

The vertebrate protein CTCF functions as an insulator in *Saccharomyces cerevisiae*

Pierre-Antoine Defossez* and Eric Gilson

CNRS UMR 5665, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, 69364 Lyon Cedex 07, France

Received August 28, 2002; Revised and Accepted September 27, 2002

ABSTRACT

Insulators are elements that shelter genes from the effects of silencers or enhancers. CTCF is the only vertebrate protein that has a recognized role in transcriptional insulation, but how it exerts its effect is unknown. In an attempt to better understand how CTCF functions, we have used an insulation assay in *Saccharomyces cerevisiae*. We show that CTCF acts as an insulator in yeast, where it can efficiently block the spreading of repressive telomeric chromatin. We identify two domains of the protein that are responsible for this activity: a short and very potent N-terminal domain, as well as the C-terminus of the protein.

INTRODUCTION

Insulators are DNA elements that protect genes from the influence of neighboring regulatory sequences. They can be subdivided into two classes: barrier elements shield genes from the encroachment of repressive chromatin, while enhancer-blockers protect genes from unwanted activation by positively acting elements (1). Insulators have been described in many different eukaryotic organisms, including vertebrates, *Drosophila*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, but the mechanisms underlying their function are still unclear (2–4). However, several results suggest that these mechanisms might be conserved between species. For instance the 5' β -globin insulator, initially characterized in chicken, can protect transgenes against position effects in *Drosophila* (5). Also, the *Drosophila* proteins BEAF and Su(Hw), known to act at the *scs'* and *gypsy* insulators, respectively, can generate barriers against repressive heterochromatin in yeast (6,7).

CTCF is the only insulator protein known in vertebrates at this time. Its activity was first evidenced at the 5' β -globin insulator (8). This locus has both barrier and enhancer-blocking properties. CTCF is necessary and sufficient for the enhancer-blocking function, while recent research suggests that another factor is responsible for the barrier activity (9). Further investigations have revealed that the role of CTCF is not limited to the β -globin locus, but is in fact very widespread. Indeed, all vertebrate enhancer-blocking sequences characterized to date seem to involve CTCF (1). Therefore, CTCF has a role of paramount importance in

insulation, yet the mechanisms by which it fulfills its function are totally unknown.

The budding yeast *S.cerevisiae* has been a very valuable model system in the study of transcriptional phenomena. Mechanisms of transcriptional activation and transcriptional repression that were first discovered in yeast are now known to be well conserved in higher eukaryotes (10,11). We reasoned that a yeast system might also be helpful in the investigation of transcriptional insulation. Using an assay we have previously well characterized (12,13), we show that CTCF has insulating properties in yeast. We use this system to map down two insulation domains in the protein. These results will guide future experiments in vertebrates.

MATERIALS AND METHODS

Yeast strains and plasmids

The yeast strain GF97 is derived from W303 and has been described earlier (13). The plasmid used to express Gal4 fusion proteins in yeast is derived from pGBT9, a 2 μ plasmid that expresses Gal4_{1–147} from the *ADHI* promoter. First, the *TRP1* marker in pGBT9 was replaced by *HIS3*. Then, recombination cloning was used to remove the coding region of *GAL4* encoding amino acids 95–147 and simultaneously insert a sequence encoding a single Myc epitope tag. The resulting plasmid is pPAD8. A plasmid containing the chicken CTCF cDNA was kindly provided by Rainer Renkawitz. Different segments of CTCF were amplified by PCR using this plasmid as a template, and inserted by conventional cloning techniques into pPAD8. All of the resulting constructs were verified by automated sequencing.

Yeast insulation assay

The assay was carried out as previously described (12,13), with the following minor variations. Yeast cells transformed with the plasmid to be tested were grown overnight at 30°C in 250 μ l of selective medium in a 96-well culture plate. Ten microliters of the undiluted culture and of serial 10-fold dilutions (ranging from 10⁻¹ to 10⁻⁵) were then spotted on the adequate selection plates. Each plasmid construct was tested in at least three independent experiments, with four independent cultures each time.

Protein extraction and western blotting

We verified the expression of the various chimeric proteins by western blotting: yeast cells harboring the different expression

*To whom correspondence should be addressed. Tel: +33 4 72 72 87 90; Fax: +33 4 72 72 80 80; Email: pierre-antoine.defossez@ens-lyon.fr

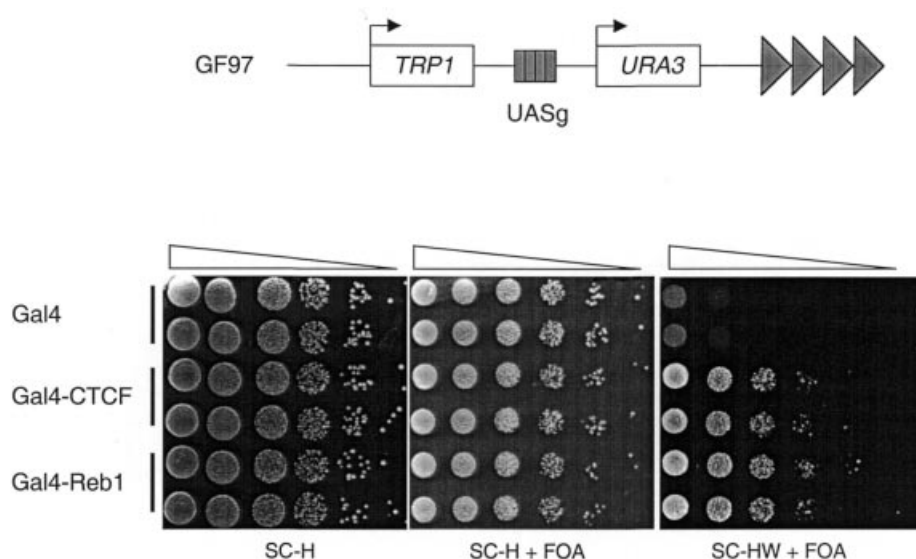


Figure 1. CTCF functions as an insulator in *S.cerevisiae*. (Top) The yeast strain used in the insulation assay, GF97. It has two reporter genes, *TRP1* and *URA3*, in proximity to a telomere (dark triangles) which represses their expression. Protein fragments fused to the Gal4DB are recruited to the UASg sites located between the reporter genes. (Bottom) A representative insulation experiment. GF97 was transformed with the indicated plasmids and two clones of each construct were tested. Serial dilutions of the cells were spotted on the indicated test media. SC-H is the control medium that allows determination of the total cell count. Growth on SC-H+FOA reflects repression of *URA3*. Growth on SC-HW+FOA indicates repression of *URA3* and simultaneous expression of *TRP1*, or in other words insulation of *TRP1*. Gal4-CTCF has no effect on *URA3* expression but greatly increases the proportion of cells where *TRP1* is insulated. The magnitude of this effect is comparable to that of Reb1, a natural yeast insulator.

plasmids were grown overnight in 2 ml of selective medium. Proteins were then extracted following the protocol of Horvath and Riezman (14). Western blotting was carried out according to standard procedures using an antibody directed against the Myc tag.

RESULTS

A genetic insulation assay

To monitor insulation we used a dual-reporter assay that we have previously described in detail (13). The relevant strain, GF97, contains the *TRP1* and *URA3* reporter genes inserted in proximity to telomere VIII_L (Fig. 1, top). Four Gal4-binding sites (UASg) placed between the reporter genes are used to recruit chimeric proteins containing the Gal4 DNA-binding domain (Gal4DB). The chimeric proteins are expressed from a *HIS3*-marked plasmid.

In this assay, three parameters are measured. First, the cells are plated on medium that lacks only histidine (SC-H) to determine the total cell count. Secondly, the cells are plated on medium lacking histidine and containing 5-FOA (SC-H+FOA). The number of cells growing on this medium is divided by the total number of cells to yield the fraction of cells that are FOA-resistant, referred to as the FOAr fraction. 5-FOA is a drug that kills only the cells expressing *URA3*, therefore, the FOAr fraction is the proportion of cells in which *URA3* is repressed. Thirdly, the cells are plated on medium lacking histidine and tryptophan, and containing 5-FOA (SC-HW+FOA). Again, the number of viable cells on these plates is divided by the total cell number to yield the fraction of cells that are simultaneously Trp⁺ and FOA-resistant. This ratio is the Trp⁺FOAr fraction. It is equal to the proportion of cells in

the population that express *TRP1* and simultaneously repress *URA3*.

In the absence of exogenous proteins, *TRP1* and *URA3* are subject to telomeric silencing and their expression is jointly repressed in a majority of cells. Therefore, the FOAr fraction is close to 1, and the Trp⁺FOAr fraction is close to 0. Recruitment of a transcriptional activation domain to the UASg increases the fraction of cells that express *URA3*. This is visualized as a decrease in the FOAr fraction. In contrast, recruitment of an insulation domain to the UASg protects *TRP1* from telomeric silencing while leaving *URA3* unaffected. This translates as an increase in the Trp⁺FOAr fraction, with no change in the FOAr fraction.

CTCF generates transcriptional barriers in *S.cerevisiae*

Strain GF97 was transformed with a vector expressing a fusion of Gal4DB to the full-length chicken CTCF protein, or the Gal4DB alone to serve as a control. The cells were first plated on medium lacking histidine (SC-H). Cells expressing Gal4DB and Gal4-CTCF grew equally well (Fig. 1). This means that expression of the Gal4-CTCF chimera is not detrimental to cell growth. The cells were then assayed on SC-H+FOA to monitor *URA3* expression. Again cells transformed with either plasmid were indistinguishable. This means that Gal4-CTCF does not activate transcription of the *URA3* reporter gene. Finally, the cells were spotted on SC-HW+FOA to detect insulation of *TRP1*. No cells expressing Gal4DB grew on this medium. In other words no cells express *TRP1* while simultaneously repressing *URA3* in these conditions. In sharp contrast, over 10% of the cells expressing Gal4-CTCF grew on SC-HW+FOA. Therefore, we conclude that Gal4-CTCF can shelter *TRP1* from telomeric repression while leaving *URA3* unaffected and behaves as an insulator.

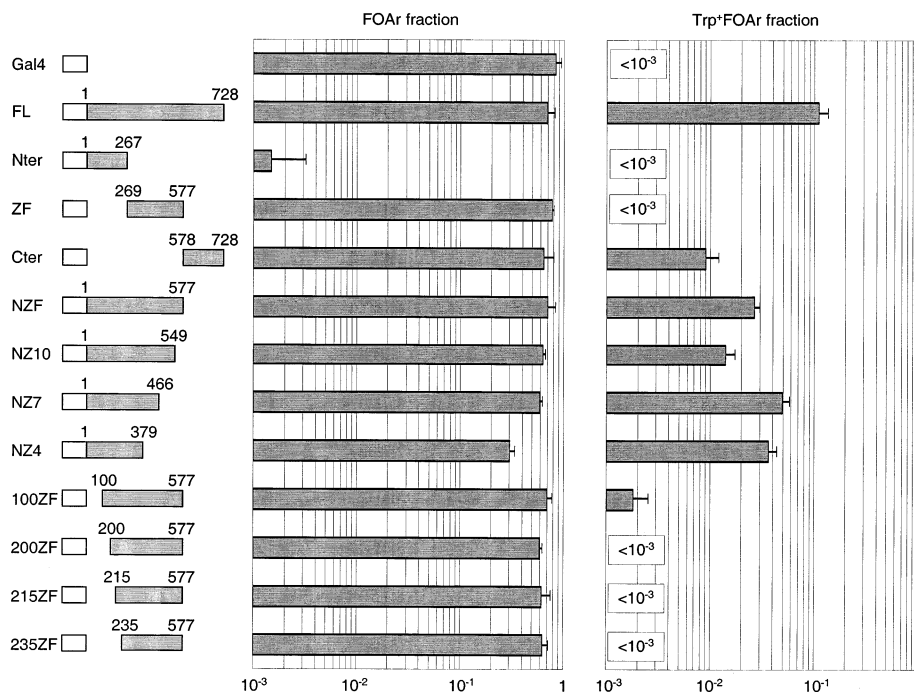


Figure 2. Two domains of CTCF are required for insulation. The indicated residues of CTCF were fused to the Gal4DB (white box) and tested in strain GF97. (Left) The FOAr fraction is the proportion of cells that grow in the presence of 5-FOA, or cells where *URA3* is repressed. (Right) The Trp⁺FOAr fraction is the proportion of cells that express *TRP1* and repress *URA3*, or cells where *TRP1* is insulated. Two regions are necessary for insulation by CTCF: the first 100 amino acids and the C-terminus.

We then sought to compare the strength of insulation by CTCF to that of an endogenous yeast insulator. Reb1 is a yeast transcription factor known to be involved in insulation (12,13). The insulation domain of Reb1 is located between its amino acids 1 and 405, and is the most potent that we have identified to date. We fused this domain to Gal4DB in the chimera Gal4–Reb1. We compared the activity of Gal4–CTCF to that of Gal4–Reb1. As can be seen in Figure 1, Gal4–CTCF is only slightly less active than Gal4–Reb1 in our assay.

From this set of experiments, we conclude that CTCF functions as a strong insulator in yeast. We then set out to use our assay to locate the insulation domain(s) of CTCF by deletion analysis.

Two domains of CTCF are necessary for insulation

We first subdivided CTCF into three domains: the N-terminus (Nter, amino acids 1–267), the central region containing the zinc fingers (ZF, amino acids 269–577), and the C-terminus (Cter, amino acids 578–728).

Each of these regions was fused to Gal4DB and tested in strain GF97 as above. Again, insulation activity in the assay is visualized as an increase in the Trp⁺FOAr fraction.

As depicted in Figure 2, the Nter and ZF regions had no insulation activity. The Cter had some activity, but it was only ~10% as high as that of the full-length protein. Expression of the Gal4–Nter chimera greatly decreased the fraction of cells that were FOA-resistant, meaning that Nter contains a transcriptional activation domain. In contrast, neither ZF nor Cter noticeably activated transcription. Expression of these chimeras and the ones mentioned later was verified by western

blotting (data not shown). All were expressed to comparable levels.

There are two possible explanations to the finding that only Cter has insulation potential, but that it is 10-fold less active than the full-length CTCF. One possibility is that CTCF contains only one insulation domain that is located in or around the Cter, and that it is truncated or improperly folded in our chimeras, resulting in poor activity. The other possibility is that Cter normally cooperates with another insulation domain in CTCF that is not detectable in this first set of constructs. If the former were true, then deletion of Cter should fully inactivate CTCF for insulation. If the latter were true, then deletion of Cter might result in only partial loss of insulation potential. Therefore, we proceeded to delete Cter from CTCF. The resulting construct is designated NZF. Gal4–NZF had clear insulating potential (Fig. 2). From this we conclude that two domains of CTCF are required for insulation: one in Cter, and another one within NZF. We tried to locate the second insulation domain with more precision and generated a series of deletions of NZF.

The DNA-binding domain of CTCF contains 11 zinc-finger motifs (15). We first engineered C-terminal deletions of NZF that removed one, four or seven of the zinc fingers. This yielded constructs NZ10, NZ7 and NZ4, respectively. All three were as potent for insulation as NZF (Fig. 2). Thus, we conclude that the insulation domain is contained within the first 379 amino acids of the protein. We then tested a series of NZF derivatives with N-terminal deletions that removed the first 100, 200, 215 or 235 amino acids. These constructs are named 100ZF, 200ZF, 215ZF and 235ZF, respectively. As can

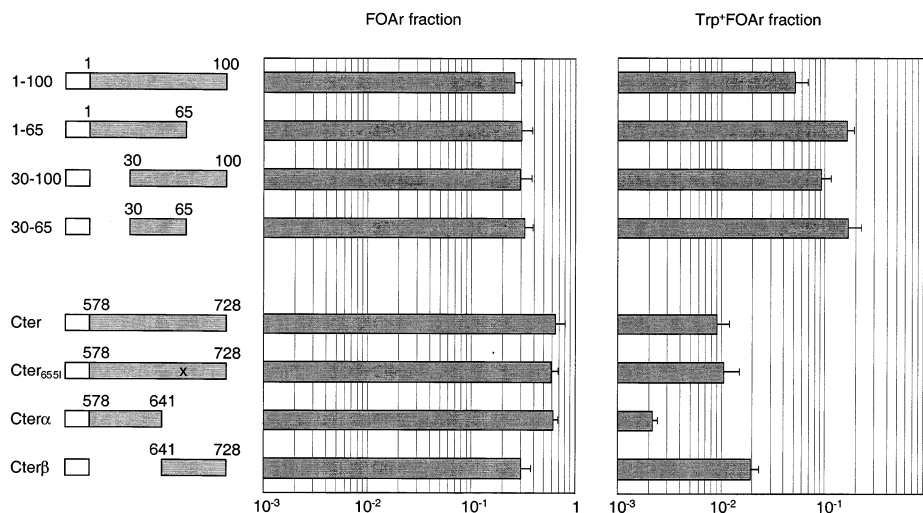


Figure 3. Delineation of the insulation domains. The indicated residues of CTCF were fused to the Gal4DB and tested in strain GF97 as in Figures 1 and 2. (Top) Residues 30–65 of CTCF form a strong insulation domain. (Bottom) The AT-hook within Cter is dispensable for insulation. Amino acids 641–728 recapitulate the activity of Cter.

be seen in Figure 2, ablation of the first 100 amino acids of NZF resulted in almost complete loss of insulation activity. Larger deletions also had the same effect. Therefore, region 1–100 is necessary for insulation by NZF.

These experiments establish that two domains are necessary for insulation by CTCF in our assay: the first 100 amino acids of the protein, as well as the Cter.

Fine-mapping of the insulation domains

The first 100 amino acids of CTCF being necessary for insulation, we tested whether they might also be sufficient for this function. This domain was fused to Gal4DB and assayed in our strain, and indeed the resulting chimera was highly active, in fact almost as potent as full-length CTCF (Fig. 3, top). The fact that the region containing amino acids 1–100 of CTCF behaves as an insulator, while the larger region Nter does not, is due to the presence in Nter of a transactivation domain (see details in Discussion). We then proceeded to try and narrow down the insulation domain in region 1–100. Deletion of the first 30, or of the last 35 amino acids of this fragment had very little effect on insulation activity. Therefore, only the region comprising amino acids 30–65 was necessary for function. To test if it was also sufficient, we fused the 35 amino acids in question to Gal4DB. This chimera was in fact slightly more potent than amino acids 1–100, and about equally as active as full-length CTCF. The primary sequence of the domain is shown in Figure 4.

We also investigated in more detail the C-terminal insulation domain. The Cter peptide contains a sequence motif, KRRGRPPG, characteristic of an AT-hook. This is a DNA-binding motif that permits interaction with AT-rich stretches of DNA (16). To determine if this motif was involved in insulation, we inactivated it by a point mutation. The core sequence GRP was changed to GIP, which should result in loss of function of the AT-hook (16). The mutant chimera Cter655I had an insulation activity similar to that of Cter, and therefore, we conclude that the AT-hook is not involved in insulation by

Chicken	GGQEDEACHI	APNQADGGEV	VQDVNSGVQM	VMMEH
Human	GGQEEDACHL	PQNQTDGGEV	VQDVNSSVQM	VMMEQ
Mouse	GGQEEDASHL	PQNQTDGGEV	VQDVNSSVQM	VMMEQ
Rat	GGQEEDACHL	PQNQTDGGEV	VQDVNSSVQM	VMMEQ
Xenopus	GGVDEENCVI	VQSQTDICEV	PHDVNSNVQM	VMMEQ
Consensus	GG cE C h	Q Dh EV	DVNS VQM	VMME

Figure 4. The N-terminal insulation domain of CTCF. The 35 amino acid insulation domain found in chicken CTCF was aligned with the corresponding regions in the human, mouse, rat and *Xenopus* proteins. h, conserved hydrophobic amino acids, c, charged amino acids.

Cter. We then subdivided Cter into two smaller domains, Cter_α (amino acids 578–641) and Cter_β (amino acids 641–728), and assayed the activity of both subregions. Cter_α only has marginal insulation potential, whereas Cter_β seems to harbor all the activity of Cter.

These data show that CTCF harbors two autonomous insulation domains. The first one is small and very active and corresponds to amino acids 30–65. The second one is 5–10-fold less active and is located at the C-terminus of the protein, within residues 641–728.

DISCUSSION

Characteristics of the insulation domains

We have identified two insulation domains within CTCF. We were able to narrow down the C-terminal insulation domain to a region of approximately 85 residues. This region contains an AT-hook, but this motif is not necessary for insulation. No other sequence motifs are salient in this domain. The N-terminal insulation domain is very active and can be narrowed down to only 35 amino acids (Fig. 4). Its sequence does not include any recognizable protein motif, and is not predicted to have an organized tridimensional structure. The sequence of CTCF has been strikingly well conserved during

vertebrate evolution, and correspondingly the N-terminal insulation domain is almost identical in the human, mouse, rat and chicken proteins (Fig. 4). Comparison with the recently described *Xenopus* CTCF (17) is more informative as this homolog is more evolutionarily distant. Twenty-one out of 35 residues are identical in the chicken and *Xenopus* proteins. This similarity, although high, is weaker than that observed over the total length of the protein (84% amino acid identity). Provided that the insulation function is conserved in *Xenopus*, this would mean that the primary sequence requirements for the insulation domain are relatively loose. This situation is generally reminiscent of that of transcriptional activation domains. Even domains that target the same protein in the transcriptional machinery only show very faint sequence resemblance (18). Also, some of the strongest known natural activators are short peptides that are unstructured in solution, such as the activation domain of the viral protein VP16 (19).

Insulation and activation domains within CTCF

It may seem paradoxical that the region containing amino acids 1–100 of CTCF has strong insulation activity, whereas the larger Nter region (amino acids 1–267) has none. The explanation for this situation is that Nter contains a strong transcriptional activation domain. This domain increases the transcription of *URA3* to such an extent that only ~1 in 1000 cells is resistant to FOA (see line Nter in Fig. 2). This of course precludes the detection of Trp⁺FOA^r cells. Given that region 1–100 does not activate transcription (see Fig. 3), the activation domain must reside between residues 100 and 267 of CTCF. This correlates closely with results recently obtained in a mammalian system. Vostrov *et al.* (20) have found that CTCF transactivates the amyloid precursor protein (APP) promoter. A CTCF truncation variant lacking the first 249 amino acids still binds the promoter, but does no longer transactivate (20). This strongly argues for the presence of a transactivating domain within the first 249 residues. Of note, this transactivation domain has not been detected by other investigators, and its activity may be promoter- or cell type-specific (21).

A related intriguing point is that the Nter region strongly activates transcription, while the larger NZF domain and its derivatives do not. The most likely explanation is that the activation domain is masked by the zinc-finger domain through intramolecular folding. It is possible that, upon binding its recognition sites, CTCF unfolds and reveals its transactivation domain. Such a mechanism has been evidenced for several mammalian transcription factors (22,23).

Relevance to vertebrate cells

Here we describe two domains of CTCF that can block the spreading of repressive telomeric heterochromatin in *S.cerevisiae*. How does this relate to the function of CTCF in vertebrate cells?

First and foremost, our experience with a number of yeast and vertebrate proteins shows that the vast majority of peptides do not insulate in our assay (12,13,24, this work). It seems unlikely, then, that the presence of very potent insulation domains in CTCF is spurious. We do not know for certain that our yeast assay is functionally equivalent to the barrier assays used in vertebrates but it could be expected to identify domains that have barrier activity. It may seem

paradoxical to find such domains in CTCF, which is thought to be an enhancer-blocker (9). However, two simple explanations for this situation can be put forward. First, it is possible that CTCF opposes telomeric heterochromatin by the same mechanisms that it blocks enhancers. How CTCF functions as an insulator is unknown, but some of the existing hypotheses are compatible with this idea (1). The second possibility is that CTCF does have barrier activity, but that this potential is not employed at the insulators examined so far. CTCF is a very versatile factor that activates transcription at certain promoters and represses it at others (15), and it could have an equally complex role in insulation. Experiments testing the activity in vertebrate cells of the two domains presented here will be necessary to directly address this question.

How do the insulation domains function?

Future directions

Recent reports in yeast have shown that many transcriptional activation domains can also be insulation domains (7,13,24). In the case of CTCF, the two insulation domains we have identified do not activate transcription, and we hypothesize that they act by recruiting other proteins. One possibility we actively consider is that they could contact a structural component of the nucleus to raise a physical barrier against silencing (4,6).

Our yeast assay has allowed us to rapidly identify candidate domains for the insulation activity of CTCF. An added advantage of the system is that it opens up opportunities to undertake a genetic analysis of insulation. Future experiments will aim to identify mutant yeast strains in which CTCF is less, or more, active than in the wild-type strain. This approach should shed light on the mechanisms of insulation by CTCF in vertebrate cells.

ACKNOWLEDGEMENTS

We are grateful to Rainer Renkawitz for the gift of the chicken CTCF cDNA. We thank members of the Gilson laboratory for reagents and entertaining discussions, special thanks go to Geneviève Fourel for the gift of useful reagents and advice. P.-A.D. thanks Allison Bardin for support. This work was supported by the Ligue Nationale contre le Cancer.

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