A Region to the N-terminal Side of the CTCF Zinc Finger Domain Is Essential for Activating Transcription from the Amyloid Precursor Protein Promoter

Alexander A. Vostrov, Michael J. Taheny, and Wolfgang W. Quitschke‡

From the Department of Psychiatry and Behavioral Science, State University of New York, Stony Brook, New York 11794-8101

Transcription from the amyloid precursor protein (APP) promoter is largely dependent on a nuclear factor binding site designated as APβ. The protein that binds to this site is the multifunctional transcription factor CTCF, which consists of 727 amino acids and contains a domain of 11 zinc finger motifs that is flanked by 267 amino acids on the N-terminal side and 150 amino acids on the C-terminal side. Deleting HeLa cell nuclear extract of endogenous CTCF specifically reduced transcriptional activity from the APP promoter. However, transcriptional activity was restored by replenishing the depleted extract with recombinant CTCF. Deleting 201 amino acids from the C-terminal end of CTCF had no detrimental effect on transcriptional activation, whereas deleting either 248 or 284 amino acids from the N-terminal end abolished transcriptional activation. Competing endogenous CTCF in vivo was accomplished by cotransfecting COS-1 cells with a plasmid overexpressing CTCF constructs and a reporter plasmid containing the APP promoter. Under these conditions, an N-terminal deletion of CTCF reduced expression from the APP promoter, whereas the C-terminal deletion had no effect. These results demonstrate that CTCF activates transcription from the APP promoter and that the activation domain is located on the N-terminal side of the zinc finger domain.

CTCF was first described as a factor that binds to the chicken c-MYC promoter (17) and to the silencer element of the chicken lysozyme gene (18, 19). CTCF binds to diverse sequences by utilizing different combinations of essential zinc fingers (16, 19). Consequently, a defined DNA recognition sequence cannot readily be recognized. The function of CTCF in gene regulation is also diverse. For example, CTCF binds to the chicken lysozyme silencer 2.4 kilobase pairs upstream from the transcriptional start site. Here it synergistically represses transcription in conjunction with the thyroid hormone receptor or the retinoic acid receptor (18, 20). Another example of synergistic repression is provided by the coordinate action of CTCF and the thyroid hormone receptor on the TRE-containing rat genomic element 144 (21, 22). Furthermore, CTCF has been found to directionally block enhancer activation by binding to the insulator element at the 5′ end of the chicken β-globin gene locus (23). Similar CTCF-binding sequences were identified in a variety of insulators from diverse vertebrate species, suggesting a widespread role for CTCF in the regulation of enhancer-activated genes (23). CTCF also binds to the proximal promoter of the chicken c-MYC gene where it acts either as a transcriptional repressor or activator (17, 24, 25). In the human and deposition in transplanted murine hippocampal tissue with trisomy 16, the mouse equivalent of Down’s syndrome (10). These observations suggest that overexpression of APP may be one of several contributing factors in the formation of amyloid deposits and in the neuropathology associated with Alzheimer’s disease.

The promoter of the APP gene is a necessary element in regulating APP transcription, and it has been shown to confer some degree of cell type-specific expression in transgenic mice (11, 12). The proximal APP promoter is devoid of CCAAT and TATA boxes but contains a prominent initiator element associated with the main transcriptional start site (+1). The integrity of this initiator element is essential for both start site selection and optimal transcriptional activity (13). In addition, an intact nuclear factor binding site designated APβ is essential for effective transcription from the APP promoter (13, 14). The core recognition sequence for this binding site is located between positions −82 and −93 (Fig. 1C), and its elimination reduces transcriptional activity by ~70–90% (13, 14). The nuclear factor that activates transcription from APβ was identified as CTCF (15), a nuclear regulatory protein comprising 727 amino acids (16). It contains a centrally located DNA binding domain with 11 zinc finger motifs that is flanked by 267 amino acids on the N-terminal side and 150 amino acids on the C-terminal side (Fig. 1, A and B). Selective deletions from the N- and C-terminal sides of the zinc finger domain showed that the N-terminal end of the zinc finger domain was aligned toward the transcriptional start site (15, 18).
CTCF Activates APP Promoter Transcription

EXPERIMENTAL PROCEDURES

Expression of Recombinant CTCF Constructs in Pichia pastoris—The cDNA encoding human CTCF was amplified by the polymerase chain reaction from a cDNA library derived from the human retinaoblastoma cell line Y79 (CLONTECH) (27). Deletions from the 5’ end were introduced at restriction sites Accl (position 965) and Bsu36I (position 1216), resulting in primary translation products with N-terminal transcriptional activity sites at positions Met-249 and Met-285, respectively. Deletions from the 3’ end were introduced at restriction sites BglII (position 2142) and PstI (position 1867), yielding translation products with C-terminal deletions at positions Asp-617 and Cys-525, respectively (Fig. 1, A and B).

Expression of CTCF and its deletion constructs in yeast was accomplished with the Pichia Expression Kit (Invitrogen) according to the manufacturer’s instructions. Briefly, cDNA encoding full-length CTCF or its N- and C-terminal deletions was excised from plasmid pGEM 7Z (−) (Promega) and cloned either into the EcoRI site of plasmid pHII-D2 or between the BamHI and NotI sites of plasmid pCIP3.5 (Invitrogen). Both are vectors that direct intracellular recombinant protein expression in P. pastoris. The vectors containing the CTCF cDNA constructs in the correct orientation were transformed into Pichia strain KM71 by electroporation. Protein clones were amplified, induced for recombinant protein expression, and screened for the presence of CTCF by mobility shift electrophoresis.

Purification of CTCF—Recombinant CTCF proteins were extracted and purified from P. pastoris as described (27) with some modifications. Pichia cells (10−15 g) were mixed with 25 ml of 0.5-mm glass beads, adjusted to a total volume of 50 ml with buffer F (40 mM HEPES, pH 7.6, 2 mM MgCl2, 1 mM EDTA, 1 mM DTT, 20% glycerol, 500 mM KCl, and 10 μM ZnSO4), and homogenized with the Bead Beater apparatus ( Biospec Products, Inc., Bartensville, OK). Cell debris was pelleted at 5,000 × g for 10 min, and the supernatant was supplemented with 3 mM KCl to a final concentration of 200 mM. The lysate was further clarified by centrifugation at 100,000 × g for 60 min.

CTCF was purified from the extracts by single step SP cation exchange chromatography. Briefly, a 1-ml Hitrap SP-Sepharose column (Amersham Biosciences) was equilibrated with buffer F containing 200 mM KCl. Cleared extract was loaded on the column, and proteins were eluted with a linear concentration gradient of KCl (0.2–1 M) in buffer F. Fractions of 0.5 ml were collected, and the CTCF binding activity was monitored by mobility shift electrophoresis. Fractions containing the peak activity were concentrated 10-fold with a Microcon-30 centrifugal device (Millipore). It must be noted that the presence of 1 M urea is necessary for successful concentration. In the absence of urea, CTCF and especially some truncated forms of the protein are prone to bind to the concentrator’s membrane.

Antibodies—Rabbit polyclonal antibodies against the N-terminal part of CTCF were prepared as described elsewhere (28). The antibodies were affinity-purified on recombinant CTCF attached to a Ni2+-chelating column (29). Specifically, recombinant full-length CTCF containing a His tag at its N terminus was expressed in P. pastoris and purified from the cell culture supernatant. CTCF binding activity was monitored by mobility shift electrophoresis. Fractions containing the peak activity were concentrated 10-fold with a Microcon-30 centrifugal device (Millipore). It must be noted that the presence of 1 M urea is necessary for successful concentration. In the absence of urea, CTCF and especially some truncated forms of the protein are prone to bind to the concentrator’s membrane.

The above examples illustrate that CTCF primarily acts as a negative regulator of transcription. In contrast, some of our previous studies (13–15, 26) have provided indirect evidence implicating CTCF as a transcriptional activator of the APP promoter. The mechanism by which CTCF exerts its diverse regulatory effects remains unclear. However, it is likely to involve interactions with specific secondary factors. We provide here direct evidence both in vitro and in vivo that CTCF activates transcription from the APP promoter and that this activation requires the N-terminal end of the molecule.
CTCF Activates APP Promoter Transcription

Depleting Nuclear Extract of CTCF Reduces Transcriptional Activity from the APP Promoter—HeLa cell nuclear extract was subjected to exhaustive overnight CHAPS treatment except in the initial incubation buffer D was used in place of the antibodies. This control preparation is referred to as whole nuclear extract.

Mobility Shift Electrophoresis—The double-stranded oligonucleotide APBß-80WT (15) was 5′ end-labeled with γ-32PATP using T4 polynucleotide kinase (30). This oligonucleotide contained the APP promoter sequence from position −110 to −84, a serum response element (SRE) and a recognition sequence for CTCF (Fig. 1C). Recombinant CTCF was diluted with buffer F containing 500 mM KCl. The binding reaction was assembled by first mixing 0.5 μl of diluted CTCF and 1 μl of CTCF-depleted nuclear extract with binding buffer (buffer D), supplemented with 5 μg of yeast tRNA, 2 μg of poly(dI-dC)/poly(dI-dC), and CHAPS to a final concentration of 2.5% in a total volume of 16 μl. Alternatively, the control reaction mixtures contained a total of 1 μl of HeLa nuclear extract (whole or depleted) with no CTCF added. The mixture was preincubated for 5 min at 25 °C followed by the addition of 1 μl of labeled double-stranded oligonucleotide (20 ng per binding reaction; 50,000–200,000 cpm) in binding buffer. The final binding reactions were incubated for 30 min at 25 °C and then supplemented with 1.5 μl of fetal calf serum and electrophoresed in 6% polyacrylamide gels containing 0.5× Trisborate/EDTA (30) at 100 V constant voltage. The gels were dried, and the amount of bound and free oligonucleotide was quantitated with a GS-250 PhosphorImager (Bio-Rad). Binding activity was expressed in mobility shift units (msu). One msu was designated as the amount of protein that completely binds 1 ng of 32P-oligonucleotide APBß-80WT during mobility shift electrophoresis.

Special conditions were employed in the binding reactions for mobility shift electrophoresis in experiments dealing with in vitro competition of native CTCF with purified recombinant CTCF proteins. In this case the binding conditions were adjusted to reproduce exactly the conditions of the corresponding in vitro transcription reaction (see below). Recombinant CTCF was diluted with buffer F containing 500 mM KCl, and 0.7 μl was mixed with 2 μl of depleted nuclear extract, 5 μg of yeast tRNA, and 2 μg of poly(dI-dC)/poly(dI-dC). The volume was adjusted to 8 μl with buffer E bringing the final KCl concentration to 100 mM, and the mixture was incubated at 25 °C for 1 min. Subsequently, 25 ng of labeled 80-mer oligonucleotide APBß-80WT was added to this mixture. This represented approximately the same molar amount of APBß CTCF-binding sequence present in 2 μg of the plasmid DNA that was used in a nuclear transcription reaction below. The mixture was preincubated for 10 min at 25 °C. During this time, in a separate tube, 2 μl of whole nuclear extract was mixed with 5 μg of yeast tRNA and 2 μg of poly(dI-dC)/poly(dI-dC) and adjusted to a total volume of 8 μl with buffer E. After an additional minute at 25 °C the mixtures were combined, and the binding reaction was continued for 30 min at 30 °C.

The samples were analyzed by mobility shift electrophoresis as described above.

In Vitro Transcription—We optimized our in vitro transcription protocol (13) to better suit the aims of this study. The reaction buffer contained 40 mM HEPES, pH 7.6, 2 mM MgCl2, 1 mM EDTA, 1 mM DTT, 75 mM KCl, and 10% glycerol. Our preliminary tests indicated that the highest in vitro transcriptional activity from the APP promoter was achieved at a 75 mM KCl concentration. We also found that CTCF activity decreased to 0.75 μl of 1 mM urea could be added to a 16-μl reaction mixture without significantly affecting the activity of either the APP or β-actin promoters. The in vitro transcription reaction mixture contained 4 μl of nuclear extract, 2 μg of the plasmid APP′−488 (13), which is based on reporter plasmid pCAT2βGAL (31), and 600 μl of each NTP in a total volume of 16 μl. Recombinant CTCF was diluted with buffer F containing 500 mM KCl. The same volume of 0.5 μl of diluted CTCF or buffer F was added to all the reaction samples to maintain a constant concentration of urea. The sample volume was balanced with buffers D and E for various concentrations of urea. The sample volume was balanced with buffers D and E for various concentrations of urea. The sample volume was balanced with buffers D and E for various concentrations of urea.

To the annealing mixture was added 10 μl of a reverse transcriptase reaction mixture containing 12.5 mM Tris, pH 7.8, 10 mM MgCl2, 12.5 mm DTT, and 6 mM of each of the dNTPs, supplemented with 20 units of avian myoblastosis virus-reverse transcriptase and 20 units of RNA-sin (both Roche Molecular Biochemicals). The reaction was continued at 48 °C for 90 min and stopped by phenol/chloroform extraction followed by ethanol precipitation. The primer extension products were separated on a 6% sequencing gel and quantitated with a GS-250 PhosphorImager (Bio-Rad).

In experiments where native CTCF was competed with purified recombinant CTCF proteins, the reaction mixture was assembled in a modified way to allow for preliminary binding of the recombinant CTCF to the APBß site. Briefly, 0.7 μl of buffer F containing 500 mM KCl and recombinant CTCF was diluted with 2 μl of depleted HeLa cell nuclear extract and 2 μg of reporter plasmid. The volume of the sample was adjusted to 8 μl with buffer E. After incubation at 25 °C for 10 min, 2 μl of whole HeLa cell nuclear extract, 3 μl of buffer D, and 2 μl of buffer E were added to each sample. The transcription reaction was carried out at 30 °C, and the products were analyzed as described above.

In Vivo Competition—The rationale for these competition experiments was to analyze the effect of intracellular recombinant CTCF expression on the expression from a cotransfected APP promoter construct. The APP promoter from position −488 to +100 was cloned into the polylinker site of plasmid pCAT2βGAL (31), which is located immediately upstream of the bacterial chloramphenicol acetyltrans- ferase (CAT) gene. This plasmid also contains the β-galactosidase gene, which is transcribed from the β-actin promoter and serves as an internal control for experimental variations. For the expression of CTCF by transient transfection, full-length CTCF, N-terminal deletion Met-285, and C-terminal deletion Cys-525 were provided with a green fluorescent protein (GFP) tag on the 5′ end of the respective CTCF cDNA constructs. The reading frame for GFP was obtained from plasmid pEGFP-N1 (CLONTECH), which was cut at restriction site EcoRI and blunt-ended with T4 DNA polymerase. This operation eliminated the stop codon and the codon for the C-terminal amino acid of the GFP reading frame. The blunt-ended fragment was then cloned in-frame to the 5′ end of the three CTCF constructs. The resulting CTCF constructs, GFP-FL, GFP-M285, and GFP-C525 were subcloned into the polylinker site of plasmid pCMV3.1 (Invitrogen), which is located downstream from the CMV promoter. As a control, the entire GFP reading frame alone was also cloned into the same position of plasmid pCMV3.1.

293T cells were grown to about 70% confluence in 25-cm² flasks and were then transfected with 2 μg of plasmid mixture per flask using the FuGENE reagent according to manufacturer's instructions (Roche Molecular Biochemicals). The plasmid mixture contained the APP reporter plasmid (APP′−488) and the CTCF expression vector (pCMV3.1) at a molar ratio of 4:1. This ratio was found to be optimal both for CTCF expression and APP promoter activity. The FuGENE/plasmid mixture was left on the cells for about 16 h. The cells were then examined by fluorescence microscopy and harvested. CAT and β-galactosidase assays were performed as described elsewhere (14). The CAT activities resulting from the APP promoter construct was normalized to identical β-galactosidase activities. Subsequently, in each experiment the APP promoter activity obtained with the cotransfected control GFP expression plasmid was assigned the value 100%.

RESULTS

Depleting Nuclear Extract of CTCF Reduces Transcriptional Activity from the APP Promoter—HeLa cell nuclear extract supports an in vitro transcription from both the β-actin and APP promoters. Transcription from the β-actin promoter depends on a core promoter element upstream of the CCAAT and TATA box as well as a serum-response element (32, 33). In contrast, the APP promoter is TATA-less, which indicates that the two promoters are transcribed by different mechanisms. Transcription from the β-actin promoter is initiated at one primary start site, whereas the APP promoter is initiated at multiple sites from position +1 to −4 (Fig. 2B, lane 3).
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Fig. 2. Depletion of CTCF from nuclear extract. A, native whole HeLa cell nuclear extract was depleted of CTCF by immunoabsorption and analyzed by mobility shift electrophoresis. The CTCF binding activity present in 1 μl of whole nuclear extract (lane 1) and in 1 μl of depleted extract (lane 5) is shown. The binding activity in whole nuclear extract was assigned the relative value of 100%. While leaving the amount of total extract in the binding reaction constant, the whole extract was mixed with increasing amounts of depleted extract (lanes 2–4) resulting in an approximately proportional decrease in CTCF binding. The positions of the APBβ–CTCF binding complex (b) and the free oligonucleotide (f) are indicated (brackets). B, in vitro transcription with the same extract combinations as in A, except that 4 μl of extract was used in the reaction (lanes 1–5). The transcriptional start sites from the control β-actin and the APP promoters are indicated (brackets). The transcriptional activities from the APP promoter were normalized to identical β-actin promoter activities.

1). When HeLa cell nuclear extract was depleted of CTCF by immunoprecipitation, transcription from the APP promoter was drastically reduced to a level corresponding to 23% of the activity observed with the non-depleted extract (Fig. 2B, lanes 1 and 5). When whole nuclear extract was mixed with increasing amounts of CTCF-depleted extract, there was a gradual decline in the transcriptional activity from the APP promoter (Fig. 2B, lanes 2–4). In all cases transcriptional activity from the β-actin promoter remained unaffected (Fig. 2B, lanes 2–4). The decrease in transcriptional activity was paralleled by a comparable decrease in binding activity to the APBβ site (Fig. 2A, lanes 1–5).

Replenishing CTCF-depleted Nuclear Extract with Purified Recombinant CTCF Restores Transcriptional Activity from the APP Promoter—To determine whether purified recombinant CTCF expressed in the yeast strain P. pastoris supports transcriptional activity from the APP promoter, depleted nuclear extract was replenished with increasing amounts of full-length recombinant CTCF. Mobility shift electrophoresis showed that binding to the APBβ site increased proportionally to the amount of CTCF added (Fig. 3A, lanes 1–5). In a similar manner, transcriptional activity from the APP promoter was restored to levels approximating those of whole nuclear extract, whereas the transcriptional activity from the β-actin promoter remained unaffected. Incidentally, no CTCF binding activity can be detected in control Pichia extracts, and such extracts do not support transcription from the APP promoter (data not shown). These results demonstrate that CTCF activates transcription from the APP promoter in vitro and that the recombinant version of the protein expressed in P. pastoris can replace endogenous CTCF depleted from HeLa cell nuclear extract.

The N-terminal Domain of CTCF Is Essential for Restoring Transcriptional Activity from the APP Promoter in Depleted Nuclear Extracts—The domains of CTCF that are located to the N- and C-terminal side of the zinc finger domain are not essential for DNA binding (27). However, they may contribute to functional activity. To address this issue, we analyzed CTCF deletions from the N- and C-terminal ends that had been purified from P. pastoris (Fig. 1, A and B). The deletions were used to supplement CTCF-depleted HeLa cell nuclear extract, and their effect on transcriptional activity was assessed.

Two N-terminal deletions were constructed that resulted in primary translational start sites at methionine residues 249 and 285 (Fig. 1A) (32). Deletion Met-249 removed much of the N-terminal domain, whereas deletion Met-285 extended into the first zinc finger. The two C-terminal deletions terminated at cysteine residue 525 and aspartic acid residue 617. C-terminal deletion Asp-617 removed 109 amino acids of the C-terminal end of CTCF without extending into the zinc finger domain, whereas in C-terminal deletion Cys-525, 201 amino acids were deleted, which additionally eliminated peripheral zinc fingers 10 and 11 (Fig. 1, A and B). All N- and C-terminal deletions displayed DNA binding activity to the APBβ sequence (Fig. 3A, lanes 6–12). However, only the C-terminal deletions restored transcriptional activity from the APP promoter proportionally to their binding activity. In contrast, transcriptional activity was not supported with the N-terminal deletions despite adequate binding activity to the APBβ sequence (Fig. 3B, lanes 6–12). This observation was confirmed by combining data from several independent experiments (Fig. 3C). CTCF-depleted extract was supplemented with different amounts of recombinant full-length CTCF, N-terminal deletion Met-249, or C-terminal deletion Cys-525. In each case only full-length CTCF and C-terminal deletion Cys-525 provided a dose-dependent transcriptional activation, whereas N-terminal deletion Met-249 showed little or no activation.

Competition of Endogenous CTCF with N-terminal Deletions of Purified Recombinant CTCF Reduces In vitro Transcriptional Activity from the APP Promoter—Because both endogenous CTCF and recombinant N- and C-terminal deletions bind to the APBβ site of the APP promoter, it should be possible to compete the binding of endogenous CTCF by supplementing the native nuclear extract with an excess of N- and C-terminal deletions. Indeed, mobility shift competition showed that adding increasing amounts of recombinant N-terminal deletion Met-249 to HeLa cell nuclear extract gradually shifted the binding equilibrium from the endogenous CTCF toward the N-terminal deletion (Fig. 4A, lanes 1–5). Similar shifts were observed with N-terminal deletion Met-285 and C-terminal deletion Cys-525 (Fig. 4A, lanes 5 and 7). When full-length recombinant CTCF was added, the resulting mobility shift complex was indistinguishable from the endogenous CTCF binding complex, and the total amount of binding complex formed was merely enhanced (Fig. 4A, lane 6).

However, in vitro transcription from the APP promoter was
recombinant CTCF constructs (msu) proportional to those delineated in the CTCF binding activity was determined by mixing 1/2/H9262 (lanes 2) were determined by mobility shift electrophoresis. Similarly, lane 2 was depleted (lanes 6) – lanes 3 – lane 4 with increasing amounts of recombinant CTCF constructs purified from P. pastoris: full-length CTCF (lanes 3–5), C-terminal deletions Cys-525 (lanes 6–8) and Asp-617 (lane 9), and N-terminal deletions Met-249 (lanes 10 and 11) and Met-285 (lane 12). The binding complexes (b) and the free oligonucleotides (f) are indicated (brackets). The binding activity is expressed here as mobility shift units (see “Experimental Procedures”). B, in vitro transcription with the same extract combination as in A except that 4 μl of whole nuclear extract (lane 1) and depleted (lane 2) extract were used in the reaction. Amounts of purified recombinant CTCF constructs (msu) proportional to those delineated in A were then added to 4 μl of CTCF-depleted extract, and the in vitro transcriptional activity was determined (lanes 3–12). The transcriptional activity resulting from the whole nuclear extract was assigned the value 100%. C, these experiments were performed essentially as described in Fig. 2B except that the graph represents a compilation of data from numerous independent experiments with recombinant full-length CTCF (FL), N-terminal deletion Met-249, and C-terminal deletion Cys-525. The curves were generated by nonlinear regression analysis, resulting in R values of 0.94 (FL), 0.92 (C525), and 0.94 (M249).

Fig. 3. Replenishment of CTCF-depleted HeLa cell nuclear extract with recombinant CTCF. A, the CTCF binding activity in 1 μl of whole nuclear extract (lane 1) and in 1 μl of CTCF-depleted extract (lane 2) were determined by mobility shift electrophoresis. Similarly, the CTCF binding activity was determined by mixing 1 μl of depleted extract with increasing amounts of recombinant CTCF constructs purified from P. pastoris: full-length CTCF (lanes 3–5), C-terminal deletions Cys-525 (lanes 6–8) and Asp-617 (lane 9), and N-terminal deletions Met-249 (lanes 10 and 11) and Met-285 (lane 12). The binding complexes (b) and the free oligonucleotides (f) are indicated (brackets). The binding activity is expressed here as mobility shift units (see “Experimental Procedures”). B, in vitro transcription with the same extract combination as in A except that 4 μl of whole nuclear extract (lane 1) and depleted (lane 2) extract were used in the reaction. Amounts of purified recombinant CTCF constructs (msu) proportional to those delineated in A were then added to 4 μl of CTCF-depleted extract, and the in vitro transcriptional activity was determined (lanes 3–12). The transcriptional activity resulting from the whole nuclear extract was assigned the value 100%. C, these experiments were performed essentially as described in Fig. 2B except that the graph represents a compilation of data from numerous independent experiments with recombinant full-length CTCF (FL), N-terminal deletion Met-249, and C-terminal deletion Cys-525. The curves were generated by nonlinear regression analysis, resulting in R values of 0.94 (FL), 0.92 (C525), and 0.94 (M249).

Fig. 4. In vitro competition of native CTCF with purified recombinant CTCF proteins. A, in this experiment, 2 μl of whole nuclear extract were mixed with 2 μl of CTCF-depleted extract (compare Fig. 1). The 4 μl of combined extract were then incubated with a molar amount of radiolabeled APB oligonucleotide (20 ng) that corresponded to the amount of the same sequence present in the reporter plasmid used for in vitro transcription (below). The binding activity was determined by mobility shift electrophoresis (lane 1). For competition purposes, the extract was then mixed with increasing amounts of recombinant N-terminal CTCF deletion Met-249 (lanes 2–4) or Met-285 (lane 5), recombinant full-length CTCF (lane 6), and recombinant C-terminal deletion Cys-525 (lane 7) (see “Experimental Procedures” for details). The various binding complexes (b) and the free oligonucleotides (f) are indicated by brackets. B, in vitro transcription was performed with 4 μl of nuclear extract consisting of 2 μl of whole extract and 2 μl of CTCF-depleted extract (lane 1). Because these are the same conditions as those described in Fig. 2B (lane 3), the normalized transcriptional activity from the APP promoter was here retained at 67% for comparison. The transcriptional activity was then determined with extracts that contained increasing amounts of recombinant CTCF N-terminal deletion Met-249 (lanes 2–4) or Met-285 (lanes 5 and 6), full-length CTCF (lane 7), and C-terminal deletion Cys-525 (lane 8).}

only reduced by the addition of N-terminal deletions of Met-249 and Met-285, and this reduction was somewhat more pronounced with deletion Met-285 (Fig. 4B, lanes 1–6). In contrast, the addition of recombinant full-length CTCF and C-terminal deletion Cys-525 increased transcriptional activity. These results confirm that CTCF activates transcription from the APP promoter and that the N-terminal domain is essential for this activation.

In Vitro Transcription from the APP Promoter Is Unaffected by Adding a GFP Tag to the N-terminal End of CTCF—The results described above provide convincing evidence that CTCF activates transcription from the APP promoter in vitro. Furthermore, the activation is mediated by a portion of the CTCF molecule that is located close to the N-terminal side of the zinc finger domain. To complement this observation, it was of interest to investigate whether similar results could be obtained in
cultured cells. Because CTCF is essential for cell survival, its elimination from the cells was precluded. We therefore devised an in vivo competition experiment to examine how the expression from a transfected APP promoter construct was affected by the concomitant expression of selected cotransfected CTCF constructs. For the purpose of this assay, issues relating to the effect of CTCF deletions on intracellular expression and nuclear translocation had to be resolved. Specifically, in the in vitro assay the transcripts derived from the N-terminal deletions contained different translational start sites than those derived from full-length CTCF and the C-terminal deletions. This was not an issue when the CTCF constructs were purified from P. pastoris, because the amount of CTCF added for extract complementation was normalized to DNA binding activity (mobility shift units). However, the utilization of inappropriate translational start sites in vivo could potentially result in variable translation efficiencies between different CTCF deletion constructs. We therefore decided to add the transcriptional unit of the GFP in frame with the N-terminal ends of the CTCF constructs. This addition of an N-terminal GFP tag served two purposes. First, all constructs contained the same translational start site, which minimized the possibility that the expression of the CTCF constructs was influenced by differential utilization of translational start sites. In addition, both the level of expression and the subcellular localization of the CTCF constructs could be evaluated by fluorescence microscopy.

For the purpose of the in vivo competition assay, we selected full-length CTCF, N-terminal deletion Met-285, and C-terminal deletion Cys-525. Initially, all constructs were provided with an N-terminal GFP tag, expressed in P. pastoris, and purified by chromatography. The purified constructs were analyzed by mobility shift electrophoresis. All GFP-tagged constructs supported binding to the APB site and their ability to activate transcription from the APP promoter remained unaffected.

Nuclear Translocation of CTCF Is Unaffected by N- or C-terminal Deletions—As a regulator of numerous gene functions CTCF unfolds its activity within the nucleus. However, following synthesis in the cytoplasm the mechanism of nuclear translocation has not been adequately examined to date. Consequently, it is conceivable that the extensive deletions of the N- and C-terminal ends might interfere with or eliminate nuclear translocation. To investigate this possibility, full-length GFP-CTCF, GFP-M285, and GFP-C525 all activated transcription in proportion to the number of mobility shift units added. In contrast, the N-terminal deletion GFP-M285 did not activate transcription (Fig. 5B). These results demonstrate that the N-terminal addition of the GFP tag did not alter the property of the CTCF constructs in the sense that their ability to bind to the APB site and their ability to activate transcription from the APP promoter remained unaffected.

In Vivo Competition Confirms that the N-terminal Domain of CTCF Is Essential for Transcriptional Activation—The above experiments established that the N-terminal GFP tag had no discernible effect on the binding of the CTCF constructs to their APB target sequence or on their properties associated with transcriptional activation in vitro. In addition, the N- and C-terminal deletions did not affect nuclear translocation. We therefore proceeded to examine whether the transfected CTCF constructs were able to compete with endogenous CTCF for the expression from the cotransfected APP promoter construct. The reporter plasmid (APP(−488)) contained the APP promoter

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![Fig. 5.](http://www.jbc.org/)

**FIG. 5.** Replenishment of CTCF-depleted HeLa cell nuclear extract with recombinant N-terminal GFP-tagged CTCF. A, the CTCF binding activity in 1 μl of whole nuclear extract (lane 1) and in 1 μl of CTCF-depleted extract (lane 2) was determined by mobility shift electrophoresis. The binding activity of recombinant CTCF protein was determined by mixing 1 μl of depleted extract with purified recombinant full-length CTCF (lane 3), full-length GFP-tagged CTCF (lane 4), GFP-tagged C-terminal deletion Cys-525 (lane 5), and GFP-tagged N-terminal deletion Met-249 (lane 6). The binding complexes (b) and the free oligonucleotides (f) are indicated (brackets). B, in vitro transcription with 4 μl of whole nuclear extract (lane 1) or CTCF-depleted nuclear extract (lane 2). In vitro transcription was then performed with CTCF-depleted extract that was supplemented with recombinant full-length CTCF (lane 3), GFP-tagged full-length CTCF (lane 4), GFP-tagged C-terminal deletion Cys-525 (lane 5), and GFP-tagged N-terminal deletion Met-285. The transcription activities from the APP promoter were normalized to those from the β-actin promoter, and the APP promoter activity in native nuclear extract was assigned the value 100%. The transcripts from the APP and β-actin promoters are indicated by brackets.

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2 A. A. Vostrov, M. J. Taheny, and W. W. Quitschke, unpublished observations.
CAT activities were normalized to identical description from the constitutive CMV promoter. The resulting GFP reading frame alone. These CTCF constructs were transfected with a plasmid containing either one of the GFP-tagged CTCF constructs or the reporter plasmid was cotransfected with a plasmid containing the GFP open reading frame alone. The results show that cotransfection of either the full-length GFP-CTCF or GFP-M285, or C-terminal deletion Cys-525. In each experiment the APP promoter activity obtained with GFP alone was assigned the relative value of 100%. Error bars represent the S.E. of the mean from 8 to 10 independent experiments.

levels of expression from the APP promoter. Specifically, progressive deletions from the 5′ end resulted in a 70–90% reduction in expression from the APP promoter upon removal of the APBβ site. This was observed both by transient transfection in HeLa cells and by in vitro transcription (13, 14). In addition, block mutations within the APBβ core recognition sequence that abolish CTCF binding have a similar effect as 5′ deletions that eliminate the site (13, 14). Furthermore, competition for endogenous CTCF in HeLa cell nuclear extract with double-stranded oligonucleotides containing two independent CTCF-binding sites specifically reduced in vitro transcription from the APP promoter (15). Investigations on embryonic mouse hippocampal neurons showed a time- and differentiation-dependent up-regulation of expression of both endogenous APP transcript and transiently transfected APP promoter constructs (26). This increase in APP expression was largely dependent on the presence of an intact CTCF-binding site (APBβ). An increase in the level of CTCF in hippocampal neurons as a function of differentiation was also observed. A plausible interpretation of these results is that the level of CTCF is a limiting factor in the expression of APP during differentiation of mouse hippocampal neurons.

CTCF is a multifunctional protein that regulates the expression of a wide variety of genes. However, in most of these cases CTCF acts as a selective repressor of transcription, as exemplified by the binding to gene silencers and insulators (see Introduction) (34). CTCF also binds to the chicken c-MYC gene between positions −180 and 210 upstream from the transcriptional start site. It may act either as a transcriptional repressor or activator depending on which cell background the promoter is analyzed (17, 24, 25). In view of these divergent functions, a demonstration that CTCF activates transcription from the APP promoter was essential. We have here provided several lines of direct evidence that this is the case. A large portion of this evidence has been provided by in vitro transcription, because in this system it is easier to control experimental parameters. In contrast, experimental perturbations in vivo are inherently more difficult to interpret because of the probability of secondary intracellular responses and interactions that are often beyond investigative control.

As a first step in demonstrating the requirement for CTCF in transcriptional activation of the APP promoter, the HeLa cell nuclear extract was selectively depleted by antibody adsorption. The depletion of CTCF was confirmed by mobility shift

DISCUSSION

We have previously presented extensive indirect evidence that the nuclear factor binding site APBβ is essential for high
electrophoresis, and in vitro transcription from the APP promoter was reduced to 23% compared with the undepleted extract (Fig. 2B). This residual level of transcription from the APP promoter is consistent with transcriptional levels observed with APP promoter constructs devoid of the APBβ site both in vivo and in vitro (13, 14). Mixing CTCF-depleted extract with increasing ratios of native extract restored the transcriptional activity from the APP promoter. The restoration of transcriptional activity was largely proportional to the increase in CTCF binding activity to the APBβ site. Although these results strongly suggest a direct role of CTCF in APP promoter activation, it could not be excluded that additional factors that interact with CTCF were also removed by the antibody depletion.

This concern was addressed by replenishing the depleted extract with CTCF from a heterologous source. CTCF was expressed in the yeast strain P. pastoris and was purified to near-homogeneity. Interestingly, recombinant CTCF was able to reactivate transcription from the APP promoter in depleted extract in the same manner as the native CTCF (Fig. 3B). This effect cannot necessarily be taken for granted because some functions of CTCF have been shown to depend on posttranslational modifications. For example, phosphorylation of CTCF is correlated with specific differentiation pathways of human myeloid cells, and this modification may alter the affinity for specific DNA-binding sites or alter the binding of cofactors (35). Furthermore, dephosphorylation of casein kinase II-dependent serine phosphorylation sites was associated with enhanced repression of c-myc promoters. However, these phosphorylation sites were located on the C terminus of the protein (36). We have shown previously that the binding properties of recombinant CTCF derived from Pichia is indistinguishable from their native counterparts (27). The observation that recombinant CTCF activates transcription indicates that either CTCF is correctly modified in Pichia or that no specific posttranslational modifications are necessary. However, the role of posttranslational modifications in transcriptional activation from the APP promoter has not yet been determined.

Because recombinant CTCF activates transcription from the APP promoter, specific deletions can be used to define the domain that is essential for activation. Similar deletions were employed to determine that the N-terminal end of the zinc finger domain is aligned toward the transcriptional start site of the APP promoter (27). In this study we investigated two deletions each from the N- (Met-249 and Met-285) and C-terminal (Cys-525 and Asp-617) ends of CTCF (Fig. 1, A and B). Of these, deletion Met-285 extended into zinc finger 2 from the N-terminal side and deletion Cys-525 extended into zinc finger 11 from the C-terminal side. Although these peripheral zinc fingers are not essential for binding to the APP promoter per se, their removal may reduce the stability of the binding complex (27). In this case the absolute amount of CTCF construct present in the reaction would not necessarily reflect its relative binding activity. In addition, it is conceivable that during the purification procedure of recombinant CTCF, a portion of the molecules might be rendered functionally inactive. It therefore became necessary to normalize the binding activity of the CTCF deletions to their potential to activate transcription. For this purpose we introduced the concept of the mobility shift unit (msu) (Fig. 3). This allowed for a direct comparison between the transcriptional activation and the binding activity achieved with each CTCF construct. The results show that the N-terminal domain is essential for transcriptional activation, whereas the C-terminal domain is dispensable. This conclusion was further corroborated by competing endogenous CTCF with the deletion fragments. However, it may be pointed out that N-terminal deletion Met-285 inhibited transcription somewhat more effectively than deletion Met-249 (Fig. 4). Although this phenomenon was not further investigated systematically, it suggested that the transcriptional activation domain of CTCF is located in close proximity to the N-terminal end of the zinc finger domain.

Because it was feasible to compete endogenous CTCF with recombinant CTCF deletions in nuclear extract, we investigated whether it was possible to achieve a similar competition in vivo. Indeed, cotransfection of an APP promoter construct with CTCF deletion Met-285 showed a dramatic decrease in APP promoter activity. In contrast, APP promoter activity was largely unaffected by cotransfection with recombinant full-length CTCF and C-terminal deletion Cys-525. These results support the in vitro observations that the N-terminal end of CTCF is essential for transcriptional activation of the APP promoter. They also suggest that in COS-1 cells the availability of endogenous CTCF is not a limiting factor in transcriptional activation, because APP promoter activity was unaffected by the expression of additional recombinant CTCF. In contrast, nuclear extract in vitro can be adjusted so that the amount of endogenous CTCF becomes a limiting factor. In such a case, providing additional recombinant CTCF results in an increase in transcription from the APP promoter (Fig. 4).

In summary, these results provide the first direct evidence that CTCF is required for APP promoter activation and that the activation domain is located on the N-terminal side of CTCF, presumably close to the zinc finger domain. The mechanism by which CTCF activates transcription is currently under further investigation. However, preliminary evidence suggests that CTCF acts by recruiting additional factors to the transcription complex. An essential step in this process would then be the binding of such a factor to the N-terminal domain of CTCF.

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