Bidirectional Silencing and DNA Methylation-sensitive Methylation-spread Properties of the \textit{Kcnq1} Imprinting Control Region Map to the Same Regions*

Noopur Thakur‡§, Meena Kanduri‡§, Claes Holmgren‡, Rituparna Mukhopadhyay‡, and Chandrasekhar Kanduri¶

From the \&Department of Development and Genetics, Evolution Biology Centre, Uppsala University, Norbyvägen 18A, S-752 36 Uppsala, Sweden

The mechanisms underlying the phenomenon of genomic imprinting are poorly understood. Accumulating evidence suggests that imprinting control regions (ICR) associated with the imprinted genes play an important role in creation of imprinted expression domains by propagating parent-of-origin-specific epigenetic modifications. We have recently documented that the \textit{Kcnq1} ICR unidirectionally blocks enhancer-promoter communications in a methylation-dependent manner in Hep-3B and Jurkat cell lines. In this report we show that the \textit{Kcnq1} ICR harbors bidirectional silencing and methylation-sensitive methylation-spread properties in a lineage-specific manner. We fine map both of these functions to two critical regions, and loss of one of these regions results in loss of silencing as well as methylation spreading. The cell type-specific functions of the \textit{Kcnq1} ICR suggest binding of cell type-specific factors to various cis elements within the ICR. Fine mapping of the silencing and methylation-spread functions to the same regions explains the fact that the silencing factors associated with this region primarily repress the neighboring genes and that methylation occurs as a consequence of silencing.

The distal end of the mouse chromosome 7 and an orthologous human chromosome 11p15.5 contain a well studied cluster of imprinted genes: \textit{Ipl}, \textit{Orel12}, \textit{p57kip2}, \textit{Kcnq1}, \textit{Kcnq1 A-SLIT1}, \textit{Mash2}, \textit{Ins2}, \textit{Igf2}, and \textit{H19} (1, 2). A differentially methylated region, located at \textit{\textminus}2 to \textit{\textminus}4 kb upstream of the \textit{H19} gene, is involved in manifesting the imprinting of the \textit{Ins2}, \textit{Igf2}, and \textit{H19} genes as well as methylation spreading. The cell type-specific functions of the \textit{Kcnq1} ICR suggest binding of cell type-specific factors to various cis elements within the ICR. Fine mapping of the silencing and methylation-spread functions to the same regions explains the fact that the silencing factors associated with this region primarily repress the neighboring genes and that methylation occurs as a consequence of silencing.

\* This work was partially funded by Vonhoffsten Foundation and Helge AX-SON Johnsons Stiftelse (to C. K.). 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.

§ N. T. and M. K. contributed equally to this work.

¶ To whom correspondence should be addressed. E-mail: kanduri.chandrasekhar@hbc uu.se.

\[ \text{This paper is available online at http://www.jbc.org} \]

\[ \text{Received for publication, December 2, 2002, and in revised form, December 26, 2002} \]

\[ \text{Published, JBC Papers in Press, January 2, 2003, DOI 10.1074/jbc.M212203200} \]

\[ \text{MATERIALS AND METHODS} \]

\[ \text{Plasmid Cloning Strategies—To facilitate cloning of the 3.6-kb \textit{Kcnq1} ICR fragment and its serial deletions into episomal plasmids, we inserted a fragment with multiple cloning sites from the parent pREP4 plasmid (3) (amplified using forward primer AAGCTGATCTATCATG-TCTGGATCCGGCC and reverse primer, AAGCTGATCTATCATG-TCTGGATCCGGCC and reverse primer, AAGCTGATCTATCATG-TCTGGATCCGGCC and reverse primer, AAGCTGATCTATCATG-TCTGGATCCGGCC and reverse primer, AAGCTGATCTATCATG-TCTGGATCCGGCC and reverse primer, AAGCTGATCTATCATG-TCTGGATCCGGCC and reverse primer, AAGCTGATCTATCATG-TCTGGATCCGGCC and reverse primer, AAGCTGATCTATCATG-TCTGGATCCGGCC and reverse primer, AAGCTGA} \]

\[ \text{This paper is available online at http://www.jbc.org} \]

\[ \text{Received for publication, December 2, 2002, and in revised form, December 26, 2002} \]

\[ \text{Published, JBC Papers in Press, January 2, 2003, DOI 10.1074/jbc.M212203200} \]
Because the imprinting of Kcnq1 and neighboring genes is tissue-specifically regulated, we were interested in knowing whether the Kcnq1 ICR function depends on the cell type used. For this purpose, we chose the trophodermally derived cell line, JEG-3. We have used an episomal based system, which comprises the SV40 enhancer and the H19 gene as a reporter gene that can discriminate between the silencing and insulating properties. We have inserted the 3.6-kb Kcnq1 ICR into both insulating (SatI) position, between the SV40 enhancer and the H19 reporter gene promoter) and silencing (ClaI position, out of the SV40 enhancer and the H19 promoter context) positions in the episomal plasmids, pREP4H19A and pREP4H19C (3), and these plasmids were transfected into JEG-3 cells. The RNA extracted from these samples was subjected to RNase protection analysis after transiently propagating for 9 days in cell culture and adjusted for episome copy numbers as has been described (3). Fig.1B shows that when the Kcnq1 ICR was positioned in the PS4 orientation (in this orientation, the antisense transcript transcribed from the Kcnq1 ICR faces toward the H19 promoter; see Fig. 1A) at the insulating position, the reporter gene activity was reduced 10-fold. We have observed 7-fold reduction in the reporter gene activity, however, when we inserted the Kcnq1 ICR in the opposite (NSI) orientation, suggesting the fact that the Kcnq1 ICR down-regulated the activity of the mouse H19 reporter gene in an orientation-independent manner at the insulating position. We next addressed whether the observed silencing is a consequence of silencing rather than insulating by analyzing the steady-state levels of the H19 transcripts in episomal plasmids with Kcnq1 ICR inserted at the silencing position (i.e., outside the reporter gene enhancer context). As can be seen in Fig. 1B, constructs with the Kcnq1 ICR in PC3 orientation showed more than a 10-fold reduction in the reporter gene activity. Conversely, the Kcnq1 ICR did not modify reporter gene activity in the opposite orientation, LC2. Down-regulation of the H19 transcripts at the insulator and silencing positions suggests that the Kcnq1 ICR acts as a silencer rather than insulator in JEG-3 cells. Orientation-dependent silencing by the Kcnq1 ICR at the silencing position, however, reveals that the Kcnq1 ICR may be harboring both silencer and insulator elements.

Because the Kcnq1 ICR is involved in bidirectional repression of neighboring genes in vivo, we wished to recapitulate a similar scenario using our episomal-based approach. The Kcnq1 ICR at the insulating position, that is, between the SV40 enhancer and the H19 reporter gene, is flanked with the H19 and hygromycin genes. Above, we have analyzed the effect of the Kcnq1 ICR on the H19 reporter gene by RNase protection analysis. The effect of the Kcnq1 ICR on the hygromycin gene activity was measured by counting the number of hygromycin-resistant colonies. As shown in Fig. 1B the 3.6-kb Kcnq1 ICR down-regulated the activity of the hygromycin gene about 10-fold in the PS4 orientation. The Kcnq1 ICR in the NS1 orientation also down-regulated the hygromycin gene activity, albeit to a lesser extent. These observations taken together suggest that the Kcnq1 ICR silences the reporter genes bidirectionally in our episomal context, thereby mimicking the in vivo situation.

Because our episomal approach recapitulates the bidirectional silencing property that is observed in vivo, we sought to fine map the regions responsible for the silencing feature. To this end, we have created several serial deletions within the 3.6-kb Kcnq1 ICR (Fig. 1A). As shown in Fig. 1B, we recapitulated the silencing property of the 3.6-kb Kcnq1 ICR with the 1.9-kb fragment, a smaller version of the 3.6-kb Kcnq1 ICR fragment. Deletions within the 1.9-kb the Kcnq1 ICR fragment

The Episome Silencer/Insulator Assay—The pREP4-based episomal vectors were transfected into Hep3B and JEG-3 cells, as has been described (3). The RNase protection expression analysis was performed as previously described using a 385-bp H19 antisense probe and a 150-bp glyceraldehyde-3-phosphate dehydrogenase antisense probe as control (3). 10 μg of RNA (including various amounts of total cell RNA depending on episome copy number and yeast tRNA) was hybridized with the antisense probes (300,000 cpm/reaction for H19 and 20,000 cpm/reaction for glyceraldehyde-3-phosphate dehydrogenase) overnight at 45 °C. All procedures were performed according to the manufacturer’s protocol of the RPAIII kit (Ambion). Quantification of individual protected fragments was done using a Fuji FLA 3000 PhosphorImager. The sample was corrected with respect to both internal control (glyceraldehyde-3-phosphate dehydrogenase) and episome copy number as determined by Southern blot analysis of Bgl II restricted DNA, hybridized with H19 (probe 4) and PDGFB (probe 3) probes (3).

To assess the Kcnq1 ICR-mediated bidirectional silencing on hygromycin- and neomycin-resistance genes, equimolar concentrations of episomal plasmids containing the various portions of the Kcnq1 ICR were transfected into JEG-3 cells. After transfection, the cells were selected with 750 μg of G418 and 150 μg of hygromycin until all of the cells in the control plate died. After selection, the drug-resistant colonies were stained with methotrexol and counted.

The Methylated Cassette Approach—A purified Kcnq1 ICR 3.6-kb fragment was methylated with 2 units/mg SsoI methyltransferase in the presence of 180 μM S-adenosyl methionine for 16 h at 37 °C. The methylation reaction was terminated by heat-inactivation at 65 °C for 15 min, and the methylation status of the purified fragment was analyzed by digestion with HhaI. The mock-methylated fragment was treated in the same way without the addition of SsoI. After linearization of pREP4H19A with Xhol and NotI, the methylated and mock-methylated ICR fragments were ligated within the vector overnight at 14 °C. Each ligation mix was then phenol/chloroform-extracted (1:1) and used directly for transfection.

Hygromycin-selected clones were individually harvested, and cells from each clone were equally divided for DNA and RNA extraction. DNA was extracted by lysing the cells overnight with 1% SDS supplemented with Proteinase K (50 μg/ml). DNA was then purified with phenol/chloroform (1:1) extraction. The RNA was extracted with an RNeasy kit (Qiagen) according to the manufacturers recommended protocol. For genotyping, DNA from cell clones was restricted with BstBI/BstZ17I, and for methylation analysis the DNA was digested with PstI and HhaI and analyzed by standard Southern blot hybridization protocols (3) using probe 2, a 3.6-kb PstI fragment covering the entire Kcnq1 ICR.

Probes—Probe 1 comprises a 2.4-kb BamHI fragment encompassing promoter and coding regions of the H19 gene. Probe 2 comprises a 3.6-kb Kcnq1 fragment covering episomal vector sequences containing the hygromycin gene. Probe 3 is a BglII fragment covering the intron portion of the human PDGFR gene and probe 4 is a linearized fragment of pREP4H19 A.

Southern Hybridization—Twenty μg of digested DNA was restricted with restriction enzymes and electrophoresed on a 1.7% gel andblotted to a Hybond N+ membrane (Amersham Biosciences) followed by hybridization with probes according to standard protocols. All probe fragments were radiolabeled using a multi-prime labeling kit (Amersham Biosciences) and [α-32P]dCTP.

Bisulfite Sequencing—Bisulfite treatment of genomic DNA extracted from episomal plasmids inserted with Kcnq1 ICR in PS4 and NSII orientation, transfected into Hep3B cells and propagated for 42 days, was done as described earlier (13). The PCR product was amplified from the bisulfite-treated DNA using forward primer 5′-AAATGGTGGGAGTATTAGGTTTTTTTTT-3′ and reverse primer 5′-AAAAACATACAAAAAACAACACTAAA-3′ from region 1(R1). The amplified product was cloned into pGEMT Easy vector (Promega) and subsequently sequenced with BigDye Terminator Cycle Sequencing Kit.

RESULTS

The results indicate that the Kcnq1 ICR faces toward the Kcnq1 promoter and coding regions of the H19 gene as a reporter gene by RNase protection analysis. The effect of the Kcnq1 ICR on the hygromycin gene activity was measured by counting the number of hygromycin-resistant colonies. As shown in Fig. 1B the 3.6-kb Kcnq1 ICR down-regulated the activity of the hygromycin gene about 10-fold in the PS4 orientation. The Kcnq1 ICR in the NS1 orientation also down-regulated the hygromycin gene activity, albeit to a lesser extent. These observations taken together suggest that the Kcnq1 ICR silences the reporter genes bidirectionally in our episomal context, thereby mimicking the in vivo situation.

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revealed two regions, region 1 (R1) and region 2 (R2), that are involved in bidirectional silencing. Removal of the 300-bp fragment comprising R1 resulted in complete loss of silencing activity. On the other hand, removal of the 600-bp R2 from the 1.9-kb fragment yielded \( \frac{1}{3} \) activation from the reporter genes. These studies suggest that both R1 and R2 are critical for achieving 100% efficient silencing and loss of one of the regions results in loss of silencing.

The bidirectional silencing activity of the Kcnq1 ICR was further tested using an episomal based system equipped with neomycin and hygromycin resistance genes as reporter genes under the control of the SV40 enhancer (Fig. 1C). We measured the effect of the Kcnq1 ICR on the reporter genes by counting neomycin- and hygromycin-resistant colonies. As shown in Fig. 1C, the 3.6-kb and 1.9-kb Kcnq1 ICR fragments at the insulating position bidirectionally silence both reporter genes in an orientation-independent manner. The serial deletions within the 1.9-kb Kcnq1 ICR fragment also uncovered that R1 and R2 are required for efficient bidirectional silencing. Taken together, these data suggest that the Kcnq1 ICR silences the reporter genes bidirectionally in an orientation-independent manner at the insulating position in JEG-3 cells. We could not carry out the methylation sensitivity of the bidirectional silencer activity of the Kcnq1 ICR in JEG-3 cells because episomal plasmids become heavily de novo-methylated in short-term cultures (14).

Having fine mapped the regions responsible for bidirectional repression in the Kcnq1 ICR, we next sought to understand the mechanisms underlying the bidirectional repression. Because DNA methylation is linked to gene silencing, we presumed that Kcnq1 ICR silences the reporter genes by spreading DNA methylation over the neighboring sequences. We therefore analyzed DNA methylation of the regions flanking the Kcnq1 ICR on episomal plasmids collected at various time points from the transiently propagated JEG-3 cells and long term-propagated Hep-3B cells (Fig. 2A) to check the role of DNA methylation in the Kcnq1 ICR-mediated silencing. As can be seen in Fig. 2, B–D, the 3.6-kb Kcnq1 ICR spreads DNA methylation in the PS4 orientation over the H19 reporter gene in JEG-3 and Hep-3b cells. The Kcnq1 ICR, however, did not spread DNA methylation over the H19 reporter gene in the PS4 orientation to the extent that has been noticed with the Kcnq1 ICR in the PS4 orientation (Fig. 2D). Taken together, the data suggest that the Kcnq1 ICR spreads DNA methylation in an orientation-specific manner.

We next addressed whether this de novo methylation spreading is unique to the Kcnq1 ICR or is a common feature among the other imprinting control regions by transiently transfecting...
the episomal plasmids containing the H19 ICR and the Kcnq1 ICR. We initiated this assay by using JEG-3 cells, which show high de novo methylation property on transfected episomal plasmids (15). Fig. 2C shows that the methylation-spreading property is orientation-dependent (i.e. methylated 2.4-kb BamH1 fragment appears only when the Kcnq1 ICR is in the PS4 orientation but not in the NSII orientation) and is unique to the Kcnq1 ICR in the episomal plasmids transfected into JEG-3 cells. D, Southern blot showing the kinetics of methylation spreading by the Kcnq1 ICR in the episomal plasmids, containing the Kcnq1 ICR in PS4 and NSII orientations, propagated for longer term in Hep-3B cells. E shows the autoradiogram of the Southern blot showing that the methylation spreading by the Kcnq1 ICR is DNA methylation-sensitive in Hep-3B cells.

the episomal plasmids containing the H19 ICR and the Kcnq1 ICR is CpG methylation-sensitive in the Hep-3B cell line (12), we were interested in knowing whether CpG methylation has any role in the de novo methylation spreading property of the Kcnq1 ICR. Paradoxically, the methylated Kcnq1 ICR inserted into the episome abrogated the de novo methylation spreading that is observed in the unmethylated version of the Kcnq1 ICR,
suggesting that the \textit{de novo} methylation spreading property of the \textit{Kcnq1} ICR is CpG methylation-sensitive (Fig. 2E).

Serial deletion experiments within the \textit{Kcnq1} ICR revealed R1 and R2 regions that are together responsible for bidirectional silencing activity. Methylation analysis on the episomes containing these serial deletions (Fig. 3A) transiently transfected into JEG-3 cells was carried out assuming that the methylation-spreading regions may also fine map to R1 and R2. Fig. 3B shows that methylation-spreading properties were recapitulated in the 1.9-kb fragment of the \textit{Kcnq1} ICR. Removal of 400 bp from the 1.9-kb fragment (PS4 1.5 and NSII 1.5) resulted in bidirectional methylation spreading, suggesting the 400-bp region may contain signals for orientation specificity. Further loss of 200 bp from the 1.5-kb \textit{Kcnq1} ICR fragment

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{The \textit{Kcnq1} ICR is methylated in an orientation-specific manner. A, diagram showing the \textit{HhaI} sites in the \textit{Kcnq1} ICR fragment. B, Southern blot containing the methylation analysis of the episomes, inserted with the \textit{Kcnq1} ICR in PS4 and NSII orientations, propagated for longer term in Hep-3 B cell line and transiently in JEG-3 cell line. C, bisulfite sequencing of CpGs in R1 of the \textit{Kcnq1} ICR.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Schematic summary of data and hypothetical models for the manifestation of tissue-specific imprinting by the \textit{Kcnq1} ICR. A, the \textit{Kcnq1} ICR is a bidirectional silencer in JEG-3 cells but an orientation and position-dependent chromatin insulator in Hep3B and Jurkat cells (12). The reporter genes were \textit{neomycin} for Jurkat cells and \textit{hygromycin}, \textit{neomycin}, and the mouse \textit{H19} for Hep3B and JEG-3 cells. B, hypothetical model of how the cell type-specific modes of repression reported here might explain manifestation of the tissue-specific imprinting status of \textit{Cdkn1c} and \textit{Kcnq1}. The \textit{Kcnq1} ICR may behave as an insulator to the hypothetical enhancers from communicating to the \textit{Cdkn1c} promoter or as a bidirectional silencer to both \textit{Cdkn1c} and \textit{Kcnq1} promoters in a lineage-specific manner. The bidirectional silencing property of the \textit{Kcnq1} ICR may be an outcome of the interaction between the R1 and R2 regions. Alternatively, the double-stranded RNA formed from \textit{Kcnq1-AS}/\textit{Kcnq1} transcripts might trigger RNA interference (RNAi), which might then initiate signals for bidirectional heterochromatinization.}
\end{figure}
resulted in loss of methylation spreading (Fig. 3, A and B). Methylation spreading was also not seen when the 300-bp R1 was deleted from the 1.9-kb Kcnq1 ICR fragment, revealing that both R1 and R2 are crucial for de novo methylation spreading. Moreover, these data suggest that the factors associated with R1 and R2 probably perform both silencing and methylation-spreading functions.

It is generally perceived that the protein factors associated with cis elements protect its own sequences from de novo methylation. As shown in Fig. 4, not the cis elements in the Kcnq1 ICR results in loss of methylation spreading (Fig. 3, A). The Kcnq1 ICR was protected from its own de novo methylation-spreading property in the PS4 orientation. Surprisingly, however, the Kcnq1 ICR was methylated in the NSI orientation. The latter observation was also noticed on transiently transfected episomes in JEG-3 cells, although there was cell type specificity in de novo methylation of various CpGs when compared with the Hep-3B cell line. Bisulfite sequencing analysis of the CpGs in the R1 of the Kcnq1 ICR on the DNA, extracted from PS4 and NSI episomal plasmids transfected into Hep-3B cells propagated for 42 days, also revealed that Kcnq1 ICR is unmethylated in the PS4 orientation but methylated in the NSI orientation (Fig. 4C).

**Discussion**

The Kcnq1 ICR, which is methylated on the maternal allele and unmethylated on the paternal allele, has been implicated in the long range control of imprinted gene expression at the distal end of the mouse chromosome 7. The bidirectional silencing property of the Kcnq1 ICR in the JEG-3 cell type at the insulating position in episomal context explains the recent observation that targeted deletion of the mouse Kcnq1 ICR on the maternal chromosome bidirectionally activates neighboring genes (11). However, the orientation-independent repression at the insulator position and the orientation-dependent repression at the silencing position by the Kcnq1 ICR in episomes in JEG-3 cells suggests that this fragment contains both silencing and insulator elements. The orientation-dependent insulator activity by the Kcnq1 ICR in Hep-3B and Jurkat cell line reveals the cell type-specific complexity of the regulatory mechanisms that are operated by the various cis elements in the Kcnq1 ICR (Fig. 5A). The cell type-specific functions of the Kcnq1 ICR reflects the suggestion that the DNA-binding factors associate with the Kcnq1 ICR tissue-specifically to confer cell type-specific functions.

Orientation-dependent, methylation-sensitive methylation spreading appears to be a consequence rather than cause of silencing by the Kcnq1 ICR, because the silencing of the reporter genes by the Kcnq1 ICR proceeds well before the de novo methylation-spreading property of the Kcnq1 ICR. For example, in Hep-3B cells, the silencing of the reporter gene by the Kcnq1 ICR is observed 9 days after transfection, however, de novo methylation occurs after 15 days of transfection, suggesting that de novo methylation of reporter genes is a consequence of silencing. Interestingly, in JEG-3 cells methylation spreading is observed in the PS4 orientation of the Kcnq1 ICR, in which pronounced silencing of the reporter gene is observed, but not in the NSI orientation. This observation suggests that de novo methylation spreading occurs only during efficient silencing conditions. More strikingly, de novo methylation-spreading and silencing properties of the Kcnq1 ICR map to the same regions, R1 and R2, suggesting that the silencing factors associated with the R1 and R2 regions may directly or indirectly recruit methyltransferases to this region. The bidirectional silencing property of the Kcnq1 ICR in JEG-3 cells is synonymous to what has been documented with the Igf2r ICR (15) because both ICRs are associated with antisense transcripts. It has recently been proposed that the antisense transcript, Air, originating from the Igf2r ICR plays a critical role in the imprinting of neighboring genes by bidirectionally silencing. It has been proposed that the Air transcript bidirectionally silences neighboring genes by initially repressing the Igf2r promoter on the overlapping side by forming repressive chromatin structure, and this gradually spreads to the other side of the ICR, thereby inactivating genes bidirectionally (16). By in vitro footprinting analysis, we have been able to uncover several cis elements required for the basal promoter in the R1 of the Kcnq1 ICR (data not shown). By analogy, in the Kcnq1 ICR, the antisense transcript originating from R1 could interact with R2 to form a repressive chromatin structure, which later spreads to bidirectionally inactivate genes. Alternatively, the dsRNA derived from the Kcnq1-AS/Kcnq1 transcripts might trigger RNA interference, with ensuing methylation of histone H3 lysine 9, as has been documented in case of the centromeric region of the fission yeast Schizosaccharomyces pombe (17). Histone modification would then signal DNA methylation to stabilize repressive chromatin structures. The repressive chromatin structure, although initially formed on the Kcnq1-AS/Kcnq1 overlapping side, might then spread to other side gradually (Fig. 5B).

The present investigation documents that the Kcnq1 ICR employs multiple, lineage-specific mechanisms to developmentally manifest the imprinted state of the neighboring genes (Fig. 5A). The methylation-sensitive spreading of methylation by the Kcnq1 ICR explains the paradox of why the loss of methylation in Beckwith-Wiedemann syndrome/Wilm’s tumor patients at the maternal Kcnq1 ICR results in spreading of methylation at the neighboring sequences, leading to pathological inactivation of neighboring genes (18).

Acknowledgment—We acknowledge valuable advice and help by Prof. Rolf Ohlsson.

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J. Biol. Chem. 2003, 278:9514-9519.
doi: 10.1074/jbc.M212203200 originally published online January 2, 2003

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