

# The Human Enhancer Blocker CTC-binding Factor Interacts with the Transcription Factor Kaiso\*

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CTC-binding factor (CTCF) is a DNA-binding protein of vertebrates that plays essential roles in regulating genome activity through its capacity to act as an enhancer blocker. We performed a yeast two-hybrid screen to identify protein partners of CTCF that could regulate its activity. Using full-length CTCF as bait we recovered Kaiso, a POZ-zinc finger transcription factor, as a specific binding partner. The interaction occurs through a C-terminal region of CTCF and the POZ domain of Kaiso. CTCF and Kaiso are co-expressed in many tissues, and CTCF was specifically co-immunoprecipitated by several Kaiso monoclonal antibodies from nuclear lysates. Kaiso is a bimodal transcription factor that recognizes methylated CpG dinucleotides or a conserved unmethylated sequence (TNGCAGGA, the Kaiso binding site). We identified one consensus unmethylated Kaiso binding site in close proximity to the CTCF binding site in the human 5'  $\beta$ -globin insulator. We found, in an insulation assay, that the presence of this Kaiso binding site reduced the enhancer-blocking activity of CTCF. These data suggest that the Kaiso-CTCF interaction negatively regulates CTCF insulator activity.

The genome of eukaryotes is partitioned into transcriptionally active and transcriptionally inactive domains (1). Insulators are DNA elements that maintain this partition, and they can be subdivided into two functional classes: barrier elements, which stop the spread of heterochromatin, and enhancer blockers, which prevent an enhancer from activating transcription in a neighboring repressed region (2). One of the best studied loci regarding long-range transcriptional regulation is the  $\beta$ -globin locus of vertebrates (3). This locus contains an enhancer, the LCR (locus control region), which acts on the globin genes. The activity of the LCR is confined by two insulators, one at the 5' boundary of the locus and another at the 3' boundary. Both insulators depend on the same protein, CTCF<sup>5</sup> (4, 5).

CTCF was originally isolated as a zinc-finger transcription factor that recognized a CTC-rich sequence in the *c-myc* promoter (6). Over the years, CTCF has been shown to have complex and important roles in the

control of gene expression (7). CTCF binds many different DNA target sequences through the combinatorial use of its 11 zinc fingers, and it is capable of both activating and repressing gene transcription. An additional role of CTCF is to act as an enhancer blocker that prevents communication between an enhancer and a target gene. This process is known as transcriptional insulation. CTCF and YY1 (8) are the only two vertebrate proteins known to act as enhancer blockers. CTCF exerts this critical function at many loci (2). For example, enhancer blocking by CTCF permits correct expression of the imprinted genes *H19* and *IGF2*. At this locus, CTCF is only active on the maternal chromosome. The CTCF target sites on the paternal chromosome are methylated, and this modification completely precludes CTCF binding (9–11).

Recently, significant advances were made in our understanding of how CTCF functions as an enhancer blocker at the 5' chicken  $\beta$ -globin insulator (12, 13). At this insulator site, CTCF interacts with nucleophosmin, a nuclear matrix protein that is concentrated at the surface of the nucleolus. This is thought to result in the formation of physically separated DNA loops, which would then prevent an enhancer element in one chromatin loop from acting on a gene in the neighboring chromatin loop. Interestingly, this finding is consistent with results obtained in *Drosophila*, where a crucial link between nuclear architecture and transcriptional insulation was discovered (1). It is likely that this model also accounts for CTCF action at other insulators, and yet alternative mechanisms cannot be ruled out at this point.

The activity of enhancer blockers is not static but can be turned on and off. Recent experiments with CTCF have shown that post-translational modification of the protein plays an important regulatory role (14). In addition, the function of CTCF can also be regulated through interacting proteins (15). In this study, we sought to identify binding partners of CTCF that could influence its activity as an enhancer blocker. We report the identification of the protein Kaiso as a specific binding partner for CTCF. Kaiso is a member of the POZ (pox virus and zinc finger) family of zinc finger (ZF) transcription factors that are implicated in cancer and development (16). To date, Kaiso is the only POZ-ZF protein that has been shown to have dual specificity DNA binding; it can bind methylated CpG dinucleotides (17) or a specific nonmethylated DNA sequence (TNGCAGGA) (18). Indeed, we identified one nonmethylated Kaiso consensus site near the CTCF binding site on the human 5'  $\beta$ -globin insulator. We show that the presence of an intact Kaiso binding site near the CTCF binding site on the 5'  $\beta$ -globin insulator inhibits the enhancer-blocking activity of CTCF. This raises the possibility that Kaiso is a negative regulator of CTCF enhancer-blocking activity.

## MATERIALS AND METHODS

**Plasmids**—The two-hybrid bait plasmid was constructed by cloning the full-length chicken CTCF cDNA (provided by Rainer Renkawitz) between the EcoRI and BglII sites of vector pGBDU-C (1) (*URA3*-

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<sup>5</sup> The abbreviations used are: CTCF, CTC-binding factor; POZ, poxvirus and zinc finger; ZF, zinc finger; TBS, Tris-buffered saline; GST, glutathione S-transferase.

## Inhibition of CTCF by Kaiso

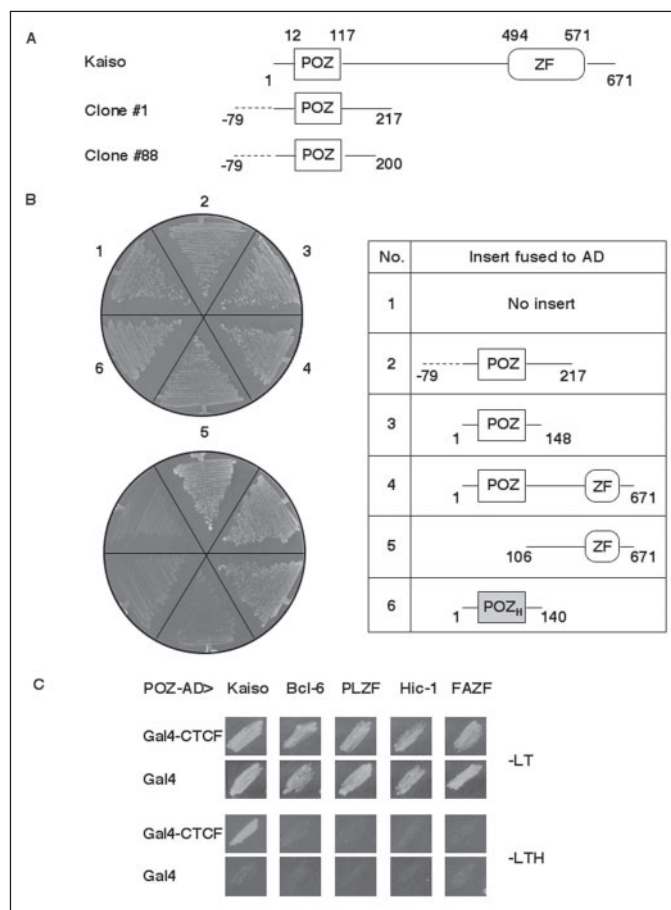
marked, multicopy, *ADH1* promoter driving expression of the bait fused to GAL4<sub>p1-147</sub> (19). The Gal4-CTCF deletion constructs used in Fig. 3 have been described previously (20). To construct the insulation reporters described in Fig. 4, we first cloned the relevant oligonucleotides into the EcoRV site of pBluescriptII KS. They were then PCR-amplified with primers containing MluI sites, digested with MluI, and inserted into the AscI site of pNI (21). All constructs were sequenced.

**Two-hybrid Screen**—We used the two-hybrid strain PJ69–4 $\alpha$  (19). The strain was first transformed with the bait plasmid and then with a library of cDNAs from 6.5–9.5 days post-coitum mouse embryos cloned into the pASV3 vector (*LEU2* marker, multicopy, *PGK1* promoter driving the expression of peptides fused to the VP16 activation domain) (22). The library was a kind gift from Régine Losson. Yeast transformation was done using lithium acetate (23). Transformation efficiency was calculated by plating an aliquot of the cells on plates lacking uracil and leucine. Interactors were selected on plates lacking uracil, leucine, and histidine and containing 5 mM 3-aminotriazole. We then tested whether growth on plates lacking histidine was dependent on the bait plasmid. Finally, cells containing the candidate interactors were mated to PJ69–4 $\alpha$  containing different bait plasmids to test the specificity of the interaction. Only two clones passed all of the screening procedures.

**Cell and Tissue Culture**—Mammalian cells used in this study were the human cervical carcinoma cell line HeLa and the human erythroleukemia K562 cells. HeLa cells were grown at 37 °C in 5% CO<sub>2</sub> in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), and fungizone (0.5  $\mu$ g/ml). K562 cells were grown in RPMI 1640 medium with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100  $\mu$ g/ml).

**Co-immunoprecipitation and Immunoblot Analysis**—HeLa cells were washed once with 5 ml PBS (pH 7.4) prior to the preparation of cytoplasmic and nuclear fractions (24). Nuclear lysates were quantified by Bradford assay, and equal amounts of total protein were used for immunoprecipitation with anti-Kaiso monoclonal antibodies 6F, 2G, 12H, 12G, and 11D (25) or with rabbit anti-CTCF polyclonal antibody (Upstate Biotechnology, catalog no. 06-917). The immune complexes were then subjected to SDS-PAGE as described previously (16). After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membrane was briefly blocked at room temperature with 3% skimmed milk powder in TBS (pH 7.4) before incubating at 4 °C overnight with rabbit anti-Kaiso polyclonal antibody at 1/12,000 dilution or rabbit anti-CTCF polyclonal antibody at 1/500 dilution in 3% milk/TBS. The primary antibodies were removed by rinsing the membranes five times with water and then once with TBS for 5 min each. The membranes were then incubated for 2 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, diluted 1:40,000 in 3% milk/TBS. Membranes were finally rinsed five times with water and once with TBS (pH 7.4) for 5 min each and processed using the enhanced chemiluminescence system (ECL, Amersham Biosciences) according to the manufacturer's protocols. The *in vitro* interaction between GST-Kaiso and CTCF was tested as described previously (26).

**Insulation Assay**—We used the method developed in the Felsenfeld laboratory (21). The various reporters were linearized with Sall, and DNA was quantified by both UV spectrophotometry and analysis on an agarose gel. One hundred nanograms of each linearized plasmid was then electroporated into  $1 \times 10^7$  K562 cells by electroporation. After 24 h of recovery, the cells were plated in 0.35% agar medium with 750  $\mu$ g/ml Geneticin (active concentration) in two 150-mm dishes. Colonies were counted after 3 weeks. Each construct was tested in duplicate



**FIGURE 1. Two-hybrid interaction between CTCF and the POZ domain of Kaiso.** *A*, clones recovered in the two-hybrid screen with CTCF. Mouse Kaiso contains a BTB/POZ domain (boxed) and three ZF domains. Two plasmids containing overlapping inserts were obtained in the screen. The peptides contain 79 additional amino acids encoded by the 5'-untranslated region of Kaiso (dashed line). *B*, the POZ domain of Kaiso mediates interaction with CTCF. Transformants containing Gal4-CTCF and the indicated prey plasmids were selected on medium lacking uracil and leucine (top). Interaction between the proteins permits growth on medium lacking histidine (bottom). POZ<sub>H</sub>, the POZ domain of the human protein HIC1. *C*, CTCF interacts with Kaiso but not with other POZ domain proteins. Two-hybrid interaction between Gal4-CTCF and the indicated mouse POZ domains was tested as in *B*.

in at least three separate experiments, using a different DNA preparation each time. Statistical analysis of the results was done using Student's *t* test.

**Immunoprecipitation of Chromatin**—HeLa cells were fixed with 1% formaldehyde for 60 min at room temperature. The cells were lysed, and the chromatin was sonicated to an average size of 600 base pairs. For each experiment the chromatin prepared from  $10^7$  cells was immunoprecipitated with 4  $\mu$ g of the relevant antibody, using a previously described protocol (27). The primers used to amplify the human  $\beta$ -globin region are TGAGGATGCCTCCTTCTCTG and CAGCAGCTTCAGCTACCTCTC.

## RESULTS

Biochemical approaches have been used to seek CTCF interactors (28). To identify additional interactors and potential regulators of CTCF function, we performed a yeast two-hybrid screen using a mouse embryo cDNA library and full-length CTCF as the bait. From 2 million transformants, we obtained only two positive clones, clones 1 and 88. The two clones contained overlapping fragments of the same cDNA, encoding a fraction of the transcription factor Kaiso (Fig. 1A). Both clones possessed 237 nucleotides upstream of the reported Kaiso ATG,

which are translated in-frame with the rest of the cDNA, and generate a 79-amino acid extension not normally present in Kaiso. The clones differed by the fact that clone 1 encoded the first 217 amino acids of Kaiso, whereas clone 88 encoded the first 200 amino acids. This N-terminal region of Kaiso contains the POZ domain, which is known to mediate homo- and heterodimerization with other POZ family proteins or non-POZ domain proteins (29, 30). This strongly suggested that the Kaiso POZ domain was involved in mediating the CTCF interaction. In fact, in an independent study, the Daniel laboratory identified CTCF as a Kaiso-specific binding partner using the Kaiso POZ domain as bait.<sup>6</sup> As seen in Fig. 1B, we found that CTCF interacted with the full-length Kaiso protein and also with the isolated Kaiso POZ domain. When the POZ domain was deleted from Kaiso, interaction with CTCF was lost (Fig. 1B). This indicates that the POZ domain is both necessary and sufficient for the CTCF-Kaiso interaction. Because POZ domains are highly conserved and are present in a large number of transcription factors (30), we tested the specificity of the interaction by asking whether CTCF would interact with the POZ domain of four other POZ-ZF proteins (BCL-6, PLZF, HIC-1, FAZF). As seen in Fig. 1B and 1C, CTCF interacted specifically with the Kaiso POZ domain and with no other POZ domain tested. We conclude that CTCF recognizes a specific feature in the POZ domain of Kaiso.

To determine whether CTCF and Kaiso interact *in vivo* in vertebrate cells, we performed co-immunoprecipitation experiments using human cervical carcinoma (HeLa) cells, which express both CTCF and Kaiso endogenously at high to moderate levels. We prepared nuclear extracts and immunoprecipitated Kaiso using five different Kaiso-specific monoclonal antibodies that have been characterized previously (25). The immunoprecipitates were subjected to SDS-PAGE and Western blot analysis using CTCF-specific antibodies. As seen in Fig. 2, endogenous CTCF was specifically co-precipitated by the various Kaiso-specific monoclonal antibodies. In contrast, the preimmune serum failed to precipitate any CTCF-containing material. We thus conclude that CTCF and Kaiso exist in a complex in vertebrate cells. To further verify the interaction, we performed the reciprocal experiment by immunoprecipitating CTCF and Western blotting with a Kaiso-specific rabbit polyclonal antibody. However we failed to detect Kaiso co-precipitating with CTCF in this reciprocal situation. This may be due to the fact that the CTCF antiserum was raised against a region of CTCF that contains the Kaiso interaction domain (see "Discussion"). The CTCF antibody may recognize only uncomplexed CTCF, or the antibody may perturb the native CTCF-Kaiso interaction. An alternative explanation for the failure of the CTCF antiserum to co-immunoprecipitate Kaiso could be stoichiometry; CTCF protein may be in large excess relative to Kaiso, with only a minor subpopulation of CTCF molecules bound to Kaiso at steady state. To determine whether the interaction between CTCF and Kaiso is direct, we used an *in vitro* interaction assay. Radioactively labeled CTCF was produced by *in vitro* transcription and translation and was incubated with bacterially expressed glutathione *S*-transferase (GST) or with GST fused to full-length Kaiso (GST-Kaiso). No CTCF was retained after incubation with GST, whereas CTCF bound the GST-Kaiso protein (Fig. 2B). From this we conclude that Kaiso and CTCF interact directly, without the involvement of a bridging factor.

To delineate the region of CTCF that interacts with Kaiso, we tested a series of CTCF deletion mutants for their ability to bind the Kaiso POZ domain in a yeast two-hybrid assay (Fig. 3A). We delineated the binding domain of Kaiso to a C-terminal region encoding amino acids 641–728 of CTCF. This domain proved necessary and sufficient for the CTCF-

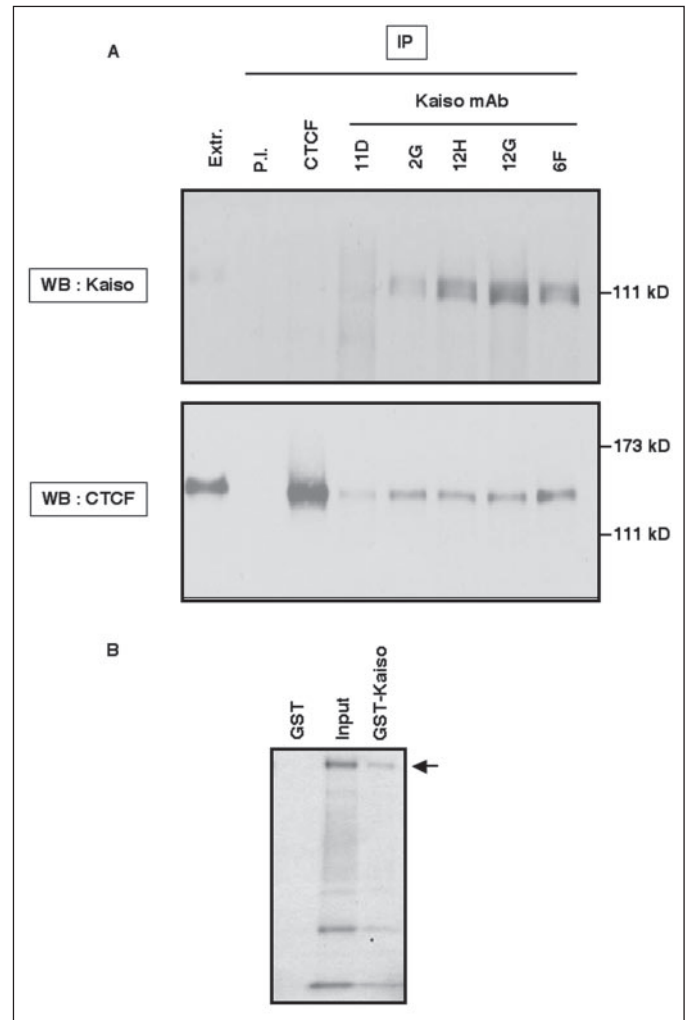


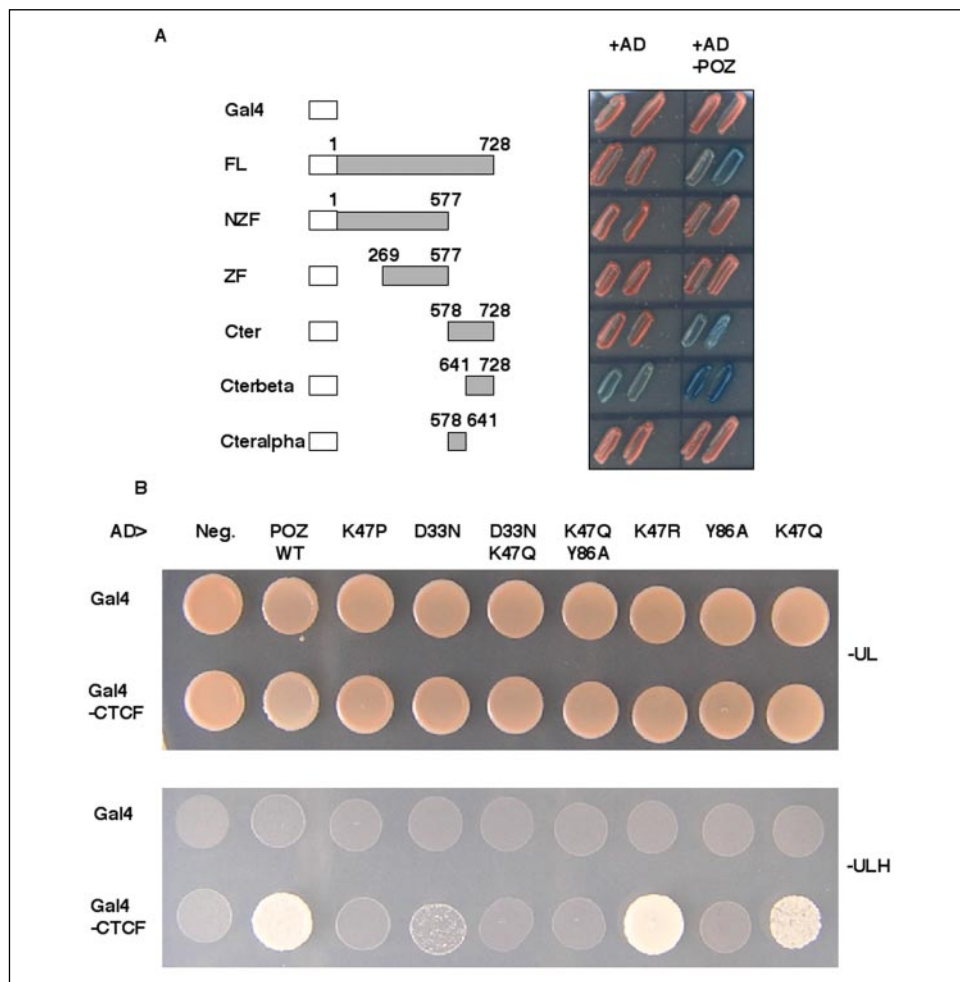
FIGURE 2. **CTCF and Kaiso interact *in vivo* and *in vitro*.** A, to determine whether Kaiso and CTCF interact *in vivo*, nuclear extracts prepared from HeLa cells were subjected to immunoprecipitation (IP) with Kaiso- and CTCF-specific antibodies as indicated. The immune complexes, resolved via SDS-PAGE, were used for Western blotting (WB) with antibodies directed against Kaiso or CTCF. Kaiso was captured using various highly specific Kaiso monoclonal antibodies (mAb). CTCF co-precipitated robustly with endogenous Kaiso from these extracts (Extr.) but not with the negative control antibody (pre-immune serum (P.I.)). B, interaction between bacterially expressed GST-Kaiso and *in vitro* transcribed and translated CTCF. Input, 5% of the input CTCF. The arrow marks the position of the full-length CTCF protein.

Kaiso interaction. Because previous studies have demonstrated that specific highly conserved POZ domain residues of BCL-6 and PLZF are crucial for mediating the homo- and heterodimerization capabilities of these transcription factors (31), we questioned whether the equivalent residues in Kaiso were crucial for the Kaiso-CTCF interaction. Hence, to more precisely define the Kaiso binding site, we generated point mutations in the Kaiso POZ domain (D33N, K47Q, K47R, Y86A, and D33N/K47Q) and tested the capacity of these mutants to interact with CTCF in the yeast two-hybrid assay (Fig. 3B). The lysine residue at position 47 (present in human Kaiso) could be substituted with arginine (present in murine Kaiso) with no apparent loss of activity. However, less conservative substitutions caused partial loss of interaction (lysine to glutamine) or totally abrogated the interaction (lysine to a proline). We also tested two other highly conserved amino acid residues that have been implicated as key determinants in POZ domain function (31, 32). We found that substituting the highly conserved aspartic acid 33 with an asparagine or tyrosine 86 with alanine also disrupted the Kaiso-CTCF interaction.

<sup>6</sup> J. M. Daniel, personal communication.

## Inhibition of CTCF by Kaiso

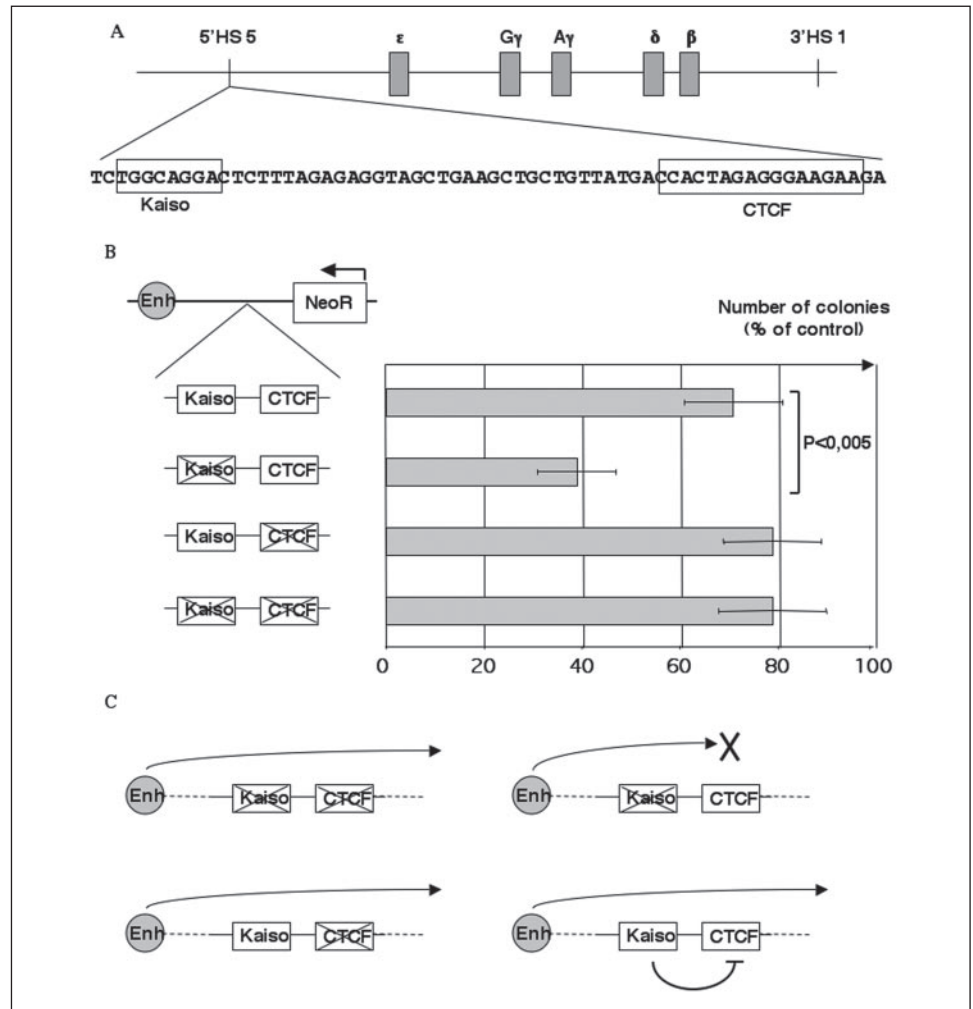
**FIGURE 3. Mapping the interaction domain in CTCF and Kaiso.** *A*, various domains of CTCF were fused to GAL4, and their interaction with the POZ domain of Kaiso was assessed in a strain in which LacZ is the reporter. *B*, point mutations were introduced in the POZ domain of Kaiso. Yeast cells containing the indicated combination of plasmids were plated on control medium (-UL) or on medium that selects for interaction (-ULH). *WT*, wild type; *AD*, activation domain; *FL*, full-length; *NZF*, N terminus and zinc fingers; *Cter*, C terminus.



Kaiso is a unique POZ-zinc finger protein with bimodal DNA-binding properties. It can recognize methyl-CpGs (17), or sequence-specific nonmethylated DNA (18). In site selection experiments, the preferred nonmethylated target of Kaiso is the sequence TNG-CAGGA (18). *In vitro*, Kaiso binds this sequence with higher affinity than methylated DNA, but it is not known whether this preference also exists *in vivo* (18). If Kaiso is a *bona fide* binding partner of CTCF, we postulated that Kaiso may cooperate with, or antagonize, CTCF regulation of target genes. Because CTCF binding to DNA is abrogated by DNA methylation (9–11), we focused our search on the nonmethylated sequence-specific Kaiso binding sites. We examined known insulator regions for the presence of this consensus site and found one Kaiso binding site 34 nucleotides upstream of the CTCF binding site in the human 5'-HS5  $\beta$ -globin insulator (Fig. 4A). Of note, this region does not contain any CpG dinucleotides and therefore cannot undergo DNA methylation. To test whether the presence of this Kaiso binding site affected insulation by CTCF, we performed an insulation assay using a characterized reporter construct, pNI (21). This plasmid contains three relevant elements: an enhancer (mouse  $\beta$ -globin HS2); a reporter gene, *NeoR*, that renders cells resistant to neomycin; and a cloning site between the enhancer and the reporter where test sequences can be inserted. After linearization the plasmid is used to stably transform mammalian cells that are then subjected to neomycin selection. If the test sequence has no enhancer-blocking activity, *NeoR* is fully activated and many neomycin-resistant colonies grow during the selection. In contrast, if the

test sequence harbors an enhancer blocker, *NeoR* is shielded from the enhancer, and few neomycin-resistant colonies appear.

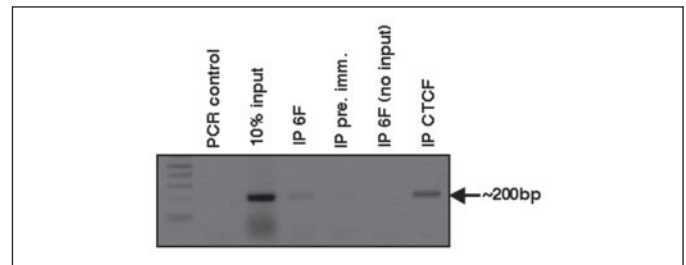
We tested four different sequences derived from the human 5'-HS5 insulator. All are 102 nucleotides in length. The first sequence simply reproduces a portion of the insulator containing both the CTCF and Kaiso binding sites. The sites are placed in the same orientation as in the endogenous locus: Kaiso is upstream on the enhancer side, and CTCF is on the target gene side. The second sequence differs in that it bears two point mutations in the Kaiso binding site. Previous studies have shown that these mutations totally abrogate Kaiso binding *in vitro* (18). The third sequence contains two mutations that have been shown to prevent CTCF binding to its target site (5). Finally, the fourth sequence contains the mutant form of both the CTCF and the Kaiso binding site. All four sequences were inserted into pNI and used for stable transformation, and the neomycin-resistant colonies were counted after selection (Fig. 4A). The experiments were performed in the human erythroleukemia cell line K562 where the HS2 enhancer is functional and CTCF is present and active. We verified by Western blotting that K562 cells also express Kaiso (data not shown). The number of colonies obtained by transfection of unmodified pNI was the reference point of each experiment and was set at 100%. When cells were transfected with an equivalent amount of pNI containing a known CTCF-dependent insulator, chicken 5'-HS4, the number of neomycin-resistant colonies was greatly decreased, to approximately only 30% of that of pNI. This is in good agreement with published values (21) and establishes



**FIGURE 4. A Kaiso binding site at the  $\beta$ -globin insulator regulates enhancer blocking by CTCF.** *A*, structure of the human globin gene locus (not to scale). A consensus Kaiso binding site is present next to the CTCF binding site in the 5'-HS5 insulator. *B*, activity of wild-type and mutant sequences in a mammalian insulation assay. Human K562 cells were transfected with the indicated constructs and seeded in soft agar in the presence of neomycin. The number of colonies was recorded 3 weeks after transformation. *C*, model explaining the behavior of the different constructs. *Enh*, enhancer.

that our test conditions are within the expected optimal parameters. The construct containing mutations in both the CTCF and Kaiso binding sites yielded a high number of neomycin-resistant colonies, about 80% that of empty pNI. This suggests that there is minimal enhancer-blocking activity in this test sequence. The construct containing a wild-type Kaiso binding site next to an inactivated CTCF binding site also produced a high number of neomycin-resistant colonies (80%). This shows that the Kaiso binding site on its own does not have enhancer-blocking potential. The construct containing a wild-type CTCF binding site and a mutant Kaiso site decreased the number of colonies to about 35% that of pNI. This reflects the known enhancer-blocking activity of CTCF in this sequence. Again, the effect is of the same magnitude as the published data (5). Finally we tested the unmodified sequence, bearing functional CTCF and Kaiso binding sites. Strikingly this sequence had little or no enhancer-blocking potential and yielded the same proportion (~75%) of neomycin-resistant colonies as the constructs lacking an intact CTCF binding site (~80%). By comparing these results with those of the other constructs, we conclude that the presence of an intact Kaiso binding site greatly inhibits the enhancer-blocking activity of CTCF at this locus.

Finally, we used chromatin immunoprecipitation to test whether Kaiso can recognize its potential binding site in the  $\beta$ -globin insulator. For these experiments we used antibodies against CTCF and Kaiso that have been validated for chromatin immunoprecipitation



**FIGURE 5. Kaiso binds the  $\beta$ -globin insulator *in vivo*.** HeLa cells were fixed with formaldehyde, and genomic DNA was isolated and fragmented by sonication. Kaiso was immunoprecipitated by using the 6F monoclonal antibody. A 200-bp fragment of the  $\beta$ -globin insulator was amplified by PCR from Kaiso immunoprecipitates (IP 6F) and from CTCF immunoprecipitates (IP CTCF). Negligible amounts of this sequence were amplified from the immunoprecipitation carried out with preimmune antibody (IP pre. imm.). To verify the lack of contamination, two other controls were used. First, the PCR reaction was done using water as the template (PCR control). Second, an immunoprecipitation reaction was done in the absence of input chromatin (no input). For the positive control (10% input), the  $\beta$ -globin insulator was amplified directly from HeLa genomic DNA, which was purified from 1/10th of the amount of lysate that was used for each Kaiso immunoprecipitation.

(15, 27, 33). As expected, the  $\beta$ -globin locus was recovered after immunoprecipitation with serum directed against CTCF (Fig. 5). The same locus could also be amplified in Kaiso immunoprecipitates but not in immunoprecipitates obtained with a nonspecific serum. This finding shows that Kaiso, like CTCF, binds the human 5'-HS5 insulator *in vivo*.

## Inhibition of CTCF by Kaiso

### DISCUSSION

Here we report an interaction between the vertebrate enhancer blocker CTCF and the transcription factor Kaiso. The interaction was also detected in an independent, reciprocal two-hybrid screen that used Kaiso as the bait.<sup>7</sup> There are at least two prerequisites for this interaction to have physiological meaning: CTCF and Kaiso should be co-expressed in some cell types, and they should have an overlapping intracellular distribution. These two criteria are fulfilled by Kaiso and CTCF; both proteins predominantly localize to the nucleus, and both proteins appear to be ubiquitously expressed (18, 34, 35). Although these two proteins may be coexpressed in many cells, this does not necessarily mean that they always function in a complex. It is likely that only a fraction of all CTCF proteins present in a cell are engaged in a complex with Kaiso and vice versa. Furthermore, it is possible that the Kaiso-CTCF interaction is regulated in a temporal manner. Clearly, further study will be required to gain insight into the upstream events or signals that regulate their *in vivo* interaction.

The interaction we have detected is highly specific; CTCF interacts with the POZ domain of Kaiso but not with the POZ domain of four other proteins (Bcl-6, PLZF, Hic-1, and FAFZ), despite the relatively high degree of conservation among their respective POZ domains. The region necessary for interaction with Kaiso is located at the CTCF C terminus. This region is absent from Boris, the closest paralogue of CTCF (36, 37). It is thus likely that Kaiso interacts specifically with CTCF but not with Boris. In addition, this domain of CTCF is particularly rich in phosphorylation sites that have been postulated to play a regulatory role in CTCF-mediated transcriptional repression and possibly protein-protein interactions (38, 39). Hence one possibility is that the phosphorylation status of CTCF regulates its interaction with Kaiso.

Kaiso is a bimodal transcription factor that can bind methylated DNA (17) or nonmethylated targets containing the sequence TNGCAGGA (18). To date, several putative Kaiso target genes have been identified on the basis of such Kaiso binding sites in their natural promoters. Kaiso activates the transcription of one target gene, *rapsyn* (27), but it represses the transcription of the other target genes, such as *matrilysin*, *Wnt-11*, and *MTA2* (33, 40, 41). For *rapsyn*, *matrilysin*, and *Wnt-11*, transcriptional regulation is mediated via the conserved TNGCAGGA binding site, whereas the *MTA2* locus is regulated through Kaiso recognition of methylated CpG sites. If Kaiso does indeed play a role in CTCF-mediated enhancer blocking, we postulated that some CTCF target genes may possess sequence-specific Kaiso binding sites or methylated CpG dinucleotides. Indeed, we found one conserved Kaiso binding site in the human 5'-HS5 insulator at the  $\beta$ -globin gene cluster. The presence of the Kaiso binding site strongly decreases the enhancer-blocking effect of CTCF and implicates Kaiso as a regulator of CTCF function.

Our data could explain the *in vivo* findings of other investigators. Grosfeld and co-workers (42) have investigated the behavior of the human  $\beta$ -globin insulator in transgenic mice. Their results show that the insulator is active in embryonic erythroid tissues but is inactive in other tissues. In tissues where the insulator is inactive, the footprint of a protein is detected over the potential Kaiso binding site TNGCAGGA; in tissues where the insulator is active, this footprint is missing. We have detected binding of Kaiso to the  $\beta$ -globin insulator in human cells, and we suggest that Kaiso is the protein that inhibits the activity of CTCF in these *in vivo* experiments.

Kaiso could act on CTCF by various mechanisms. The simplest possibility would be that Kaiso inhibits the binding of CTCF to its target

site. Such a situation has been described in the case of FBI-1, a POZ domain transcriptional repressor. The POZ domain of FBI-1 binds the Zinc fingers of Sp1 and impedes its DNA binding activity (43). We do not believe that this mechanism applies here, as CTCF is readily detected at the 5'-HS5 insulator both in cultured cells (this work) and *in vivo* (42). An alternative hypothesis relates to the proposed mode of action of CTCF. Felsenfeld and co-workers (12, 13) have shown that CTCF recruits insulated loci to the nuclear matrix at the surface of the nucleolus. It may be that Kaiso interferes with this recruitment step, possibly by redirecting the locus to another nuclear site.

What could be the biological relevance of this regulation? We hypothesize that Kaiso might be used to down-regulate the activity of CTCF at certain times or in certain cell types when enhancer blocking is not desirable. Interestingly, the Kaiso target site is not conserved in the mouse genome. It is possible that Kaiso binds a different sequence on the mouse chromosome or that regulation of the insulator by the Kaiso site is specific to humans. This could relate to the fact that the human 5'-HS5 sequence functions as an insulator *in vivo*, whereas the mouse sequence does not (5, 44, 45). Finally, we note that the interaction described here, in addition to its effect on CTCF function, could also regulate the activity of Kaiso. The adhesion molecule p120<sup>cas</sup> can translocate from the cytosol to the nucleus, where it inhibits transcriptional repression by Kaiso (18, 26). The POZ domain of Kaiso mediates interaction with CTCF and also permits homodimerization (16). Interaction with CTCF could possibly influence Kaiso homodimerization, which in turn could modify the transcriptional activity of Kaiso.

The regulation of CTCF activity by Kaiso is reminiscent of another situation; it has been shown that a subset of CTCF target sites are adjacent to binding sites for the thyroid hormone receptor and that, in this context, the insulating activity of CTCF is modulated by the activity of the thyroid hormone receptor (15). Although the mechanism of this regulation is not understood, it could indicate that CTCF-dependent insulators are not generally constitutive but can be fine-tuned to regulate the transcriptional activity of the genome.

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<sup>7</sup> C. L. Nordgaard and J. M. Daniel, unpublished results.

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