

# BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma

Dmitri I. Loukinov<sup>a</sup>, Elena Pugacheva<sup>a</sup>, Sergei Vatolin<sup>a</sup>, Svetlana D. Pack<sup>b</sup>, Hanlim Moon<sup>a</sup>, Igor Chernukhin<sup>c</sup>, Poonam Mannan<sup>b</sup>, Erik Larsson<sup>d</sup>, Chandrasekhar Kanduri<sup>e</sup>, Alexander A. Vostrov<sup>f</sup>, Hengmi Cui<sup>g</sup>, Emily L. Niemitz<sup>g</sup>, John E. J. Rasko<sup>h</sup>, France M. Docquier<sup>c</sup>, Malathi Kistler<sup>i</sup>, Joseph J. Breen<sup>a,j</sup>, Zhengping Zhuang<sup>b</sup>, Wolfgang W. Quitschke<sup>f</sup>, Rainer Renkawitz<sup>k</sup>, Elena M. Klenova<sup>c</sup>, Andrew P. Feinberg<sup>g</sup>, Rolf Ohlsson<sup>e</sup>, Herbert C. Morse III<sup>a</sup>, and Victor V. Lobanekov<sup>a,l</sup>

<sup>a</sup>Laboratory of Immunopathology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892-0760; <sup>b</sup>Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892; <sup>c</sup>Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester CQ4 3SQ, United Kingdom; <sup>d</sup>Departments of <sup>e</sup>Genetics and Pathology and <sup>e</sup>Development and Genetics, Uppsala University, Norbyvägen 18A, S-752 36 Uppsala, Sweden; <sup>f</sup>Department of Psychiatry and Behavioral Science, State University of New York, Stony Brook, NY 11794-8101; <sup>g</sup>Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Ross 1064, 720 Rutland Avenue, Baltimore, MD 21205; <sup>h</sup>Gene Therapy Research Unit, Centenary Institute and Sydney Cancer Centre, Newtown NSW 2042, Australia; <sup>i</sup>Department of Chemistry and Biochemistry and the School of Medicine, University of South Carolina, Columbia, SC 29208; and <sup>k</sup>Genetisches Institut der Justus-Liebig-Universität, Heinrich-Buff-Ring 58-62, 35392 Giessen, Germany

Communicated by George Klein, Karolinska Institute, Stockholm, Sweden, March 4, 2002 (received for review February 6, 2002)

**CTCF, a conserved, ubiquitous, and highly versatile 11-zinc-finger factor involved in various aspects of gene regulation, forms methylation-sensitive insulators that regulate X chromosome inactivation and expression of imprinted genes. We document here the existence of a paralogous gene with the same exons encoding the 11-zinc-finger domain as mammalian CTCF genes and thus the same DNA-binding potential, but with distinct amino and carboxy termini. We named this gene *BORIS* for Brother of the Regulator of Imprinted Sites. *BORIS* is present only in the testis, and expressed in a mutually exclusive manner with CTCF during male germ cell development. We show here that erasure of methylation marks during male germ-line development is associated with dramatic up-regulation of *BORIS* and down-regulation of CTCF expression. Because *BORIS* bears the same DNA-binding domain that CTCF employs for recognition of methylation marks in soma, *BORIS* is a candidate protein for the elusive epigenetic reprogramming factor acting in the male germ line.**

The ubiquitous, highly conserved, multivalent 11-zinc-finger (ZF) factor CTCF plays multiple roles in gene regulation, depending on the combinatorial utilization of different ZFs to bind varying CTCF target sites (1). CTCF–DNA complex formation can be regulated by CpG-specific DNA methylation (2, 3) if the involved CpG nucleotides are present within 50–60-bp-long CTCF target sequences precisely at DNA base positions required for recognition by the ZFs (2). Among the multitude of normal CTCF functions (1), its involvement in partitioning the genome into active or inactive domains by means of a chromatin insulator function (4) has received particular attention. This feature creates functionally autonomous gene cluster/expression domains by preventing unscheduled activation or silencing by neighboring regulatory elements. For example, the methylation-sensitive chromatin insulator within the imprinting control region (ICR) between *IGF2* and *H19* genes (5) regulates its interaction with CTCF *in vivo* (2), which likely results in maternal-specific repression of the *IGF2* gene.

Strikingly, all vertebrate chromatin insulators identified so far interact with CTCF (1, 3, 6). Moreover, sites with proven or proposed potential to interact with CTCF include other epigenetic centers, such as the ICR within the Prader-Willi/Angelman syndrome locus<sup>m</sup> conserved regions within reciprocally imprinted *Dlk1/Gtl2* genes (7, 8) and the *Kcnq1* ICR (ref. 9; and G.

Fitzpatrick, E.P., C.K., R.O., M. Higgins, and V.V.L., unpublished data). Finally, the choice/imprinting center of the *XIST* antisense gene, *Tsix*, contains several tandem CTCF-binding sites, which collectively function as CTCF-driven chromatin insulators (10).

Although the mechanism for reading of at least some imprinting marks in somatic cells may be explained by allele-specific interactions between CTCF and ICRs, there is as yet no explanation for the targeting mechanism by which the differentially methylated states of the CTCF-dependent ICRs are established *de novo*. By screening testes nuclear extracts for proteins binding to CTCF target sites, we identified and cloned a previously uncharacterized factor that shares all 11 ZFs with CTCF while being different in the regions flanking this DNA-binding domain. This CTCF-paralogue gene, termed Brother Of the Regulator of Imprinted Sites, or *BORIS*, may provide a conceptual framework for understanding epigenetic reprogramming events that go beyond the imprinting phenomenon.

## Material and Methods

**Nuclear Extracts (NE) and Electrophoretic Mobility Shift Assays (EMSA).** NE from tissues were prepared as described (11), and used in EMSA with DNA probes and antibodies as described (2, 12, 13).

Abbreviations: ZF, zinc finger; ICR, imprinting control region; EMSA, electrophoretic mobility shift assay; Ab, antibodies; RT, reverse transcription; NE, nuclear extracts.

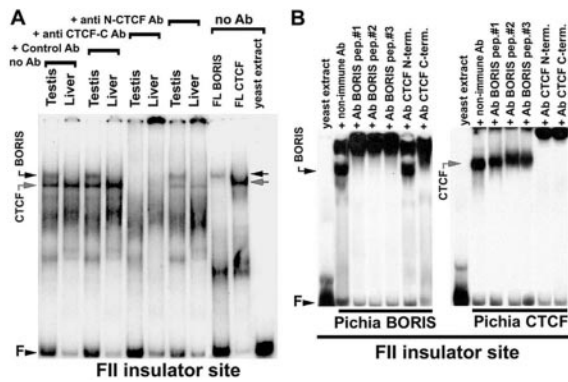
Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession no. AF336042).

<sup>l</sup>Present address: Transgenomic Inc., 11 Firstfield Road, Suite E, Gaithersburg, MD 20878.

<sup>m</sup>To whom reprint requests should be addressed at: Laboratory of Immunopathology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 7, Room 303, 7 Center Drive, MSC 0760, Bethesda, MD 20892. E-mail: vlobanekov@niaid.nih.gov.

<sup>n</sup>Ohta, T., Khadake, J. R., Rodriguez-Jato, S., Mione, C., Knepper, J. L., Svoboda, P., McCarrey, J. R., Schultz, R. M., Yang, T. P. & Nicholls, R. D. (2001) *Am. J. Hum. Genet.* **69**, 52 (abstr.).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.



**Fig. 1.** EMSA analyses. (A) EMSA analyses of nuclear extracts (NEs) from rat liver versus testis tissues. no Ab, marked by downwards brackets, no antibodies were added to the EMSA reactions; anti CTCF-C Ab and anti CTCF-N, reactions contained affinity-purified antibodies against either the C-terminal CTCF region or the N-terminal CTCF region, respectively; Control Ab, equal amounts of the rabbit irrelevant polyclonal IgGs (in this case against GATA-1 protein). FL BORIS and FL CTCF, EMSA results with the full-length BORIS and CTCF proteins. Positions of protein-free FII DNA probe (F), and of the protein-CTCF- or BORIS-bound probe—are indicated by the arrowheads. (B) “Supershifting” EMSA with three anti-BORIS-peptide chicken antibodies and two anti-CTCF antibodies (marked at the top of the gel) and recombinant full-length (FL) CTCF and FL BORIS proteins produced in *Pichia pastoris*.

**PCR-Mediated Screening for CTCF-Like cDNA Sequences and Isolation of Human and Mouse BORIS cDNAs.** PCR-screening primers are listed in *Supporting Text*, which is published as supporting information on the PNAS web site, www.pnas.org. Following cloning and sequencing of PCR products obtained using the Marathon-Ready human testis cDNA (CLONTECH), one such fragment displayed a human cDNA sequence containing an ORF encoding CTCF-like ZFs. This was used to design the specific pairs of primers for PCR analyses of the Rapid Screen Arrayed human testis cDNA Library Panel (OriGene Technologies, Rockville, MD), as well as for the 5' and 3' rapid amplification of cDNA ends (RACE) with the Marathon-Ready testis cDNA and adaptor primers from the Marathon cDNA Amplification kit (CLONTECH). 5' RACE was performed using the GeneRacer kit (Invitrogen). The mouse *BORIS* homologue was similarly amplified using the Marathon-Ready mouse testes cDNA library (CLONTECH).

**RNA Isolation, Northern Blots, and Reverse Transcription (RT)-PCR.** Northern blots of isolated RNA (14) were probed with the *NdeI*-*AccI* fragment of the 5' end of human *CTCF* cDNA clone p7.1 (14) and the *XhoI*-*XhoI* fragment of the *BORIS* cDNA (Fig. 2A). Expression patterns were determined by RT-PCR using human *BORIS*-specific primers (forward, 5'-cagccctacaagtgaacgactgca-3'; reverse, 5'-gcatctgaaggctctcacctgagtg-3') in parallel with amplification of human  $\beta$ -actin (primers from OriGene) as an internal control. Mouse *BORIS*-specific primers (forward, 5'-gagagacagacaagagagaagagaggtgctc-3'; reverse, 5'-cctgtgtgggtgttcacatggttctaagaag-3') were used together with the primers for mouse  $\beta$ -actin. For detection of CTCF mRNA, primers E5E6up (5'-tcgcaagtggaccacaaatc-3') and E4E5down (5'-gaaccattcaggggaaagc-3') were used. For both *CTCF* and *BORIS*, the primers used for RT-PCR were chosen so as not to amplify genomic DNA.

**Expression of CTCF and BORIS in *Pichia pastoris*.** CTCF was purified as originally described (15) with modifications outlined recently (12). Expression of BORIS in yeast was accomplished using the *Pichia* Expression Kit (Invitrogen) according to the manufacturer's instructions, with chromatography steps similar to those described for CTCF (12).

**Antibodies (Ab).** Rabbit antiserum against the bacterially produced N-terminal domain of CTCF (16) was produced as previously described for Ab against synthetic CTCF peptides (17). Chicken Ab against synthetic BORIS peptides (Fig. 2B) were produced by Aves Labs (Tigard, OR), purified, and characterized as described in Fig. 6, which is published as supporting information on the PNAS web site.

**In Situ Analyses of mRNA, Protein, and Cytosine-Methylation.** Digoxygenin-11-dUTP or biotin-16-dUTP-labeled (Boehringer) 5' end of the *BORIS* cDNA were hybridized to formaldehyde-fixed or frozen sections of mouse and human testis as described (18). For immunostaining, the slides were boiled for 10 min in 1× Citra (InnoGenex, San Ramon, CA), followed by incubation with affinity-purified chicken anti-BORIS, rabbit anti-CTCF, or sheep anti-5-methylcytosine (anti-5mC) Ab (Maine Biotechnology Services). The BORIS and CTCF epitopes were visualized by FITC-conjugated rabbit anti-chicken (Dako) and alkaline phosphatase (AP)-conjugated (using the 5-bromo-4-chloro-3-indolyl-phosphate substrate; Roche) goat anti-chicken or goat anti-rabbit (Promega) secondary Ab. For double-staining of chicken anti-BORIS and sheep anti-5mC Ab, a goat anti-chicken secondary Ab conjugated to biotin (Vector), with subsequent detection by avidin-Rhodamine (Vector), was used for BORIS detection, and rabbit anti-sheep Ab conjugated to FITC (Vector) for detection of 5mC.

## Results

**Detection of a CTCF-Like DNA-Binding Protein in Testes NE.** Using EMSA analyses of several well characterized CTCF-target sequences (1), we could document that testis NE produced a DNA-protein complex with a mobility slightly slower than that for the CTCF complex (Fig. 1A). This activity was not detected in NEs from a variety of somatic tissues from rats and mice (Fig. 1; data not shown). The binding activity in testis NEs could be competed with an excess of unlabeled DNA fragments bearing other CTCF targets, but not with the same fragments mutagenized at specific CTCF-contacting bases described previously (2, 3, 14, 17, 19). Fig. 1A also shows that the testis-specific factor could be “supershifted” in EMSAs with an excess of anti-CTCF-C Ab against the C-terminal region of CTCF downstream from the middle of the 11-th ZF. However, in contrast to DNA-bound CTCF, this testis-specific activity could not be supershifted by Ab against the N-terminal region upstream of the first ZF. These results suggested that in addition to CTCF, testis NE contained a different form of CTCF or a protein highly related to CTCF.

**Cloning of Human cDNA Encoding BORIS.** Conserved regions within the vertebrate *CTCF* cDNAs were used to design a variety of primers for PCR amplification of *CTCF*-like sequence fragments from a human testis cDNA library that were then cloned and sequenced. Of more than 100 fragments, one PCR product contained a *CTCF*-like sequence encoding an amino acid sequence practically identical to that of ZF 4, 5, and 6 of CTCF. After additional PCR-mediated screening and 5' rapid amplification of cDNA ends (RACE), we identified an  $\approx$ 4-kb-long cDNA encoding a previously uncharacterized protein with all 11 CTCF-like ZFs (see Fig. 6). An alignment with CTCF sequence (Fig. 2A) revealed a remarkable similarity between the two 11-ZF domains, including most of the major DNA-base-recognition residues at positions -1, 2, 3, and 6 (1, 3) within each ZF. Referring to the CTCF function in reading imprinting marks in soma, we termed this previously unknown protein BORIS.

To verify that the cloned *BORIS* cDNA encodes the same CTCF-site-binding activity, and the same pattern of recognition by anti-CTCF Ab, as initially detected in testis NE by EMSA, the clone was used as a template for coupled *in vitro* transcription/translation and to produce a full-length recombinant BORIS in *Pichia* (12, 15).

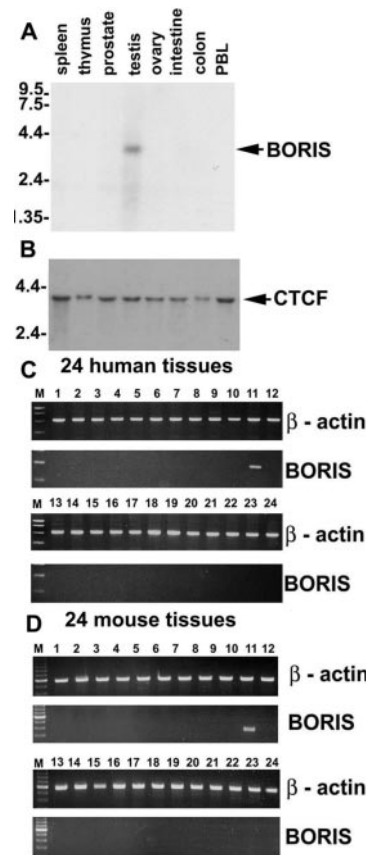


**BORIS Is a Paralogue of CTCF.** We cloned the mouse *BORIS* counterpart with the entire ORF followed by the 3'-UTR with the poly(A)-tail (see Fig. 7, which is published as supporting information on the PNAS web site). The Bestfit alignment of mouse and human *BORIS* amino acid sequences (Fig. 2B) shows that, although all 11 ZFs are practically identical, the regions outside the ZFs are overall only similar but do contain several highly conserved motifs. Although CTCF is overall 93% conserved in avian and mammalian species (1), the regions of mouse and human *BORIS* and CTCF proteins outside of the "shared" 11 ZFs display no similarity to each other or to other known protein signature motifs.

A comparison of the overall genomic organization (Fig. 2C) and of the intron-exon junction sequences (see Table 1, which is published as supporting information on the PNAS web site) of avian (20), mouse, and human *CTCF* (13) with that of *BORIS* revealed striking similarities between *BORIS* and mammalian but not avian *CTCF*. This finding suggests that the *CTCF* and *BORIS* ZF exons have descended with divergence from a common ancestral gene. The idea that *BORIS* evolved through duplication and transposition events during mammalian radiation is further supported by our analyses of chromosome localizations of the two genes. A DNA fluorescence *in situ* hybridization (FISH) probe containing human *BORIS* exons revealed a single spot on chromosome segment 20q13.2 without any crosshybridization to other chromosomes (data not shown). This agrees perfectly with the Human Genome mapping data (<http://genome.ucsc.edu/goldenPath/hgTracks.html>), which placed contigs with *BORIS* exons (AL160176-AL035541) to the distal half of the chromosomal band 20q13.2. The conclusion that *BORIS* is a paralogue of *CTCF* is also supported by a "human genome paralogy" map (21) that relates a region flanking the *BORIS*-containing locus at 20q13 to the *CTCF*-containing region of chromosome 16q22 (ref. 30; <http://u119.marseille.inserm.fr/Db/paralogy.html>). A duplication event involving a larger chromosome region harboring several genes in addition to *CTCF* was likely to have occurred first, followed by further divergence of the initially duplicated region to yield *BORIS* with a testis-specific promoter (see below).

**BORIS Expression Is Testis-Specific and Confined to CTCF-Negative Male Germ Cells Undergoing Genome Remethylation.** The pattern of *BORIS* expression was analyzed by Northern blots with poly(A)+RNA (Fig. 3A) and by RT-PCR with cDNAs from 24 human and 24 mouse tissues (Fig. 3C and D). Internal controls were  $\beta$ -actin (Fig. 3C Upper and D Upper) and *CTCF* (Fig. 3B; data not shown). These studies showed that expression of *BORIS* transcripts was testis-specific in both species and that even with the high sensitivity of RT-PCR, *BORIS* expression was below the limits of detection in mouse or human ovaries, and in tissues of 8.5-day to 19-day mouse embryos.

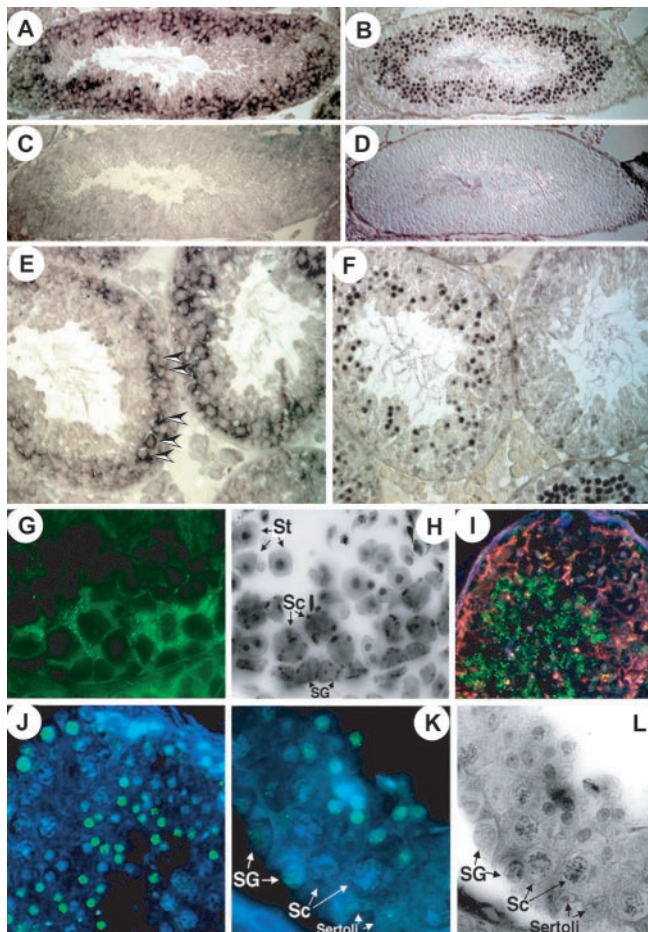
To identify *BORIS*-positive cells in testis, affinity-purified chicken Ab "ap-2-Ab" against the peptide 2, which contains a motif conserved in the mouse and human proteins (Fig. 2C), was generated. Western blot analyses showed that the ap-2-Ab bound specifically to the endogenous *BORIS* polypeptide in testis NE (see Fig. 8, which is published as supporting information on the PNAS web site). Visualization with alkaline phosphatase-conjugated secondary Abs showed that the *BORIS*- and *CTCF*-specific signals distribute to different populations of germ-line cells (Fig. 4A, B, E, and F). The control slides showed only low background signals (Fig. 4C and D). Whereas *CTCF* expression was detected in almost exclusively postmeiotic cells (round spermatids), expression of *BORIS* was restricted to primarily spermatocytes in adjacent sections (Fig. 4E and F; see also Fig. 4G and H). On these sections, most of the signal specific for the *BORIS* peptide-2 epitope appeared in cytoplasm rather than in nuclei (marked by arrowheads in Fig. 4E). Because DNA-binding activity of *BORIS* was initially detected in testis NE (Fig. 1A), we analyzed *BORIS*-positive cells for subcellular distribution of *BORIS* and *CTCF* by Western



**Fig. 3.** Normal expression of human and mouse *BORIS* mRNA is restricted to testis. (A) Northern blot containing 2 mg of poly(A)+RNA per lane from each tissue, indicated at the top of the panel, hybridized to a *BORIS* 5'-terminal probe. (B) The same blot probed with the human *CTCF* "no-ZF" probe. (C) Results of RT-PCR analyses performed using Rapid Scan Panel of cDNAs prepared from 24 human tissues including: 1, brain; 2, heart; 3, kidney; 4, spleen; 5, liver; 6, colon; 7, lung; 8, small intestine; 9, muscle; 10, stomach; 11, testis; 12, placenta; 13, salivary gland; 14, thyroid; 15, adrenal; 16, pancreas; 17, ovary; 18, uterus; 19, prostate; 20, skin; 21, PBL; 22, total bone marrow; 23, fetal brain; and 24, fetal liver. (D) The RT-PCR-mediated detection of mouse *BORIS* mRNA carried out with the Rapid Scan Panel of cDNAs from 24 mouse tissues including: 1, brain; 2, heart; 3, kidney; 4, spleen; 5, thymus; 6, liver; 7, stomach; 8, small intestine; 9, skeletal muscle; 10, lung; 11, testis; 12, skin; 13, adrenal gland; 14, ovary; 15, uterus; 16, prostate gland; 17, 8.5-day embryo; 18, 9.5-day embryo; 19, 12.5-day embryo/12.5; 20, 19-day embryo; 21, breast (virgin mice); 22, breast (pregnant mice); 23, lactating breast; and 24, involuting breast.

blotting of cytoplasmic and nuclear fractions obtained by the PEG-Dextran-Triton-extraction method that preserves natural nuclear-cytoplasmic partitioning patterns of fractionated proteins (described and illustrated in Fig. 8). These analyses indicated that approximately 10–30% of *BORIS* was present in nucleus with the rest in the cytoplasm. In contrast, *CTCF* was, as expected (9, 15) and shown for testis cells in Fig. 4F, found only in the nucleus.

The same cell-type-specific pattern of *BORIS* expression as in mouse testes (Fig. 4) was also observed in human testis by *in situ* RT-PCR and by *in situ* hybridization with a "no-ZFs" cDNA probe (data not shown). Moreover, compared with mouse testes sections, the presence of nuclear-localized *BORIS* in spermatocytes was seen somewhat better in human samples (data not shown), perhaps because of a higher affinity of the ap-2-Ab for the region of human *BORIS* peptide-2 with amino acid sequence that is similar, but not identical, to the mouse sequence (Fig. 2B). Although dual immunostaining for *CTCF* and *BORIS* was not possible (because the same pretreatments that were necessary to reveal the *BORIS*

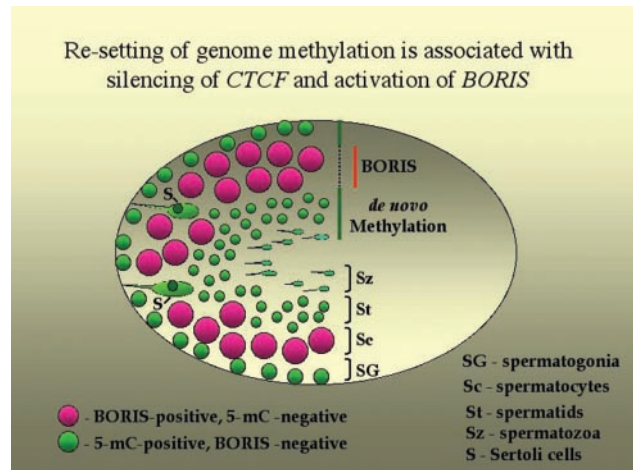


**Fig. 4.** Mutually exclusive expression patterns of BORIS and CTCF correlate with epigenetic reprogramming in testis. (A and E) Immunostaining of thin (5-mm) sections of adult mouse testis with the affinity-purified anti-BORIS antibodies visualized by secondary alkaline phosphatase-conjugated rabbit anti-IgY antibodies. (B and F) Expression in adult mouse testis sections visualized using affinity-purified anti-N-CTCF antibodies and secondary alkaline phosphatase-conjugated antibodies. (C and D) Control staining obtained with normal chicken and rabbit serum, respectively. (G) BORIS-expressing cells in mouse tubule visualized by secondary FITC-labeled antibodies. (H) 4',6-diamidino-2-phenylindole (DAPI)-inverted image of G. (I) Dual immunohistochemical staining by chicken ap-2-Ab anti-BORIS antibodies (Rhodamine fluorescence, red) and anti-5mC antibodies (FITC fluorescence, green). (J) Anti-5mC staining (FITC with DAPI counterstaining) at lower magnification. (K) Identification of the cell types with methylated DNA within the tubule, SG, Sertoli cells, and St. Note methylation free Sc. (L) An inverted DAPI image of K. (Magnification: A–D, 12-fold; E and F, 50-fold; G, H, K, and L, 106-fold; I and J, 44-fold.)

epitope have also resulted in a severe loss of CTCF detection), it is clear that BORIS and CTCF expression patterns overlap very little, perhaps not at all. Moreover, because population of BORIS-positive cells includes primary spermatocytes (shown by the arrows in Fig. 4E), activation of *BORIS* transcription seems to be tightly linked with the final round of mitosis of the male germ-line cells. Fig. 5 summarizes the results as follows: *BORIS* is up-regulated in primary spermatocytes to become silenced on activation of *CTCF* in postmeiotic germ-line cells. Strikingly, this sequential up-regulation of BORIS (in CTCF-negative cells) and of CTCF (in BORIS-negative cells) takes place in association with erasure and re-establishment of methylation marks, respectively, as visualized by the Ab against 5mC (Fig. 4 I–L).

## Discussion

The major result of this work is the identification of a previously uncharacterized testis-specific gene that has the potential to reshape



**Fig. 5.** Schematic representation of the overlapping patterns for BORIS-CTCF expression switch and for resetting of genome-wide DNA methylation during spermatogenesis.

our thinking about site-specific epigenetic events in the male germ line. The gene has been named *BORIS* for Brother of the Regulator of Imprinted Sites. The sibling referred to is CTCF, the 11-ZF protein well known for its many roles in gene regulation (reviewed in ref. 1) including functional “reading” of allele-specific methylation marks (2, 10, 22, 23). BORIS shares an identical 11-ZF DNA-binding domain with CTCF as the basis for familial similarity while being completely divergent at the amino and carboxy termini. This finding alone indicates that nucleoprotein complexes generated by BORIS and CTCF bound to the same DNA sites are likely to have distinct functions. For the ZF-coding regions, we noted remarkable similarity in genomic sequence between *BORIS* and mammalian but not avian *CTCF* at each splice site, as well as similar overall arrangement of the ZF-coding exons. We therefore suggest that at some point of mammalian evolution, all of the ZF exons of *CTCF* were duplicated together with other surrounding regions and transposed to a different chromosome.

As a result of their stunning sameness in the ZF domain, BORIS and CTCF demonstrate currently indistinguishable DNA-binding specificity *in vitro*. In development, differentiation, and function of normal cells, this sharing of specificity cannot result in sibling rivalry because expression of *BORIS* is restricted to a select cell population in testis that is the only normal cell type known that does *not* express *CTCF*. Because inhibition of *CTCF* expression in cultured cells leads to apoptosis (S.V., D.I.L., E.P., H.M., H.C.M., and V.V.L., unpublished results), it is reasonable to assume that *BORIS* is activated to maintain some of the vital *CTCF* functions in these particular cells. Also, switching to BORIS may allow these cells to perform such specific functions that otherwise are inhibited in the presence of CTCF. With respect to the latter proposition, it is striking that the genome-wide erasure of methylation overlaps extensively with BORIS expression, and that remethylation of DNA in round spermatids is associated with the subsequent silencing of *BORIS* and reactivation of *CTCF* expression as depicted in Fig. 5. Thus, BORIS could be associated with demethylases that participate in the erasure of methylation marks.

It is also possible that the BORIS–CTCF switching is intimately linked with initiating (or regional targeting) *de novo* DNA methylation. In this case, reactivation of CTCF would serve to target *de novo* methylation to the paternal imprinting marks. Marking of chromatin for *de novo* DNA-methylation appears to involve histone H3 lysine methylases, such as the *Suv39h1* and *Suv39h2* (24). Because the expression pattern of testis-specific *Suv39h* (25) is remarkably similar to the pattern of BORIS–CTCF switching (Fig. 5), it would therefore be of great interest to test whether

BORIS and CTCF may interact with the Suv39h histone H3 methyltransferases.

The finding of large amounts of BORIS protein in cytoplasm (Fig. 4), confirmed by Western blot analyses of sub fractionated cells (see Fig. 6), is in stark contrast to the strictly nuclear localization of CTCF. However, significant portion of DNA-binding BORIS is localized in nuclei as shown by EMSA with testes NE (Fig. 1A). Moreover, chromatin immunopurification (ChIP) experiments with cells from adult mouse testes showed that BORIS interacts *in vivo* with certain CTCF-target DNA sites (L. Liu, C.K., and R.O., unpublished observations). We propose therefore that the nuclear import of BORIS may have a regulatory role not shared with its sibling, CTCF; and that BORIS may be capable of selective *in vivo* occupation of CTCF sites in the maternally imprinted ICR rather than sites in the paternally imprinted ICRs. This important prediction is now being tested based on our knowledge of CTCF "footprints" within these two classes of ICRs exemplified by the *Igf2/H19* (2) and *Kcnq1* (ref. 9; G. Fitzpatrick, E.P., C.K., R.O., M. Higgins, and V.V.L., unpublished data) loci.

Finally, we showed here that human *BORIS* maps to 20q13.2. It has been shown that this chromosomal region is amplified in many cancers and may contain a dominant immortalizing or transforming gene(s) (26, 27). Our ongoing studies demonstrate that *BORIS* transcripts are abnormally activated in varying proportions of a wide variety of cancers (unpublished data). This defines *BORIS* as a previously unknown member of the cancer-testis (CT) gene family that comprises genes normally expressed only in testis but abnormally activated in different malignancies (28, 29). A conjunction of DNA-demethylation and CTCF-silencing in BORIS-positive cells

that express other CT genes may indicate a common mechanism for transcriptional regulation of *BORIS* and other CT genes. However, *BORIS* is a unique member of the CT family because, unlike the others, it has a somatic counterpart with the same DNA-binding domain but with the properties of a tumor suppressor (13, 30, 31). Expression of BORIS in addition to CTCF, observed only in cancer cells, can set the stage for competition at *IGF2/H19* ICR, and at some other CTCF target sites (1), resulting in cell growth deregulation (unpublished data). We suggest that *BORIS* and *CTCF* may act successively to govern epigenetic states in normal male germ cell development, while their rivalry caused by aberrant activation of *BORIS* in soma is associated with cancer. Therefore, characterization of mouse and human *BORIS* genes described here is likely to provide opportunities for understanding molecular mechanics of epigenetic reprogramming, both in normal development and in tumor genesis.

We thank Mrs. Anita Mattsson, Drs. Vladimir Dobrovitsky, David Lee, Zied Abdullaev, and Helena Malmikumpu for excellent technical assistance with preparing and analyzing human and mouse testis specimens; Mr. Hare Varnon, Mrs. Deborah Curtis, and Dr. Nancy Wolford for the assistance with preparing this manuscript for publication; and Valentina Lobanenkova for great patience and encouragement during the course of the work presented here. This work was supported in part by National Institutes of Health Grants RO1 CA65145 and R37 CA54358 (to A.P.F.), NS30994 (to W.W.Q.), and HD-10793 (to M.K.), as well as grants from the Swedish Cancer Research Foundation and Swedish Pediatric Cancer Foundation (to R.O. and E.L.), the Association for International Cancer Research (to I.C. and E.M.K.), the UK Breast Cancer Campaign (to F.M.D. and E.M.K.), and the Deutsche Forschungsgemeinschaft (to R.R.).

- Ohlsson, R., Renkawitz, R. & Lobanenkova, V. (2001) *Trends Genet.* **17**, 520–527.
- Kanduri, C., Pant, V., Loukinov, D., Pugacheva, E., Qi, C. F., Wolffe, A., Ohlsson, R. & Lobanenkova, V. V. (2000) *Curr. Biol.* **10**, 853–856.
- Filippova, G. N., Thienes, C. P., Penn, B. H., Cho, D. H., Hu, Y. J., Moore, J. M., Klesert, T. R., Lobanenkova, V. V. & Tapscott, S. J. (2001) *Nat. Genet.* **28**, 335–343.
- Bell, A. C., West, A. G. & Felsenfeld, G. (2001) *Science* **291**, 447–450.
- Holmgren, C., Kanduri, C., Dell, G., Ward, A., Mukhopadhyay, R., Kanduri, M., Lobanenkova, V. & Ohlsson, R. (2001) *Curr. Biol.* **11**, 1128–1130.
- West, A. G., Gaszner, M. & Felsenfeld, G. (2002) *Genes Dev.* **16**, 271–288.
- Paulsen, M., Takada, S., Youngson, N. A., Benchaib, M., Charlier, C., Segers, K., Georges, M. & Ferguson-Smith, A. C. (2001) *Genome Res.* **11**, 2085–2094.
- Takada, S., Paulsen, M., Tevendale, M., Tsai, C. E., Kelsey, G., Cattanch, B. M. & Ferguson-Smith, A. C. (2002) *Hum. Mol. Genet.* **11**, 77–86.
- Kanduri, C., Fitzpatrick, G., Mukhopadhyay, R., Kanduri, M., Lobanenkova, V., Higgins, M. & Ohlsson, R. (2002) *J. Biol. Chem.*, 10.1074/jbc.M200031200.
- Chao, W., Huynh, K. D., Spencer, R. J., Davidow, L. S. & Lee, J. T. (2002) *Science* **295**, 345–347.
- Lobanenkova, V. V., Nicolas, R. H., Adler, V. V., Paterson, H., Klenova, E. M., Polotskaja, A. V. & Goodwin, G. H. (1990) *Oncogene* **5**, 1743–1753.
- Vostrov, A. A., Taheny, M. J. & Quitschke, W. W. (2002) *J. Biol. Chem.* **277**, 1619–1627.
- Filippova, G. N., Qi, C. F., Ulmer, J. E., Moore, J. M., Ward, M. D., Hu, Y. J., Loukinov, D. I., Pugacheva, E. M., Klenova, E. M., Grundy, P. E., *et al.* (2002) *Cancer Res.* **62**, 48–52.
- Filippova, G. N., Fagerlie, S., Klenova, E. M., Myers, C., Dehner, Y., Goodwin, G., Neiman, P. E., Collins, S. J. & Lobanenkova, V. V. (1996) *Mol. Cell. Biol.* **16**, 2802–2813.
- Quitschke, W. W., Taheny, M. J., Fochtmann, L. J. & Vostrov, A. A. (2000) *Nucleic Acids Res.* **28**, 3370–3378.
- Klenova, E. M., Nicolas, R. H., U, S., Carne, A. F., Lee, R. E., Lobanenkova, V. V. & Goodwin, G. H. (1997) *Nucleic Acids Res.* **25**, 466–474.
- Klenova, E. M., Nicolas, R. H., Paterson, H. F., Carne, A. F., Heath, C. M., Goodwin, G. H., Neiman, P. E. & Lobanenkova, V. V. (1993) *Mol. Cell. Biol.* **13**, 7612–7624.
- Pack, S. D., Zbar, B., Pak, E., Ault, D. O., Humphrey, J. S., Pham, T., Hurley, K., Weil, R. J., Park, W. S., Kuzmin, I., *et al.* (1999) *Cancer Res.* **59**, 5560–5564.
- Awad, T. A., Bigler, J., Ulmer, J. E., Hu, Y. J., Moore, J. M., Lutz, M., Neiman, P. E., Collins, S. J., Renkawitz, R., Lobanenkova, V. V. & Filippova, G. N. (1999) *J. Biol. Chem.* **274**, 27092–27098.
- Klenova, E. M., Fagerlie, S., Filippova, G. N., Kretzner, L., Goodwin, G. H., Loring, G., Neiman, P. E. & Lobanenkova, V. V. (1998) *J. Biol. Chem.* **273**, 26571–26579.
- Popovici, C., Leveugle, M., Birnbaum, D. & Coulier, F. (2001) *Biochem. Biophys. Res. Commun.* **288**, 362–370.
- Bell, A. C. & Felsenfeld, G. (2000) *Nature (London)* **405**, 482–485.
- Hark, A. T., Schoenherr, C. J., Katz, D. J., Ingram, R. S., Levorse, J. M. & Tilghman, S. M. (2000) *Nature (London)* **405**, 486–489.
- Bird, A. (2002) *Genes Dev.* **16**, 6–21.
- O'Carroll, D., Scherthan, H., Peters, A. H., Opravil, S., Haynes, A. R., Laible, G., Rea, S., Schmid, M., Lebersorger, A., Jerratsch, M., *et al.* (2000) *Mol. Cell. Biol.* **20**, 9423–9433.
- Tanner, M. M., Tirkkonen, M., Kallioniemi, A., Collins, C., Stokke, T., Karhu, R., Kowbel, D., Shadravan, F., Hintz, M., Kuo, W. L., *et al.* (1994) *Cancer Res.* **54**, 4257–4260.
- Cuthill, S., Agarwal, P., Sarkar, S., Savelieva, E. & Reznikoff, C. A. (1999) *Genes Chromosomes Cancer* **26**, 304–311.
- Chen, Y. T., Gure, A. O., Tsang, S., Stockert, E., Jager, E., Knuth, A. & Old, L. J. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 6919–6923.
- Renkvist, N., Castelli, C., Robbins, P. F. & Parmiani, G. (2001) *Cancer Immunol. Immunother.* **50**, 3–15.
- Filippova, G. N., Lindblom, A., Meincke, L. J., Klenova, E. M., Neiman, P. E., Collins, S. J., Doggett, N. A. & Lobanenkova, V. V. (1998) *Genes Chromosomes Cancer* **22**, 26–36.
- Rasko, J. E., Klenova, E. M., Leon, J., Filippova, G. N., Loukinov, D. I., Vatolin, S., Robinson, A. F., Hu, Y. J., Ulmer, J., Ward, M. D., *et al.* (2001) *Cancer Res.* **61**, 6002–6007.
- Aravind, L. & Landsman, D. (1998) *Nucleic Acids Res.* **26**, 4413–4421.