tk*, thymidine kinase gene; TSA, trichostatin A; ZNF3 (etc.), zinc finger 3 (etc.).

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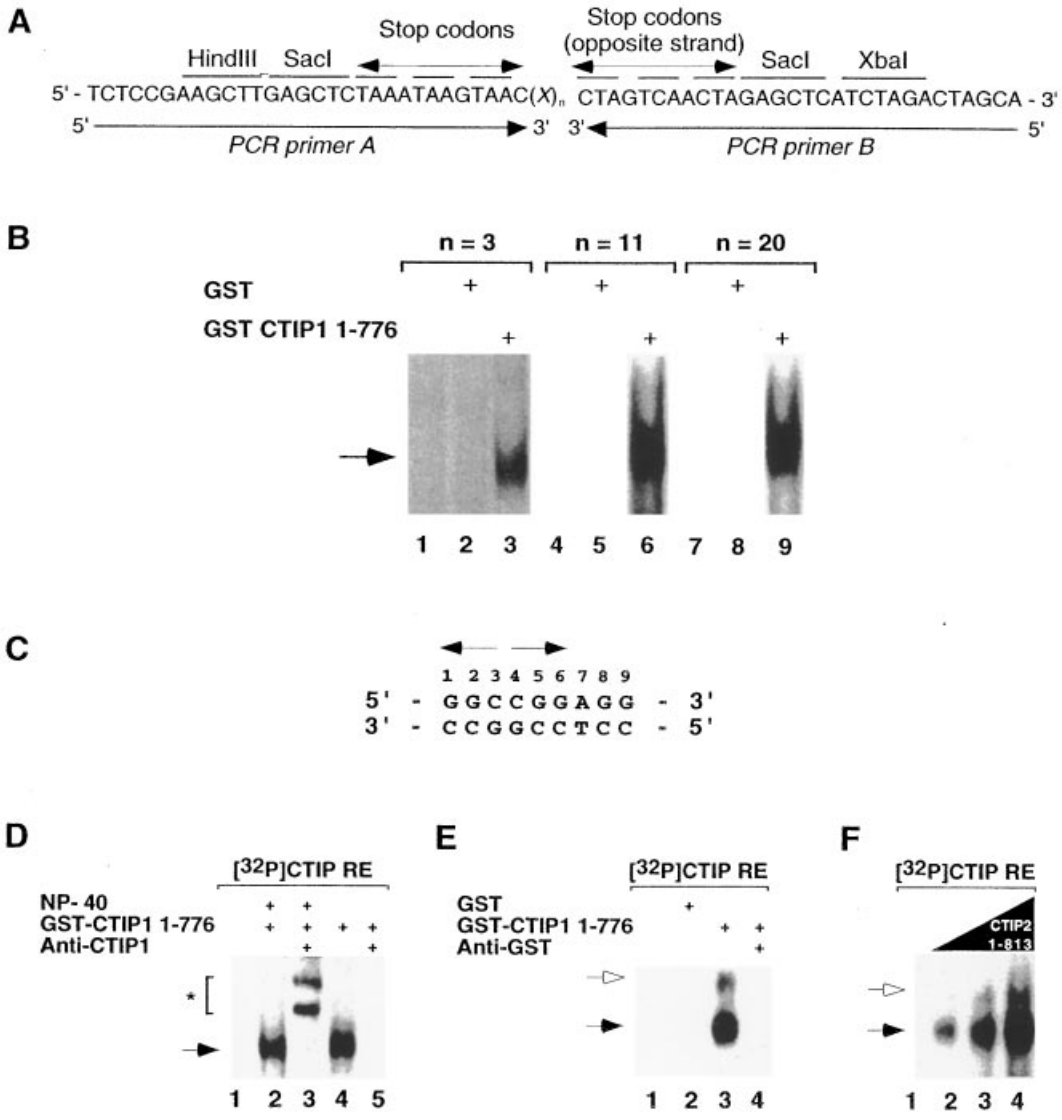


Figure 1 Binding of purified GST–CTIP1 to selected pools of oligonucleotides from independent libraries: consensus DNA binding site for CTIP1

(**A**) Probes used for CTIP1 DNA binding site selection studies. Libraries of double-stranded binding sites containing 3, 11 or 20 bp of random sequence (*n*), flanked by 30 bases of defined sequence, were prepared by extending from PCR primer B using the Klenow fragment of DNA polymerase I. The flanking, defined sequences contained restriction sites for subsequent cloning and stop codons in all three frames. (**B**) EMSA showing binding of GST–CTIP1 to the selected pools of oligonucleotides from the three libraries. Lanes 1, 4 and 7, 'probe only'; lanes 2, 5 and 8, binding reaction containing 5 pmol of purified GST; lanes 3, 6 and 9, binding reaction containing 5 pmol of purified GST–CTIP1-(1–776). Note that lanes 1–3 were derived from a gel different from that shown in lanes 4–9. In all cases, only the retarded complexes are shown (closed arrow), for simplicity. (**C**) Consensus CTIP1 binding site, as determined from sequence analysis of clones from the random 20 bp oligonucleotide library. This sequence was 100% conserved in 12 of 15 clones analysed from this library. (**D**) Anti-CTIP1 antibodies supershift or inhibit CTIP1 · DNA complexes in EMSA studies. Lane 1 contains the ^{32}P-labelled CTIP RE but no protein, and lanes 2–5 contain GST–CTIP1-(1–776) (5 pmol) incubated with the probe. Antiserum raised against amino acids 1–171 of CTIP1 was added as indicated in lanes 3 and 5. Reactions in lanes 2 and 3 contained 0.17% (v/v) NP40. CTIP1 · (CTIP RE) complexes are indicated by an arrow, while the antibody-supershifted complexes are indicated by a bracket and an asterisk. The free probe is not shown for simplicity. (**E**) Inhibition of GST–CTIP1 DNA binding by anti-GST antibodies. EMSA studies were conducted as described above with the indicated reagents in the absence of NP40. Preimmune serum did not affect CTIP1 · (CTIP RE) complex formation (results not shown). CTIP1 · (CTIP RE) complexes are indicated by open and closed arrows, which possibly represent different CTIP1 oligomerization states. (**F**) Binding of GST–CTIP2 to the CTIP1 RE. Increasing amounts (1.25, 2.5 and 5 pmol) of affinity-purified GST–CTIP2-(1–813) (lanes 2–4) were incubated with the ^{32}P-labelled CTIP RE as described above. Lane 1 contains probe only. CTIP2 · (CTIP RE) complexes of different electrophoretic mobility were formed (closed and open arrows), possibly suggesting the formation of multimeric complexes.

centrally located, C_2H_2 zinc fingers, zinc fingers 3 and 4 (ZNF3 and ZNF4 respectively) of both proteins, that exhibit 94% identity [23]. CTIP2 harbours three additional C_2H_2 zinc fingers, ZNF5, ZNF6 and ZNF7, at the C-terminus of the protein, two of which are related to the central zinc fingers of both CTIP1 and CTIP2 (71% identity). CTIP1 is distinctive among mammalian C_2H_2 zinc finger proteins, as only two of its C_2H_2 zinc fingers are grouped, which rather resembles the majority of C_2H_2 zinc finger proteins in yeast [29]. In contrast, three or more zinc fingers are usually grouped in tandem in multicellular eukaryotes and, in this configuration, these motifs may participate in sequence-specific DNA binding [20,21,30,31].

In the present study, a DNA binding site selection technique was employed to determine if CTIP1 binds DNA directly and, if

so, to identify high-affinity binding sites. Herein the identification of a consensus binding site for CTIP1, the CTIP response element (RE), is described. The association of both CTIP1 and CTIP2 with this RE is shown to be functional, as demonstrated by the finding that CTIP1 and CTIP2 repressed transcription from a promoter harbouring the CTIP RE in a TSA-insensitive manner. Also, we demonstrate that CTIP1 participates in homotypic interactions, both in solution and on the CTIP RE.

MATERIALS AND METHODS

Binding site selection

Three libraries of double-stranded DNA containing 3, 11 or 20 bp of random sequence, flanked by 30 bases of defined sequence, were prepared by extending from PCR primer B (see Figure 1A) using the Klenow fragment of DNA polymerase I. The defined sequences contained restriction sites for subsequent cloning, and stop codons in all three frames on both strands to facilitate identification of recombinants using the blue/white selection technique. The resulting random oligomers were then incubated with glutathione S-transferase (GST)–CTIP1-(1–776) bound to glutathione–Sepharose-4B in binding buffer [32,33]. Double-stranded fragments retained on the GST–CTIP1 affinity column were amplified using primers A and B (see Figure 1A), and five additional rounds of selection and amplification were performed. The final, selected pool of oligonucleotides was labelled by amplification using primers A and B (see Figure 1A) in the presence of [α-^{32}P]dCTP, and the product was gel purified and used directly in electrophoretic mobility shift assays (EMSAs). The selected oligonucleotide pools were also subcloned into pCR2.1 (Invitrogen), and random clones from each selected library were subjected to sequence analysis.

EMSAs

DNA binding experiments were conducted using a ^{32}P-labelled, double-stranded probe corresponding to the identified CTIP RE and approx. 5 pmol of affinity-purified proteins. The reactions were carried out in binding buffer consisting of 25 mM Hepes (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM dithiothreitol, 10 % (v/v) glycerol, 100 μg/ml BSA and 2 μg of poly(dI · dC), with or without 0.17 % (v/v) Nonidet P40 (NP40). After a 10 min incubation on ice, samples were loaded on to a 5 % (w/v) acrylamide gel prepared in 0.5 × TBE (1 × TBE = 45 mM Tris/borate, 1 mM EDTA), and electrophoresis was conducted at 4 °C under constant voltage conditions. Antibody supershift experiments were performed by addition of anti-CTIP1 or anti-GST (Bethyl Laboratories Inc., Montgomery, TX, U.S.A.) antibodies or preimmune serum to the reactions after the 10 min incubation on ice. The samples were then incubated for an additional 5 min prior to gel electrophoresis, as described above. All GST fusion proteins were expressed in the BL21 (DE3) plysS strain of *Escherichia coli* and purified on glutathione–Sepharose-4B (Pharmacia) using standard techniques.

GST pull-down experiments

These studies were conducted as previously described [34,35]. Briefly, bait proteins (equimolar quantities of GST or GST–CTIP1 fusion proteins) were bound to gluthathione–Sepharose (Pharmacia) and incubated with [^{35}S]methionine-labelled proteins (CTIP1 or CTIP1 truncation mutants) prepared using the TNT transcription–translation system (Promega). After extensive washing, [^{35}S]methionine-labelled proteins remaining bound to the affinity matrices were eluted under denaturing conditions and separated on SDS/polyacrylamide gels that were then exposed to X-ray film to generate the autoradiographic images shown.

Transfections and reporter assays

Human embryonic kidney (HEK) 293 cells were grown in 10 cm plates and transfected at approx. 60 % confluence using the calcium phosphate method. Cells were harvested 48 h after transfection, and extracts were prepared using standard techniques [36]. Transfection efficiency was normalized using a co-transfected β-galactosidase expression vector, and relative chloramphenicol acetyltransferase (CAT) activity was determined as described previously [23,36].

Site-directed mutagenesis

Site-directed mutagenesis was performed using the Quik-Change mutagenesis kit (Stratagene) following the manufacturer's instructions, and mutants were verified by sequence analyses.

RESULTS

Identification of the consensus DNA binding site for CTIP1

The centrally located, C_2H_2 zinc binding motifs in CTIP1 and CTIP2 are highly conserved, possibly suggesting a conservation of function. We hypothesized that such a region could mediate sequence-specific DNA recognition by the CTIPs, as has been demonstrated for numerous proteins containing similar motifs (reviewed in [22]). To test this hypothesis directly, a DNA binding site selection procedure [32] was employed. Three independent libraries were prepared harbouring 3, 11 and 20 random nucleotides flanked by known sequence, as shown in Figure 1(A). Binding of purified GST–CTIP1 to the selected pools of oligonucleotides from the three libraries is shown in Figure 1(B). The selected pools of oligonucleotides from six rounds of selection were cloned and sequenced. Sequence data have been obtained for 15 independent clones from the selected 20-mer library. Of these 15 clones, 12 were found to harbour an identical sequence, 5′-GGCCGGAGG-3′ (upper strand shown for clarity; Figure 1C), at various positions within the randomized region. This consensus CTIP1 binding site is clearly GC-rich and contains a core motif, 5′-GGCCGG-3′, resembling a canonical GC box (5′-GGGCGG-3′). Clones of similar sequence were obtained from both the selected 3- and 11-mer libraries (results not shown). The consensus CTIP1 binding site [upper strand: 5′-GGCCGGAGG-3′] (Figure 1C) is hereafter referred to as the CTIP RE.

Both CTIP1 and CTIP2 bind to the CTIP RE *in vitro*

The specificity of DNA binding by CTIP1 was confirmed using anti-CTIP1 and anti-GST antibodies to demonstrate that GST–CTIP1 was a component of the observed protein–DNA complexes. For these experiments, an affinity-purified anti-CTIP1 antibody (raised against amino acids 1–171 of CTIP1) was utilized. This antibody, but not preimmune serum or an unrelated affinity-purified antibody {raised against general receptor for phosphoinositides-1 (GRP1)-associated scaffold protein [37]; results not shown}, supershifted the GST–CTIP1 · DNA complex, indicating the presence of CTIP1 in the retarded complex (Figure 1D, compare lanes 2 and 3). However, this antibody-

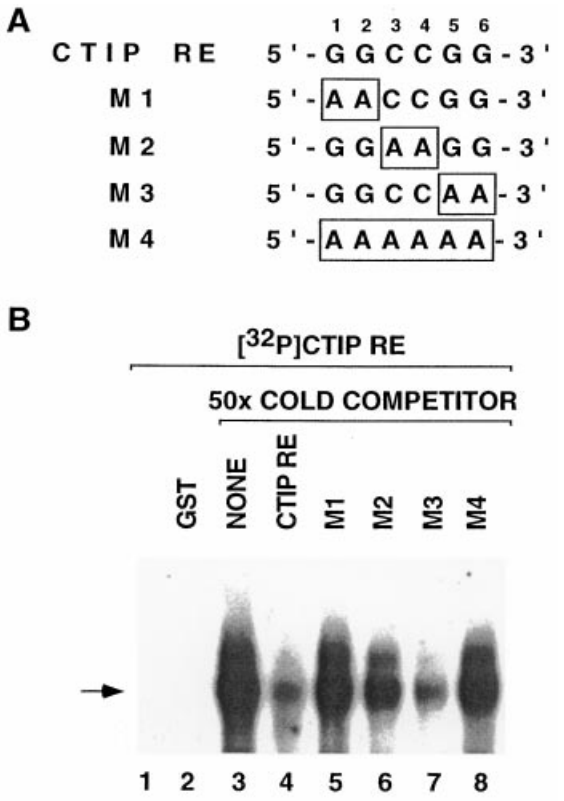


Figure 2 Definition of the specificity of the CTIP1 RE

(**A**) Sequences of wild-type CTIP RE core and mutants M1–M4. The nucleotides that are mutated in each oligonucleotide are indicated with boxes. Only the core of the upper strand is shown for clarity, and the 5′ and 3′ flanking regions were identical in all five probes. (**B**) EMSA studies with competitor CTIP REs. Lanes 1 and 2 correspond to the probe in the absence and presence of GST respectively. Lanes 3–8 contain GST–CTIP1-(1–776) and the indicated unlabelled ('cold') competitor in 50-fold excess over the concentration of [^{32}P]CTIP. Complexes between CTIP1-(1–776) and ^{32}P-labelled CTIP RE are indicated by an arrow.

mediated supershift was dependent on the presence of NP40 (0.17 % v/v) in the binding reaction, because the same antibody completely inhibited GST–CTIP1 · (CTIP RE) complex formation in the absence of the detergent (Figure 1D, compare lanes 4 and 5). The region of CTIP1 that is recognized by this antibody is outside the central ZNF3–ZNF4 region that putatively harbours the DNA binding domain (see below). Antibody-mediated inhibition of DNA binding by CTIP1 in the absence of detergent appears to be a general phenomenon, as formation of the CTIP1 · (CTIP RE) complex was similarly inhibited by another anti-CTIP1 antiserum (raised against amino acids 407–776; results not shown), as well as by the anti-GST antibody (Figure 1E, lanes 3 and 4). The molecular basis for antibody-mediated inhibition of DNA binding by GST–CTIP1 in the absence of detergent is not clear. However, this effect was specific, as neither preimmune serum nor an unrelated antibody inhibited CTIP1 · (CTIP RE) complex formation (results not shown). These findings suggest that the GST–CTIP1 fusion protein may exist in a tightly compacted structure that, when decorated with an antibody, is precluded from binding to the CTIP RE. In this case, NP40 may serve to unfold the protein, at least partially, resulting in positioning of the epitope in a region sufficiently removed from the DNA binding domain that decoration with the antibody no longer sterically hinders DNA binding activity.

The formation of two CTIP1 · (CTIP RE) complexes with different electrophoretic mobilities was observed (Figure 1E, open and closed arrows). The appearance of the slower migrating complex was dependent on protein concentration (results not shown; but see Figure 1F for CTIP2), perhaps suggesting the formation of CTIP multimers on the CTIP RE. Overall, these results demonstrate that GST–CTIP1 binds directly to the CTIP RE *in vitro*.

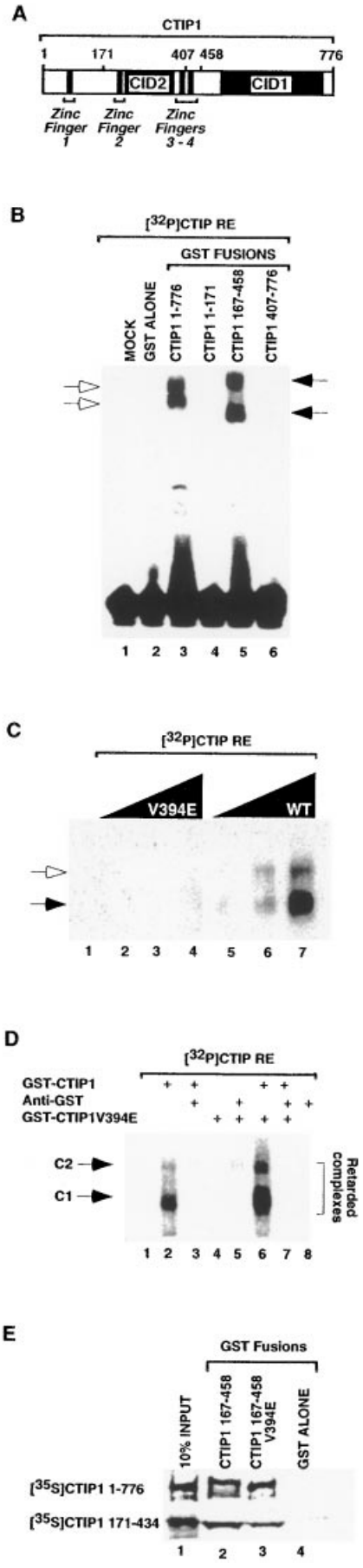
Based on the high degree of sequence similarity between CTIP1 and CTIP2 in the zinc finger core (ZNF3–ZNF4 of both proteins), we tested the ability of GST–CTIP2 to bind to the ^{32}P-labelled CTIP RE *in vitro*. Purified GST–CTIP2 bound strongly and in a concentration-dependent manner to the ^{32}P-labelled CTIP RE (Figure 1F). As observed previously for CTIP1 (see Figure 1E), two CTIP2 · (CTIP RE) complexes with different mobilities were observed (Figure 1F, open and closed arrows). The slower migrating species was particularly evident at higher protein concentrations (open arrow; Figure 1F, lane 4), again suggesting the possibility of CTIP2 homo-oligomerization on the CTIP RE.

Specificity of CTIP1 binding to the CTIP RE

To determine the sequence specificity of DNA binding by CTIP1, we examined the ability of various unlabelled oligonucleotides to inhibit binding of purified GST–CTIP1 to the ^{32}P-labelled CTIP RE. The competitor oligonucleotides, wild-type CTIP RE and mutants M1–M4, that were used in these experiments are shown in Figure 2(A). The wild-type CTIP RE inhibited the binding of CTIP1 to the [^{32}P]CTIP RE, as expected (Figure 2B, lane 4). Mutant binding site oligonucleotide M3 also inhibited binding of CTIP1 to the [^{32}P]CTIP RE to an extent similar to that with the wild-type CTIP RE (compare lanes 3 and 7 of Figure 2B). M3 is mutated at positions 5 and 6 (see Figure 2A), suggesting that these positions are not critical for the interaction of CTIP1 with the CTIP RE. Mutant M1 (mutation of positions 1 and 2; Figure 2A) did not appreciably inhibit binding of CTIP1 complexes to the [^{32}P]CTIP RE (Figure 2B, lane 5), suggesting that these bases are crucial for CTIP1 binding. Similarly M4 (mutation in all six core positions; Figure 2A) did not diminish the formation of GST–CTIP1 · ([^{32}P]CTIP RE) complexes. Finally, mutant M2 (mutation of positions 3 and 4; Figure 2A) weakly inhibited the binding of CTIP1 to the [^{32}P]CTIP RE (Figure 2B, lane 6). Positions 7–9 of the CTIP RE (Figure 2A) were intact in all of the mutants tested and, at the present time, the contribution of these nucleotides to the interaction between CTIP1 and the CTIP RE is not known. These results demonstrate that positions 1 and 2 and, to a lesser extent, positions 3 and 4 of the CTIP RE (Figure 2A) are critical determinants for CTIP1 binding. Moreover, these findings further prove that CTIP1 binds DNA in a sequence-specific manner *in vitro*.

The module comprising ZNF3 and ZNF4 confers CTIP1 sequence-specific DNA binding activity

Isolated CTIP1 regions were tested for their ability to bind to the consensus CTIP RE, towards the goal of identifying the determinants of sequence-specific DNA recognition by CTIP1. Full-length CTIP1-(1–776) and a fragment spanning ZNF3–ZNF4 [CTIP1-(167–458)] both bound DNA to a similar extent, but yielded complexes with different electrophoretic mobilities (Figure 3B, lanes 3 and 5 respectively). In contrast, a fragment lacking the ZNF3–ZNF4 domain [CTIP1-(1–171)] and another fragment containing only ZNF4 [CTIP1-(407–776)] did not bind to the CTIP RE *in vitro* (Figure 3B, lanes 4 and 6 respectively). These findings are consistent with our hypothesis that the



ZNF3–ZNF4 module confers CTIP1 sequence-specific DNA binding activity.

In order to verify the role of the ZNF3–ZNF4 module in sequence-specific DNA recognition by CTIP1, a point mutant was constructed and tested for its ability to bind to the CTIP RE *in vitro*. Based on the general structure of C_2H_2 zinc finger proteins, it is likely that both ZNF3 and ZNF4 may make direct DNA contacts [22]. However, because ZNF4 may be involved in CTIP1 self-association (see below), we focused our attention on ZNF3, within which amino acids Phe^{388}, Ser^{390}, Asn^{391} and Val^{394} are predicted to contact DNA directly [22]. Val^{394} would be predicted to interact with position 1 or 4 of the CTIP1 RE [22], both of which are among the first four positions critical for CTIP1 binding (Figure 2B). Thus Val^{394} of CTIP1 was mutated to glutamic acid, generating CTIP1 V394E, which was then tested for DNA binding activity in EMSA studies. CTIP1 V394E exhibited a dramatically reduced DNA binding activity when compared with wild-type CTIP1 (Figure 3C, compare lanes 2–4 with 5–7). This finding demonstrates that the structural integrity of ZNF3 is crucial for the DNA binding activity of CTIP1, and that Val^{394} may make direct contact with discriminatory nucleotides within the CTIP RE.

CTIP1 forms homotypic complexes on the CTIP RE and self-associates in solution

In general, C_2H_2 zinc finger proteins are known to bind DNA as monomers (reviewed in [20,22]). Dimerization, although not required for DNA binding, has been observed to enhance the DNA binding activity of some C_2H_2 zinc finger proteins, such as the Ikaros family members [38]; however, it is not clear if both partners contact DNA directly. Formation of slower migrating complexes was observed for both CTIP1 and CTIP2 (Figures 1E and 1F, open arrows), suggesting the possibility that both CTIP1 and CTIP2 form oligomers on the CTIP RE *in vitro*. To determine if CTIP1 forms oligomeric complexes on the CTIP RE, we performed EMSAs utilizing DNA-binding-competent and -incompetent forms of CTIP1 [GST–CTIP1-(167–458) and GST–CTIP1-(167–458) V394E respectively; see Figure 3C]. In the event that self-association plays a role in DNA binding by CTIP1, we would expect to observe an enhancement of DNA binding when these two forms are mixed together. GST–CTIP1-(167–458) again formed a strong complex with the CTIP RE (C1; Figure 3D, lane 2), and a fainter complex with reduced electrophoretic mobility was also observed (C2; Figure 3D, lane 2) that may represent a multimeric form of CTIP1-(167–458) (see

Figure 3 Mapping the CTIP1 DNA binding domain

(**A**) Schematic diagram of CTIP1. The two COUP-interaction domains (CID1 and CID2) and ZNF1–ZNF4 (vertical bars) are indicated [23]. (**B**) EMSA studies with CTIP1 fragments. The ^{32}P-labelled CTIP RE was incubated with 5 pmol of affinity-purified GST (lane 2) or GST fusion protein (lanes 3–6) as indicated. The open and closed arrows indicate CTIP RE complexes containing CTIP1-(1–776) and CTIP1-(167–458) respectively. (**C**) EMSA studies with increasing amounts (1.25, 2.5 and 5 pmol) of GST–CTIP1-(167–458) (lanes 5–7) or the V394E mutant (lanes 2–4). The free probe is not shown for simplicity. (**D**) Homotypic interaction of CTIP1 on the CTIP RE. EMSA studies using 2.5 pmol of GST–CTIP1-(167–458) or GST–CTIP1-(167–458) V394E (lanes 2–3 and 4–5 respectively). Lanes 6–8 correspond to reactions in which GST–CTIP1-(167–458) and GST–CTIP1-(167–458) V394E were incubated in equal amounts (2.5 pmol each) with the ^{32}P-labelled CTIP RE. CTIP1 · (CTIP RE) complexes (C1 and C2) are indicated by arrows. Addition of purified anti-GST antiserum (lanes 3 and 7) inhibited DNA–protein complex formation in these experiments, which were conducted without the addition of NP40. (**E**) GST pull-down experiments using CTIP1-(167–458) (CTIP1 WT; lane 2) and CTIP1-(167–458) V394E (CTIP1 V394E; lane 3) both fused to GST. Input [^{35}S]methionine-labelled proteins are shown in lane 1.

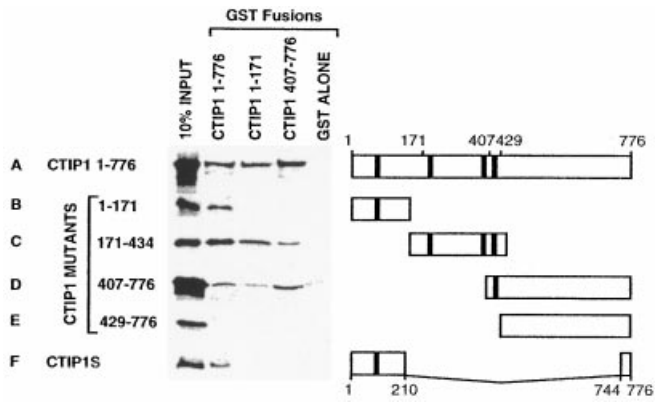


Figure 4 Self-association of CTIP1 in solution

GST pull-down experiments were carried out using the indicated GST fusion proteins as bait and [^{35}S]methionine-labelled proteins as prey. In all cases, lane 1 represents 10% of the [^{35}S]methionine-labelled input protein, and lanes 2–4 reflect the amount of [^{35}S]methionine-labelled input protein that bound to the indicated GST fusion protein. The CTIP1 proteins used in these studies (**A**–**E**) are represented schematically on the right, with zinc finger motifs being represented by vertical bars.

above). GST–CTIP1-(167–458) V394E again did not form a detectable complex on the CTIP RE (Figure 3D, lane 4). However, when the two proteins were mixed together, an enhancement of the GST–CTIP1-(167–458) · (CTIP RE) complexes was observed (C1 and C2; Figure 3D, lane 6), suggesting that association with CTIP1-(167–458) V394E enhanced the DNA binding activity of CTIP1-(167–458). Because addition of GST–CTIP1-(167–458) V394E enhanced the interaction between GST–CTIP1-(167–458) and the CTIP RE without affecting migration of the complexes, we assume that, in the absence of GST–CTIP1-(167–458) V394E, both C1 and C2 correspond to homo-oligomers of CTIP1-(167–458). All CTIP1 · (CTIP RE) complexes were specific, as demonstrated by inhibition of complex formation by the addition of antiserum (Figure 3D, lanes 3 and 7). These experiments were conducted using a submaximal amount of wild-type protein, to facilitate observation of the enhancement of DNA binding activity by the DNA-binding-incompentent mutant CTIP1-(167–458) V394E.

The above results suggested that CTIP1 may form oligomeric complexes on the CTIP RE, implying direct protein–protein interaction between CTIP1 monomers. GST pull-down experiments were conducted to address this possibility directly. [^{35}S]Methionine-labelled CTIP1-(1–776) interacted with GST fusions of both CTIP1-(167–458) and CTIP1-(167–458) V394E (Figure 3E, upper panel, lanes 2 and 3 respectively), but not with GST (lane 4). Similarly, CTIP1-(171–434) also interacted with both GST–CTIP1-(167–458) and GST–CTIP1-(167–458) V394E (lower panel of Figure 3E, lanes 2 and 3). GST–CTIP1-(167–458) V394E interacted with both CTIP1-(1–776) and CTIP1-(171–434) in a manner that was indistinguishable from that of GST–CTIP1-(167–458) (compare lanes 2 and 3 of the upper and lower panels of Figure 3E). This result indicates that: (i) GST–CTIP1-(167–458) V394E may enhance the DNA binding activity of GST–CTIP1-(167–458) by direct protein–protein interaction that is independent of DNA binding, and (ii) the structural integrity of ZNF3 is not essential for the self-interaction of CTIP1.

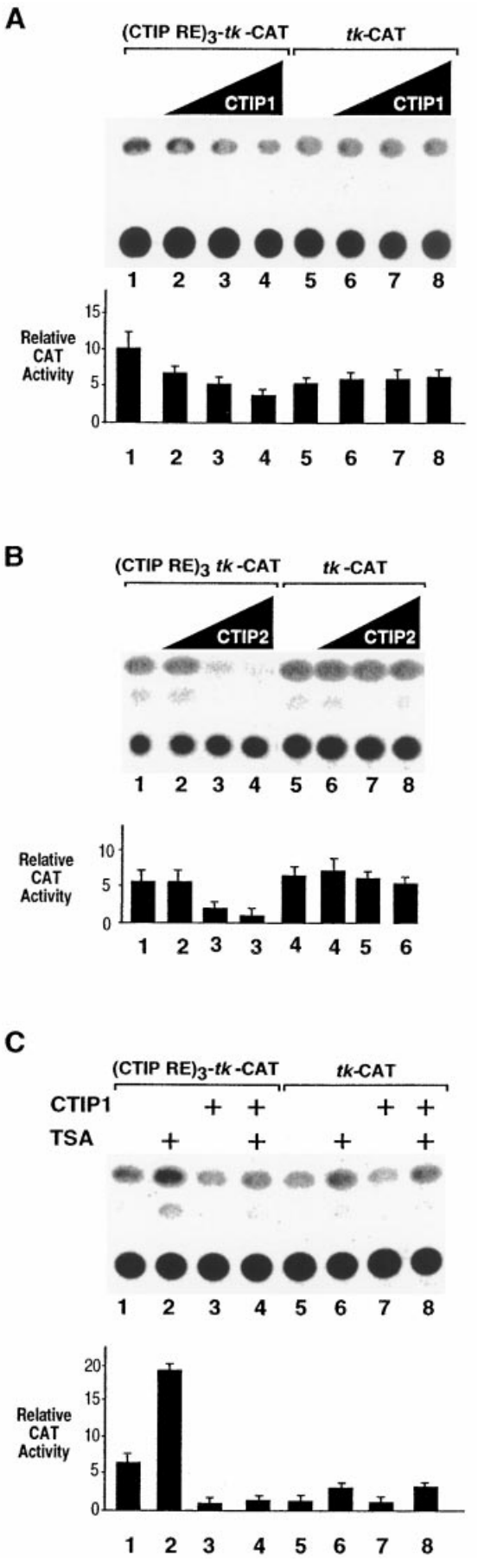
Additional GST pull-down experiments were conducted to map the CTIP1 self-association interface. Full-length CTIP1-(1–776) was found to associate with itself (Figure 4A, lane 2), and with both the isolated N-terminus [CTIP1-(1–171); lanes 3 and 2 of Figures 4A and 4B respectively) and C-terminus [CTIP1-(407–776); lanes 4 and 2 of Figures 4A and 4D respectively], but not with GST (Figure 4A, lane 5). CTIP1-(1–171) did not interact with either itself (Figure 4B, lane 3) or the C-terminus of CTIP1 (lane 4), but did interact with the central core of the protein [CTIP1-(171–434); Figure 4C, lane 3]. Similarly, a naturally occurring splice variant comprising amino acids 1–210 fused to residues 744–776 [24] behaved in a manner to similar to that of CTIP1-(1–171) (compare Figures 4B and 4F). CTIP1-(171–434) also interacted with full-length CTIP1 (Figure 4C, lane 2) and, to a lesser extent, with the C-terminus (Figure 4C, lane 4).

The C-terminal region of CTIP1 [CTIP1-(407–776)], which includes ZNF4, interacted with itself (Figure 4D, lane 4) and with full-length CTIP1 (lane 2), but not appreciably with the N-terminal region [GST–CTIP1-(1–171); Figure 4D, lane 3]. Self-association of the CTIP1 C-terminal region appeared to require ZNF4, as deletion of this domain resulted in a completely inactive mutant [CTIP1-(429–776); Figure 4E, lane 4] that also failed to interact with full-length CTIP1 (lane 2) or the isolated N-terminal region (lane 3).

Considered together, the above results suggest that CTIP1 oligomerization in solution appears to occur through at least two interaction interfaces, the N-terminal region (amino acids 1–171) and the central core of the protein (amino acids 171–434), and that these two domains probably contact each other in the context of the full-length protein. ZNF4 of CTIP1 would appear to be essential for interaction of the C-terminal region [CTIP1-(407–776)] with itself, but the significance of this interaction in the context of the full-length protein is unknown. For example, ZNF4 is unlikely to be sufficient to mediate the self-association of CTIP1 in solution, as two fragments harbouring ZNF4 [CTIP1-(171–434) and CTIP1-(407–776)] interacted only weakly (Figure 4C, lane 4), suggesting that structural determinants outside ZNF4 may contribute to the stability of the CTIP1 · CTIP1 complexes *in vitro*.

CTIP1 and CTIP2 repress transcription from the consensus CTIP RE in mammalian cells

The above results demonstrate that CTIP1 and CTIP2 bind DNA in a sequence-specific manner *in vitro*. We next sought to determine if CTIP1 and CTIP2 regulate the expression of a reporter gene harbouring a multimerized CTIP RE in the context of the thymidine kinase (*tk*) promoter. CTIP1-(1–776) repressed transcription from a $(CTIP\ RE)_3$-*tk*-CAT reporter in a concentration-dependent manner (Figure 5A, lanes 1–4), but not from the empty *tk*-CAT reporter (lanes 5–8), in transiently transfected HEK293 cells. Similar results were obtained using CTIP2-(1–813), except that CTIP2 appeared to be a much stronger transcriptional repressor than CTIP1 (Figure 5B, compare lane 1 with lanes 2–4 and lane 5 with lanes 6–8). These data confirm that the consensus CTIP RE is functional in mammalian cells, and that both CTIP1 and CTIP2 are *bona fide* transcription factors that bind directly to the CTIP RE and mediate transcriptional repression. Transcriptional repression mediated by CTIP1 (Figure 5C, compare lanes 1 and 2 with lanes 3 and 4) or CTIP2 (results not shown) was only minimally inhibited by TSA, suggesting a mechanism independent of class I or II histone deacetylases. In addition, co-transfection of the orphan nuclear receptor ARP1 did not enhance either CTIP1- or CTIP2-mediated transcriptional repression (results not shown), suggesting that CTIP-mediated transcriptional repression may be



independent of COUP-TF family members. In contrast, we observed previously that CTIP1 stimulated ARP1-mediated transcriptional repression in HEK293 cells in a TSA-insensitive manner [23].

DISCUSSION

CTIP1 was initially identified as a mediator of the transcriptional repression activity of the orphan nuclear receptor ARP1 [23] and as an interaction partner of proto-oncogene product Bcl6 [24]. In the case of ARP1, the transcriptional repression activity of CTIP1 was rendered functional by the tethering of the protein to DNA-bound ARP1 complexes [23]. However, CTIPs are C_2H_2 zinc finger proteins, which are generally known to bind DNA directly and regulate the expression of target genes. The present study provides direct evidence that CTIP1 is a sequence-specific DNA binding protein, and describes the identification of a consensus DNA binding site for CTIP1, the CTIP RE. Purified CTIP2 also bound directly to the CTIP RE *in vitro*; however, a detailed analysis of the DNA binding properties of this protein has not been conducted, and these results should be considered as preliminary at the present time. Nonetheless, CTIP1 and CTIP2 repressed transcription of a reporter gene in a manner that was dependent on the presence of the CTIP RE, indicating the functionality of both CTIPs and of the CTIP RE in mammalian cells.

The sequence of the CTIP RE is highly related to the canonical GC box, which is associated with transcriptional initiation in the context of TATA-less promoters [39,40], and also with the regulation of gene expression mediated by different transcriptional regulatory factors, such as the Sp1 family of proteins [41–44]. In this context, the CTIP RE may function as a rather promiscuous binding site for a large family of transcriptional regulatory proteins. Indeed, we have observed that the basal level of transcription of a reporter construct containing the CTIP RE is somewhat higher than that of the same reporter lacking the CTIP RE (see Figures 5A and 5C). Thus CTIP-mediated transcriptional repression may result, at least in part, from competition with transcriptional activators that bind to and function through the CTIP RE to dictate the level of expression of a target gene.

Transcriptional repression mediated by CTIP1 through the CTIP RE did not appear to involve the recruitment of TSA-sensitive class I or II histone deacetylases to the template. We previously observed that transcriptional repression mediated by CTIP1 · ARP1 complexes similarly did not require recruitment of TSA-sensitive histone deacetylases [23]. These findings suggest

Figure 5 CTIPs repress transcription from the consensus CTIP RE in mammalian cells in a TSA-insensitive manner

(**A**) CAT assays were carried out using extracts from HEK293 cells transiently co-transfected with increasing amounts (0.5, 1.0 and 2.0 μg) of an expression vector encoding haemagglutinin-epitope-tagged CTIP1 (lanes 2–4) and a reporter construct consisting of the CAT gene downstream of the herpes simplex virus *tk* promoter with (lanes 1–4) or without (lanes 5–8) the trimerized CTIP RE. Cells were harvested 48 h after transfection, and transfection efficiency was normalized by co-transfection with an expression vector for β-galactosidase. The quantification shown in the lower panel reflects the mean relative CAT activity ($\pm$S.E.M.) of three independent experiments. (**B**) Transcriptional repression mediated by Flag–CTIP2. Transfections, CAT assays and quantifications were conducted as described for (**A**). (**C**) Effect of TSA on CTIP1-mediated transcriptional repression. HEK293 cells were co-transfected with the indicated plasmids as described for (**A**). At 24 h after transfection, cells were treated with TSA (100 ng/ml; lanes 2, 4, 6 and 8) for an additional 24 h. Transfections, CAT assays and quantifications were conducted as described for (**A**).

that alternative mechanisms, perhaps recruitment of members of the TSA-insensitive and NAD^+-dependent histone deacetylases of the silent information regulator 2 (Sir2) family [45] and/or association with heterochromatin, may underlie CTIP1-mediated transcriptional repression in mammalian cells.

Although CTIPs act as transcriptional repressors when bound directly to the consensus CTIP RE in transient transfection experiments, we cannot exclude the possibility that CTIPs may also act as transcriptional activators in some promoter and cellular contexts. Both CTIP1 and CTIP2 harbour C-terminal acidic regions that may mediate transcriptional activation [46–48]. Indeed, we observed that the acidic domain of CTIP1 modestly activated transcription when fused to the GAL4 DNA binding domain [23]. Other C_2H_2 zinc finger proteins, such as YY1 (Yin and Yang 1) and Ikaros, have also been shown to behave as bifunctional modulators of transcription, promoting either transcriptional activation or repression as a function of the promoter and cellular context [49–53].

CTIP1 was found to self-associate in solution and on DNA, and this appeared to facilitate interaction of the protein with the CTIP RE *in vitro* in a manner similar to that observed for Ikaros [38]. In the present study, we found that only one partner in the DNA binding complex is required to make direct contact with the DNA (see Figures 3C and 3D). Conceivably, this could facilitate the assembly of large complexes, such as the nuclear substructures observed in nuclei of transfected cells ([23] and results not shown). It is presently unknown if CTIP1 self-associates prior to or as a consequence of DNA binding. In solution, CTIP1 clearly associates with itself and with at least one naturally occurring splice variant (Figure 4 and results not shown), suggesting that oligomerization may occur before DNA binding. The physiological relevance of this is presently unknown; however, it is conceivable that CTIP1 splice variants may interact with and alter the subcellular distribution, DNA binding and/or transcriptional regulatory properties of full-length CTIP1 proteins. This form of 'self' regulation by proteins encoded by alternatively spliced transcripts has been described previously among members of the Ikaros family of transcriptional regulators. The Ikaros proteins harbour a dimerization function comprising a cluster of C_2H_2 zinc finger motifs within the C-terminal region that is common to the full-length proteins and all splice variants [54]. These C_2H_2 zinc fingers mediate homodimerization of the Ikaros proteins and heterodimerization with other members of the members of the family, such as Aiolos [55], Helios, [56,57], Eos and Pegasus [58]. Consistent with this, Ikaros complexes have been found to contain multiple Ikaros proteins [52].

The results of these studies demonstrate that CTIP1 and CTIP2 are sequence-specific DNA binding proteins that can function independently of COUP-TF family members. The relative contributions of COUP-TF-dependent and -independent pathways to the overall transcriptional regulatory activities of the CTIPs in mammalian cells are unknown. Isolation of CTIP1 and CTIP2 target genes is under way in this laboratory, and will further help to elucidate the biological functions of these transcription factors in various cellular and tissue contexts during embryonic development and in adult life.

This work was supported NIH grants AR02194 (to D.A.) and GM60852 (to M.L.). Additional support was provided by the Oregon State University Environmental Health Sciences Center (under National Institutes of Health grant ES00210). We thank Valerie Peterson for technical advice, and the Central Services Laboratory of the Oregon State University Center for Gene Research and Biotechnology for DNA sequencing. M.L. is grateful to Dr David Shepherd for critical reading of the manuscript and providing useful suggestions before submission.

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Received 29 March 2002/22 August 2002; accepted 27 August 2002
Published as BJ Immediate Publication 27 August 2002, DOI 10.1042/BJ20020496