Multiple cis Elements within the Igf2/H19 Insulator Domain Organize a Distance-dependent Silencer

A CAUTIONARY NOTE*  
Received for publication, September 26, 2001, and in revised form, December 27, 2001  
Published, JBC Papers in Press, January 2, 2002,  
DOI 10.1074/jbc.C100552200  

Vasudeva Ginjalaṣ, Claes Holmgrenṣ,  
Erik Ulleräs§, Chandrasekhar Kanduri‡,  
Vinod Pant§, Victor Lobanenkov§,  
Gary Franklin‡, and Rolf Ohlsson***  

From the †Department of Development & Genetics,  
Evolution Biology Centre, Uppsala University,  
Nörbyvägen 18A, S-752 36 Uppsala, Sweden  
and the§Laboratory of Immunopathology, NIAID, National  
Institutes of Health, Bethesda, Maryland 20892  

The 5′-flank of the H19 gene harbors a differentially methylated imprinted control region that represses the maternally derived Igf2 and paternally derived H19 alleles. Here we show that the H19 imprinting control region (ICR) is a potent silencer when positioned in a promoter-proximal position. The silencing effect is not alleviated by trichostatin A treatment, suggesting that it does not involve histone deacetylase functions. When the H19 ICR is separated from the promoter by more than 1.2 ± 0.3 kb, however, trichostatin A stimulates promoter activity 10-fold. Deletion analyses revealed that the silencing feature extended throughout the ICR segment. Finally, chromatin immunopurification analyses revealed that the H19 ICR prevented trichostatin A-dependent reacetylation of histones in the promoter region in a proximal but not in a distal position. We argue that these features are likely to be side effects of the H19 ICR, rather than explaining the mechanism of silencing of the paternal H19 allele. We issue a cautionary note, therefore, that the interpretation of insulator/silencer data could be erroneous should the distance issue not be taken into consideration.

The differentially methylated 5′-flank of the H19 gene (1) is central to our understanding of how the neighboring Igf2 and H19 genes are repressed in a parent of origin-dependent manner. Genetic experiments have demonstrated its involvement in the repression of the maternal Igf2 and paternal H19 alleles (2). The silenced maternal Igf2 allele requires the continuous presence of the H19 imprinting control region (ICR) (3), which has been proposed to function as a chromatin insulator by default (4–7). In line with this supposition, the H19 ICR has no insulator function when methylated (8).

The notion that the methylated status of the H19 ICR involves recruitment of repressive factors that propagate an inactive chromatin toward the H19 promoter (3) is supported by the observation that MeCP2 and MBD 2/3 are associated preferentially with the paternal H19 ICR allele in chromatin immunopurification assays.2 However, because the unmethylated H19 ICR performed as an efficient silencer in transgenic Drosophila assays, it has been claimed that the H19 ICR contains silencing features by default (9). To resolve this paradox, we set out to examine if the unmethylated H19 ICR can act as a silencer under certain circumstances.

EXPERIMENTAL PROCEDURES  

Cell Culture  
The JEG-3 human choriocarcinoma cell line was maintained in modified Glutamax Eagle’s medium (Life Technologies, Inc.), supplemented with 10% fetal bovine serum, glutamate, and penicillin/streptomycin (Life Technologies, Inc.) as described previously (10). Trichostatin A (TSA, Wako GmbH, Neuss, Germany) was added to the culture medium at a final concentration of 3.0 × 10−6 m, 28 h after transfection. The cells were harvested after a 14-h exposure to TSA.

Plasmid Constructs  
The construction of the pSIS vector (based upon the PDGF-B promoter) with CAT gene has been described previously (10). pSISICR1 (in + or − Orientation)—The 3.35-kb H19 ICR, including the natural “spacers,” which refers to the endogenous H19 sequence that lies between the ICR and promoter, was inserted into the multiple cloning site of pSIS at BamHI-XbaI using non-directional A/T cloning strategy. pSISICR1 (+/−)—The 1.4-kb AvrII-XbaI fragment of H19 ICR (4) was ligated into the XbaI-digested pSIS vector in either orientation. pSISICR1S1 was generated by digesting with AccI-EcoRI and removing a 470-bp fragment from the parent ICR natural spacer construct and religating the two ends by blunt-end cloning. pSISICR1S2 was made by digestion with PstI/EcoRI and removing a 1020-bp fragment from the parent construct and religating the two ends after a fill-in reaction to create blunt ends. pSISICR1S3 was created by deleting 550 bp by PstI/PstI restriction digestion followed by blunt-end ligation. pSISICR1S4 was generated by inserting a 550-bp fragment of PstI/EcoRI from the H19 natural spacer. pSISICR1S4 plasmids were generated by inserting a 1.94-kb neutral fragment of the ePrGR (11) with XbaI-SotI digestion followed by blunt-end ligation into the XbaI site of the pSIS and pSISICR plasmids. The pSISICR1S2 construct (with mutated CTCF target sites) was generated by cloning a 1.4-kb KpnI/AvrII blunt-end fragment from pCR2.1-SISH2 (7) into BamHI, the Klenow-filled site of pSISCAT vector. The pSISH1, pSISHII, and pSISNH constructs were generated by PCR amplification of the different parts of the ICR using the following primer pairs (see map of Fig. 4A): H1, 5′-AAAAACCCGCGGCTATCGCTCAATGTCGAT-3′ and 5′-AAAAACCGCGGTGTTAGGAAAAATGTC-3′; HIII, 5′-AAAAACCCGCGGCTATCGCTCAATGTCGAT-3′ and 5′-AAAAACCCGCGGCTATCGCTCAATGTCGAT-3′. The amplified fragments were (AT)-subcloned into pCR2.1 (Invitrogen).

* This work was supported by the Swedish Natural Science Research Council (NFR), the Swedish Pediatric Cancer Research Foundation (BCF), the Lundberg Foundation, and the Swedish Cancer Research Foundation (UFS). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Current address: Dept. of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, S-751 85 Uppsala, Sweden.
§ Current address: Biocare AB, Rapsgatan 7, S-754 50, Uppsala, Sweden.
** To whom correspondence and requests for materials should be addressed. Tel.: 46-184712660; Fax: 46-184712683; E-mail address: Rolf.Ohlsson@ebc.uu.se.

1 The abbreviations used are: ICR, imprinting control region; CAT, chloramphenicol acetyltransferase; PDGF, platelet-derived growth factor; TSA, trichostatin A.
2 C. Kanduri, A. Wolffe, and R. Ohlsson, unpublished data.
The H19 Imprinting Control Region and Gene Silencing

*Transfections and CAT Assays*

Transfections were carried out with equimolar amounts of the plasmid DNAs (5 μg for the basal promoter controls) and 0.5 μg of a reference plasmid containing a β-galactosidase reporter gene under the control of the SV40 promoter-enhancer (pSVβGal), as has been described previously (10). The amount of protein extracts taken for CAT control of the SV40 promoter-enhancer (pSVβGal), as has been described previously (10). The amount of protein extracts taken for CAT analysis on plasmids, see the legend of Fig. 1 and "Experimental Procedures." The results of transient transfection analyses are shown in the right-hand part of the panel and are represented by colored bars that refer to basal activity (blue) versus TSA-induced activity (red). The expression levels were normalized with respect to the activity obtained with the TSA-activated pSIS construct. The S.E. are based on 3–16 independent transfection analyses. B, strategy of multiplex PCR analysis of transfected plasmids with or without the H19 spacer segment. C, chromatin immunopurification analysis of the histone acetylation status at the PDGF-B promoter with or without TSA treatment. The image shows the results of multiplex PCR analyses of immunopurified DNA from cells transfected with equimolar amounts of constructs with or without the H19 spacer, as indicated in the image. Antibodies specific for acetylated forms of histone H3 and H4 were used to assess the histone acetylation status in the promoter region. Rabbit normal serum (norm. ser.) served as a negative control. The antibody against non-acetylated histone H4 was used as a positive control.

*Chromatin Immunopurification Assay*

Transfected cells were harvested and formaldehyde-cross-linked, as has been described (12). Following isolation of nuclei and sonication to shear the DNA, the histone-containing DNA-protein complexes were immunopurified using antibodies to acetylated histones H3 and H4 as well as unacetylated H4 (Upstate Biotechnology Inc., Lake Placid, NY) and protein A4 Fast Flow-Sepharose beads (Pharmacia-Upjohn). The immunopurified DNA was PCR-amplified using forward primers 5′-GGTGAACAGCCTGTAGATCCC-3′ and 5′-GACGCTTCAGTGTGGCAG-3′ for ICR and spacer, respectively, and the common reverse primer 5′-TCAGGGGAGAGTGCAC-3′. The PCR conditions were 1 × 94 °C for 5 min, 1 × 94 °C for 30 s, 1 × 58 °C for 30 s, 1 × 72 °C for 90 s, 24 × (94 °C for 30 s, 58 °C for 30 s, 72 °C for 90 s), and 1 × 72 °C for 5 min. The PCR products were visualized on 2% agarose gels stained with SYBRGreen (Molecular Probes, Eugene, OR), and images were analyzed with Fuji-film Image Reader LAS-1000.

**RESULTS**

We focused on a 1.2-kb segment of the H19 ICR that encompasses a major portion of the differentially methylated domain (Fig. 1A) and displays strong insulator activity (4). This fragment was inserted into CAT reporter vectors that were equipped with either the H19 or PDGF-B promoter. Fig. 1B shows that the basal activity of the PDGF-B promoter was reduced 5-fold when the H19 ICR was inserted in either orientation in a promoter-proximal position. This effect was neutralized when the natural spacer was inserted between the H19 ICR and the PDGF-B promoter. Similar results were obtained using the mouse H19 promoter (data not shown). Because the repressive effect reappeared when the spacer-H19 ICR fragment was reversed, i.e. ICR-proximal, spacer-distal, the effect of the spacer on the repressive properties of the ICR is position-dependent.

Given that gene silencing functions frequently involve histone deacetylation (13), we also examined the performance of these constructs following treatment with TSA, which is an inhibitor of several histone deacetylases (14) and will activate these constructs following treatment with TSA, which is an inhibitor of several histone deacetylases (14) and will activate the paternal H19 allele in mouse embryos (15). TSA treatment of transfected cells enhanced the activity of the basal PDGF-B promoter 10-fold but had little effect on the activity of the PDGF-B promoter when this was juxtaposed to the H19 ICR (Fig. 2A). To determine whether these effects could be directly related to the histone acetylation status of the PDGF-B promoter, we employed chromatin immunopurification assays. Plasmids with the H19 ICR either separated from the promoter by the spacer or juxtaposed to the PDGF-B promoter were mixed in equimolar proportions and transfected into JEG-3 cells. Following administration of TSA during the last 14 h before cell harvest and formaldehyde fixation, cross-linked DNA-protein complexes were immunopurified followed by multiplex PCR analysis (see strategy in Fig. 2B). Fig. 2C shows a mixing experiment to ensure that the promoter configurations in the two plasmids could be amplified without ratio distortions. Fig. 2C also shows that the antibodies against the acetylated forms of histones H3 and H4 pulled down sequences primarily representative of the plasmid with a 1.8-kb natural spacer element separating the H19 ICR from the promoter. As...
a positive control we used histone H4 antibodies that pulled down sequences from both plasmids with a slight preference for the plasmid without the spacer segment (Fig. 2C). We conclude that the proximity of the H19 ICR directly controlled the histone acetylation status at the promoter of the reporter gene.

To further examine the distance-dependent effects, we generated deletion mutants of the spacer that placed the H19 ICR closer to the PDGF-B promoter (Fig. 3A). Fig. 3B shows that repression via the H19 ICR was abrogated when located more distally from the promoter, with the critical distance mapping somewhere between 856 and 1456 bp. To deal with an argument that this distance dependence might be due to the deletion of an additional cis element in the spacer region, we inserted a 1.94-kb exonic fragment derived from the glucocorticoid receptor gene between the PDGF-B promoter and the H19 ICR. Fig. 3B shows that the repressor function was eliminated in this construct (pSISICRA). We conclude, therefore, that the H19 ICR represses cis regulatory elements in a distance-dependent manner but independent of the sequence of the intervening DNA.

Next we examined whether or not the CTCF target sites that organize the insulator function of the H19 ICR (7) were responsible for the repressive features. Fig. 4B shows that the two fragments that harbor CTCF target sites (Fig. 4A) indeed act as silencers in promoter-proximal positions with and without TSA. However, the pSISNH (covering a region 5’ of the CTCF target sites) and pSISS1S2 (with mutated CTCF target sites) constructs revealed that the silencer features do not require the CTCF target sites (Fig. 4B). We conclude that minimally three different and non-overlapping cis elements within the H19 ICR organize a distance-dependent silencer.

**DISCUSSION**

The major point of this report is that a distance-dependent silencing function is a side effect of the H19 ICR. If proven general for insulator and silencer elements, this feature might complicate interpretations on both position-dependent and independent silencing functions should the distance issue not be taken into consideration. The reasons underlying our conclusions are 3-fold. Firstly, despite our demonstration of the H19 ICR silencer function, the H19 gene in the 3’-flank of the H19 ICR is transcriptionally active on the maternal chromosome. Secondly, whereas the minimum distance for neutralizing the silencer function was 1.34 kb between the H19 ICR and the promoter of the reporter gene, both the mouse and human H19 ICRs are separated by 2 kb despite no similarity in the intervening sequence. Hence, the H19 ICR appears to be separated from the H19 promoter by an obligatory “spac ing” region in order to avoid adverse effects on H19 expression. Thirdly, whereas the H19 ICR-induced silencing of the paternal H19 allele could be neutralized by TSA treatment during in vitro mouse embryogenesis (15), the distance-dependent H19 ICR silencer was TSA-insensitive.

What could be the cause for the distance-dependent repressor effect by the H19 ICR? One possibility that comes to mind
is an H19 ICR-specific recruitment of a TSA-insensitive histone deacetylase that locally changes the histone acetylation status to form a repressive chromatin conformation. This possibility is seemingly supported by the demonstration that CTCF, which interacts with two different binding sites within the H19 ICR fragment analyzed in this report, is able to recruit histone deacetylases (16). Given that deletion and mutation analyses reveal that the silencing features might include but clearly do not depend on CTCF target sites, however, this scenario is less likely. Instead, we favor the interpretation that multiple cis elements within the H19 ICR collaborate to organize a chromatin conformation that sterically prevents accessibility of regulatory factors to juxtaposed promoter and enhancer elements. Such cis elements, including the CTCF target sites, might jointly organize a normally protective shield around the maternal H19 ICR allele against de novo methylation.

REFERENCES
1. Olek, A., and Walter, J. (1997) Nat. Genet. 17, 275–276
2. Thorvaldsen, J. L., Duran, K. L., and Bartolomei, M. S. (1998) Genes Dev. 12, 3693–3702
3. Kaffer, C. R., Srivastava, M., Park, K. Y., Ives, E., Hsieh, S., Battle, J., Grinberg, A., Huang, S. P., and Pfeifer, K. (2000) Genes Dev. 14, 1908–1919
4. Kanduri, C., Holmgren, C., Franklin, G., Pilartz, M., Ulleräs, E., Kanduri, M., Liu, L., Ginjala, V., Mattsson, R., and Ohlsson, R. (2000) Curr. Biol. 10, 449–457
5. Bell, A. C., and Felsenfeld, G. (2000) Nature 405, 482–485
6. Hark, A. T., Schoenberr, C. J., Katz, D. J., Ingram, R. S., Levorise, J. M., and Tilghman, S. M. (2000) Nature 405, 486–489
7. Kanduri, C., Pant, V., Loukinov, D., Pugaacheva, E., Qi, C.-F., Wolfe, A., and Lobanenkov, A. (2000) Curr. Biol. 10, 855–856
8. Holmgren, C., Kanduri, K., Dell, G., Ward, A., Mukhopadhyya, R., Kanduri, M., Lobanenkov, V., and Ohlsson, R. (2001) Curr. Biol. 11, 1128–1130
9. Lyko, F., Brenn, J. D., Surani, M. A., and Paro, R. (1997) Nat. Genet. 16, 171–173
10. Franklin, G., Donovan, M., Adam, G., Holmgren, L., Specht, A., Pfeifer-Ohsllson, S., and Ohlsson, R. (1991) EMBO J. 10, 1365–1373
11. Ulleräs, E., Miller, S., Adam, G., Kanduri, C., Wilcock, A., and Franklin, G. (2001) Exp. Cell Res. 270, 188–198
12. Kanduri, M., Kanduri, C., Vostrov, A., Quitschke, W., Lobanenkov, V., and Ohlsson, R. (2001) Mol. Cell. Biol. in press
13. Grunstein, M. (1997) Nature 389, 349–352
14. Yoshida, M., Horinouchi, S., and Beppu, T. (1995) Bioessays 17, 423–430
15. Svensson, K., Mattsson, R., James, T., Wentzel, P., Pilartz, M., MacLaughlin, J., Miller, S., Olsson, T., Eriksson, U., and Ohlsson, R. (1998) Development 125, 61–69
16. Lutz, M., Burke, L., Barrete, G., Goeman, F., Greb, H., Arnold, R., Schulteis, H., Brehm, A., Kouzarides, T., Lobanenkov, V., and Renkawitz, R. (2000) Nucleic Acids Res. 28, 1707–1713
Multiple cis Elements within the Igf2/H19 Insulator Domain Organize a Distance-dependent Silencer: A CAUTIONARY NOTE
Vasudeva Ginjala, Claes Holmgren, Erik Ullerås, Chandrasekhar Kanduri, Vinod Pant, Victor Lobanenkov, Gary Franklin and Rolf Ohlsson

J. Biol. Chem. 2002, 277:5707-5710.
doi: 10.1074/jbc.C100552200 originally published online January 2, 2002

Access the most updated version of this article at doi: 10.1074/jbc.C100552200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 15 references, 3 of which can be accessed free at http://www.jbc.org/content/277/8/5707.full.html#ref-list-1