Biochemical and Biophysical Research Communications 430 (2013) 26–31

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

An upstream promoter element blocks the reverse transcription of the mouse *insulin-degrading enzyme* gene

Lang Zhang, Qingyang Ding, Pan Wang, Zhao Wang *

Protein Science Key Laboratory of the Ministry of Education, Department of Biological Sciences and Biotechnology, School of Medicine, Tsinghua University, Beijing 100084, China

ARTICLE INFO

Article history:
Received 17 October 2012
Available online 27 November 2012

Keywords:
Bidirectional promoter
CpG Island
Insulin-degrading enzyme
Unidirectional promoter

ABSTRACT

Despite the prevalence of bidirectional promoters among the mammalian genomes, the majority of promoters are unidirectional. The mechanism through which unidirectional promoters are prevented from reverse transcription remains to be clarified. Here we investigate the transcriptional directionality of the mouse *insulin-degrading enzyme* (*IDE*) promoter, which contains a CpG island and has dispersed transcription initiation sites. Although *IDE* is unidirectionally transcribed according to its genomic context, the basic promoter region of mouse *IDE* has bidirectional transcriptional properties. The region between −219 and +133 of mouse *IDE* relative to its first transcription initiation site has bidirectional transcriptional activities, but the region between −350 and +133 can only be transcribed from the normal direction, implying that an upstream promoter element locating between −350 and −219 blocks the reverse transcription of mouse *IDE*. We further mapped this upstream promoter element to the region between −243 and −287. Promoter mutation analysis showed that the upstream promoter element contains two functional sub-regions. In conclusion, we identified an upstream promoter element which blocks the reverse transcription of mouse *IDE*. Our studies are suggestive for the transcriptional mechanism of bidirectional promoters in mammalian genomes.

1. Introduction

In the mammalian genome, some genes are arranged in a divergent (head-to-head) fashion regulated by a bidirectional promoter [1]. Approximately 11% of human genes are divergently transcribed, whose transcription initiation sites are separated by less than 1000 base pairs [2]. Gene pairs are usually coordinately regulated by a bidirectional promoter, whereas a small fraction of bidirectional genes are anti-regulated [2]. Bidirectional promoters are usually GC-rich and TATA-less [3–6]. Trinklein and Myers showed that 77% of the bidirectional promoters are associated with a CpG Island, compared to 38% of nonbidirectional promoters [2]. CpG Island methylation in promoters is known to silence individual genes. Similarly, DNA methylation within bidirectional promoters can silence gene pairs [7]. By contrast with the TATA box, GABPA, NRF-1, YY1 and NF-Y binding sites are over represented in bidirectional promoters, suggesting that these transcription factors are important for the transcriptional regulation of bidirectional promoters [8].

Despite the prevalence of bidirectional promoters among the mammalian genomes, the majority of promoters are unidirectional. The mechanism through which unidirectional promoters are prevented from reverse transcription remains to be unknown. Bidirectional promoters contain few TATA boxes, suggesting that the TATA box regulates the orientation of transcription. However, the direction of transcription initiation is not always explained by the presence of a TATA box, because some TATA-less promoters show strong directional activity, while some TATA-containing promoters show activity in both directions [2]. Besides the TATA box, CCAAT boxes were also reported to control the directionality of bidirectional promoters [9].

Insulin-degrading enzyme (*IDE*) is a ubiquitously expressed zinc metalloprotease which degrades a variety of substrates, including insulin [10]. Another important substrate of *IDE* is β-amyloid (Aβ) [11–13], whose accumulation in the brain is the primary cause of Alzheimer's disease (AD) [14]. *IDE* knockout mice show accumulation of endogenous Aβ and the diabetic phenotype, such as hyperinsulinemia and glucose intolerance [15,16]. Transgenic overexpression of IDE in neurons decreases brain Aβ levels, and rescues the premature lethality of amyloid precursor protein (APP) transgenic mice. In addition, human genetic studies suggest that *IDE* polymorphisms are associated with the pathogenesis of both type II diabetes mellitus (DM) [17] and AD [18]. These studies indicate that *IDE* is closely related to both DM and AD.

We have previously showed that *IDE* has a CpG-Island promoter which is dispersedly transcribed and nuclear respiratory factor 1

* Corresponding author.
E-mail addresses: zhanglang08@mails.tsinghua.edu.cn (L. Zhang), dingqy0909@gmail.com (Q. Ding), wpanda@yahoo.cn (P. Wang), zwang@tsinghua.edu.cn, tcm@tsinghua.edu.cn (Z. Wang).

0006-291X/$ - see front matter Crown Copyright © 2012 Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.11.052

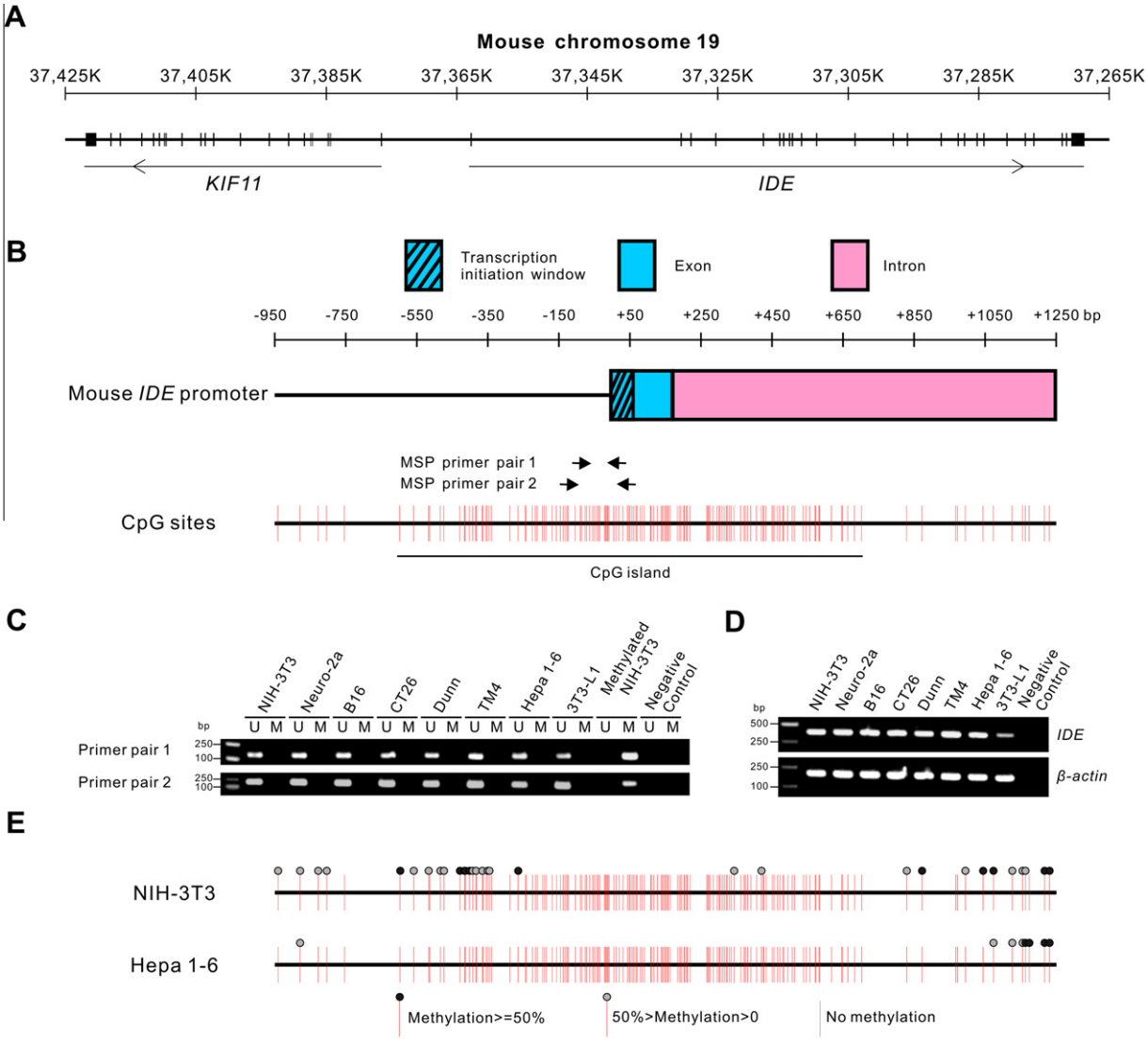

Fig. 1. The mouse *IDE* promoter contains a CpG island which is free of DNA methylation. (A) Genomic context of mouse *IDE*. *IDE* and *KIF11* are located head-to-head in mouse chromosome 19. Their transcription initiation sites are approximately 14 kb away. (B) Representation of the mouse *IDE* promoter. The mouse *IDE* promoter has dispersed transcription initiation sites and contains a CpG island with a length of approximately 1300 bp. (C) Methylation-specific PCR. Genomic DNA from eight different mouse cell lines was bisulfite-treated, and then used as template for methylation-specific PCR. Genomic DNA from NIH-3T3 cells which is methylated in vitro by CpG methyltransferase acts as a positive control for DNA methylation. Two different primer sets (Fig. 1A and Supplementary Table S2) were used to detect the methylation status of the plus and minus strands of the mouse *IDE* promoter, respectively. U = unmethylated; M = methylated. (D) *IDE* is ubiquitously expressed in different mouse cell lines, as detected by RT-PCR. *β-actin* was used as an internal control. (E) The detailed methylation status of the mouse *IDE* promoter in NIH-3T3 and Hepa 1-6 cells, as determined by bisulfite sequencing.

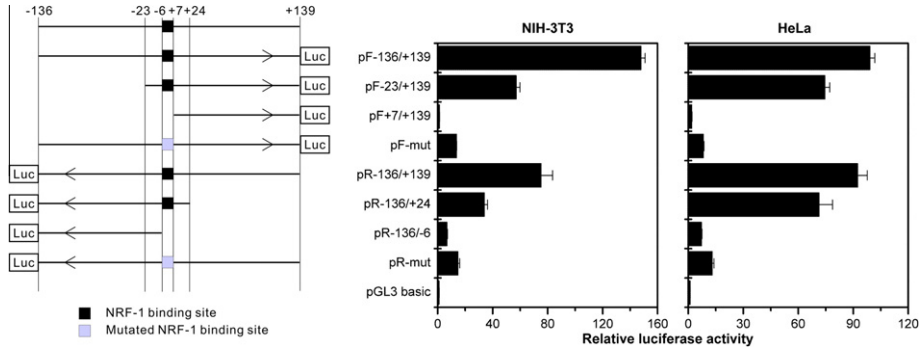

Fig. 2. The basic promoter region of mouse *IDE* has bidirectional transcriptional activities in a NRF-1-dependent manner. Different truncations of mouse *IDE* promoter were inserted into the pGL3 basic vector in either forward or reverse orientations. The NRF-1 binding site in the mouse *IDE* promoter is mutated through site-directed mutagenesis experiments. Reporter plasmids were transfected into NIH-3T3 or HeLa cells, and their luciferase activities were detected 24 h after transfection. Data are represented as fold of the luciferase activity of the pGL3 basic vector.

(NRF-1) is critical for *IDE* transcription initiation [19]. In this study, we focus on the transcriptional directionality of the mouse *IDE* promoter. Although the *IDE* promoter is unidirectionally transcribed according to its genomic context, the basic promoter region of *IDE* has bidirectional transcriptional activities which are NRF-1-dependent. Through promoter deletion and mutation analysis, we identified an upstream promoter element which blocks the reverse transcription of the mouse *IDE* promoter.

2. Materials and methods

2.1. Plasmid constructs

Different truncations of the mouse *IDE* promoter were cloned into the *Xho* I and *Hind* III restriction sites of the pGL3-basic vector (Promega, Madison, WI) in either normal or inverted directions. PCR primers for plasmid construction are shown in Supplementary Table S1. Site-directed mutagenesis experiments were performed to generate mutated *IDE* reporter plasmids. The NRF-1 binding site in the mouse *IDE* promoter was mutated from TGGGCATGCGCA to TGGGCAGCGACA.

2.2. Cell culture and transfection

All cells were maintained in Dulbecco's modified Eagle's medium (Thermo Scientific HyClone, Logan, UT) containing 10% fetal bovine serum (Thermo Scientific HyClone), except for TM4 cells, which were cultured in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (Thermo Scientific HyClone) containing 10% fetal bovine serum. For dual-luciferase reporter assays, NIH-3T3 and HeLa cells were seeded in 24-well plate at a density of 1×10^5 cells per well in antibiotic-free medium the day before transfection. Each well of cells were transiently co-transfected with 0.8 μg of *IDE* reporter plasmids and 8 ng of *Renilla* reporter plasmid (pCMV-RL, Promega) as an internal control using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA).

2.3. Dual-luciferase reporter assays

Twenty-four hours after transfection, the luciferase acitvity was detected using the Dual-luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Briefly, each well of cells were lysed with 100 μL 1 × passive lysis buffer for 15 min at room temperature. Samples (20 μL) were transferred to a white 96-well plate (NUNC, Roskilde, Denmark). Then, 25 μL of the firefly substrate (LAR II) was added, and firefly luminescence signal was read. Finally, 25 μL of the *Renilla* substrate (Stop & Glo) was added, and *Renilla* luminescence signal was read. The injection and reading process were done automatically in the GloMax®-Multi Microplate Multimode Reader (Promega). Firefly luminescence signal was normalized based on the *Renilla* luminescence signal.

2.4. Methylation-specific PCR

Genomic DNA was extracted using the ZR Genomic DNA II Kit™ (Zymo Research Corp., Irvine, CA), and then bisulfite-treated using the EZ DNA Methylation-Gold Kit™ (Zymo Research Corp.) as described in the manufacturer's protocols. As a positive control for DNA methylation, genomic DNA from NIH-3T3 cells was methyalted in vitro by CpG Methyltransferase (New England Biolabs, Ipswich, MA) and bisulfite-treated. Methylation-specific PCR was performed using the ZymoTaq™ PreMix (Zymo Research Corp.), with an annealing temperature of 50 °C for 40 cycles. Two different primer sets (shown in Supplementary Table S2) were used to detect the methylation status of the plus and minus strands of the mouse *IDE* promoter, respectively.

2.5. Bisulfite sequencing

For bisulfite sequencing, genomic DNA from NIH-3T3 and Hepa 1-6 cells was extracted and bisulfite-treated as mentioned above. PCR was performed using the ZymoTaq™ PreMix and the primers shown in Supplementary Table S3. PCR products were cloned into the pMD19-T vector, and at least six positive clones were sequenced for each PCR product. CpG sites which were not converted to TpGs were regarded to be methylated.

2.6. RNA isolation and RT-PCR

RNA was isolated using Trizol reagent (Invitrogen), and treated with DNase I (Promega) before cDNA synthesis. cDNA was synthesized with 2 μg of total RNA with anchored oligo (dT)20 primers using the Transcript First-Strand cDNA Synthesis Kit (Transgene Biotechnology Inc., Beijing, China). PCR primers for mouse *β-actin* were forward: 5′-ATCGTGCGTGACATCAAAGAG-3′ and reverse: 5′-ATGCCACAGGATTCCATACCC-3′. PCR primers for mouse *IDE* were forward: 5′-ACTGACCGCACAGAGCAGTGGT-3′ and reverse: 5′-AGGCCTGCTAGCTCTGCTGCAT-3′. Twenty-six cycles of PCR were performed for *β-actin*, and 30 cycles of PCR were performed for *IDE*, both with an annealing temperature of 60 °C.

3. Results and discussion

3.1. The mouse IDE promoter contains a CpG island which is free of DNA methylation

In the mouse genome, *IDE* and *KIF* are located head-to-head (Fig. 1A). The mouse *IDE* promoter has dispersed transcription ini-

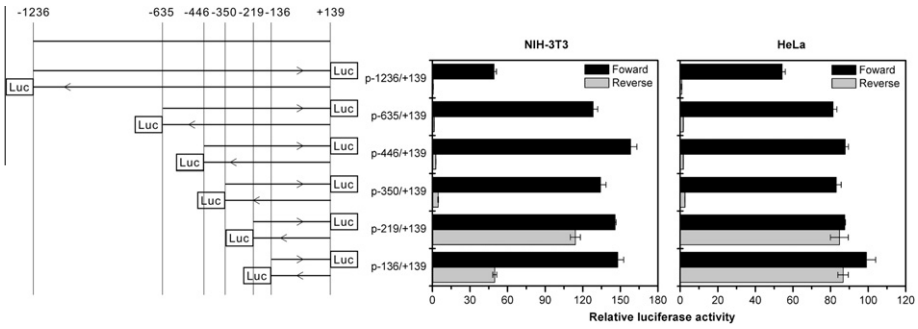

Fig. 3. An upstream promoter element blocks the reverse transcription of the mouse *IDE* promoter. Different truncations (−1236 to +139, −635 to +139, −446 to +139, −350 to +139, −219 to +139, −136 to +139) of the mouse *IDE* promoter were inserted into the pGL3 basic vector in either forward or reverse orientations. Luciferase activities of reporter plasmids in NIH-3T3 and HeLa cells are represented as fold of the luciferase activity of the pGL3 basic vector, respectively.

tiation sites and contains a CpG island approximately 1300 bp long (Fig. 1B). To investigate whether *IDE* expression was affected by promoter methylation, we first studied the DNA methylation status of the *IDE* promoter. Methylation-specific PCR experiments using two different primer sets suggested that CpG sites around the *IDE* transcription initiation sites are unmethylated in the eight mouse cell lines (Fig. 1C), which is consistent with the ubiquitous expression of *IDE* in these cell lines (Fig. 1D). The detailed methylation status of the *IDE* promoter was determined by bisulfite sequencing. In mouse NIH-3T3 cells, CpG sites inside the CpG Island are generally unmethylated, while CpG sites on the periphery of or outside the CpG Island are methylated to different extents (Fig. 1E). The methylation level of the *IDE* promoter in mouse Hepa 1-6 cells is significantly lower than in NIH-3T3 cells (Fig. 1E). The variance of DNA methylation in different cells may influence *IDE* transcription. However, silencing of *IDE* expression by promoter methylation was not seen.

3.2. The basic promoter region of mouse IDE has bidirectional transcriptional activities in a NRF-1-dependent manner

Although *IDE* and *KIF* are located head-to-head in the mouse genome (Fig. 1 A), the transcription initiation sites of *IDE* and *KIF* are approximately 14 kb away, indicating that these two genes

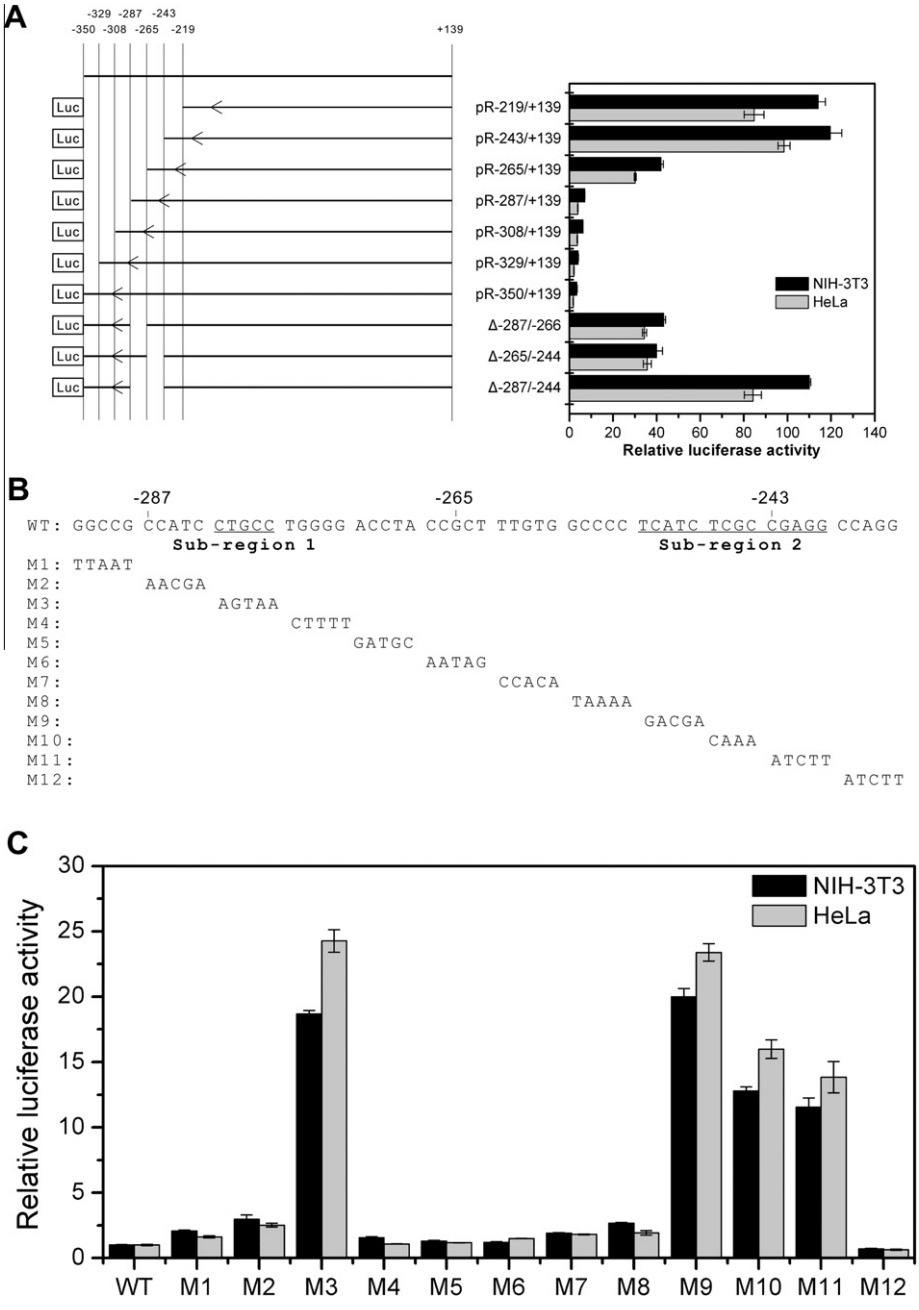

Fig. 4. Precisely mapping of the upstream promoter element. (A) Promoter deletion analysis. To precisely locate the upstream promoter element, different regions (−350 to +139, −329 to +139, −308 to +139, −287 to +139, −265 to +139, −243 to +139, −219 to +139) of the mouse *IDE* promoter were cloned into the pGL3 basic vector in the reverse direction. Plasmids Δ-287/−266, Δ-265/−244 and Δ-287/−244 represent the pR-350/+139 plasmid which lack the corresponding regions. Luciferase activities of reporter plasmids in NIH-3T3 and HeLa cells are represented as fold of the luciferase activity of the pGL3 basic vector, respectively. (B) Mutation of the mouse *IDE* promoter. The pR-350/+139 plasmid (WT) was used as the template to generate a serious of mutation plasmids (M1–M12). The region between −287 and −243 was sequentially mutated to sequences displayed in the figure. (C) Promoter mutation analysis. Reporter plasmids (WT and M1–M12) were transfected into NIH-3T3 or HeLa cells, and their luciferase activities were detected 24 h after transfection. Data are represented as fold of the luciferase activity of the WT plasmid (pR-350/+139).

do not share a bidirectional promoter and the *IDE* promoter is not transcribed from the reverse orientation. Strikingly, the region between −136 and +139 of the mouse *IDE* promoter relative to the first transcription initiation site can be transcribed from both orientations (pF-136/+139 and pR-136/+139) (Fig. 2). We previously showed that NRF-1 is critical for the transcription initiation of the mouse *IDE* promoter [19]. Therefore, we wondered whether the NRF-1 binding site is required for the reverse transcription of *IDE*. Indeed, deletion or mutation of the NRF-1 binding site abolished both the normal and reverse transcription of *IDE* (Fig. 2). These results indicate that the basic promoter region of mouse *IDE* has bidirectional transcriptional activities which are NRF-1-dependent.

3.3. An upstream promoter element blocks the reverse transcription of mouse IDE

We speculated that there is an upstream promoter element which blocks the reverse transcription of mouse *IDE*. To test this hypothesis, we constructed luciferase reporter plasmids containing different truncations of the mouse *IDE* promoter in either normal or inverted orientation. Just like p-136/+139, the region between −219 and +139 of the mouse *IDE* promoter (p-219/+139) also has bidirectional transcriptional activities (Fig. 3). However, when more upstream regions were included (p-350/+139, p-446/+139, p-635/+139 and p-1236/+139), the *IDE* promoter could not be transcribed from the reverse direction any longer (Fig. 3), indicating that an upstream region between −219 and −350 of the mouse *IDE* promoter may block its reverse transcription.

3.4. Precisely mapping of the upstream promoter element

Next, we aimed to map the upstream promoter element precisely through promoter deletion and mutation analysis. The region between −243 and +139 of the mouse *IDE* promoter (pR-243/+139) could still be transcribed from the reverse direction (Fig. 4A). The reverse transcription activity of pR-265/+139 was much lower, while pR-287/+139 had little reverse transcription activity (Fig. 4A), indicating that the region between −243 and −287 is critical to block the reverse transcription of the mouse *IDE* promoter. We further confirmed this conclusion by deleting this region from pR-350/−139. Deletion of the region between −244 and −265 or the region between −266 and −287 increased the reverse transcription activity remarkably, and a combined deletion of these two regions had even stronger effects (Fig. 4A). Then, we performed site-directed mutagenesis of the region between −292 and −234 using pR-350/−139 as the template. The mutation analysis revealed two sub-regions which are important for blocking the revere transcription of pR-350/−139. The first sub-region locates between −282 and −278 (Sub-region 1), and the second sub-region locates between −252 and −239 (Sub-region 2) (Fig. 4B). Mutation of either sub-region restored the reverse transcriptional activity of pR-350/−139 (Fig. 4C).

In this study, we investigated the transcriptional directionality of the mouse *IDE* promoter, which contains a CpG island and has dispersed transcription initiation sites. The basic region of the mouse *IDE* promoter can be transcribed from both directions. However, an upstream promoter element blocks its reverse transcription. Interestingly, this upstream element can be divided into two sub-regions, and mutation of either sub-region enables the reverse transcription of the *IDE* promoter. However, the mechanism through which this upstream element functions remains to be investigated. Some transcriptional repressors probably bind to this element. Another possibility is that this element influences the DNA methylation status and the chromatin structure of the *IDE* promoter. Finally, this element may work in a manner similar to an insulator [20].

Unidirectional promoters predominate among the mammalian genomes. However, the mechanism through which unidirectional promoters are prevented from reverse transcription is unclear. The TATA box may determine the direction of transcription, because bidirectional promoters generally lack the TATA box. However, the direction of transcription is not always correlated with the presence of the TATA box [2]. It is possible that other promoter elements, such as the Inr (initiator) element and CCAAT boxes, also contribute to the promoter direction [9,21]. Our studies provide a new explanation to this question—a portion of promoters have bidirectional transcriptional potentials, but an upstream promoter element blocks their reverse transcription.

Acknowledgments

This work was financially supported by the National Basic Research Program (973 Project) of China (No. 2007CB507406), and the Tsinghua University Initiative Scientific Research Program (20111081143).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.052.

References

[1] N. Adachi, M.R. Lieber, Bidirectional gene organization: a common architectural feature of the human genome, Cell 109 (2002) 807–809.
[2] N.D. Trinklein, S.F. Aldred, S.J. Hartman, D.I. Schroeder, R.P. Otillar, R.M. Myers, An abundance of bidirectional promoters in the human genome, Genome Res. 14 (2004) 62–66.
[3] W.C. Liao, J. Ash, L.F. Johnson, Bidirectional promoter of the mouse thymidylate synthase gene, Nucleic Acids Res. 22 (1994) 4044–4049.
[4] H. Qvist, H. Sjostrom, O. Noren, The TATA-less, GC-rich porcine dipeptidyl-peptidase IV (DPPIV) promoter shows bidirectional activity, Biol. Chem. 379 (1998) 75–81.
[5] S. Dong, L. Lester, L.F. Johnson, Transcriptional control elements and complex initiation pattern of the TATA-less bidirectional human thymidylate synthase promoter, J. Cell Biochem. 77 (2000) 50–64.
[6] Y. Kawai, K. Asai, Y. Miura, Y. Inoue, M. Yamamoto, A. Moriyama, N. Yamamoto, T. Kato, Structure and promoter activity of the human glia maturation factor-gamma gene: a TATA-less GC-rich and bidirectional promoter, Biochim. Biophys. Acta 1625 (2003) 246–252.
[7] J. Shu, J. Jelinek, H. Chang, L. Shen, T. Qin, W. Chung, Y. Oki, J.P. Issa, Silencing of bidirectional promoters by DNA methylation in tumorigenesis, Cancer Res. 66 (2006) 5077–5084.
[8] J.M. Lin, P.J. Collins, N.D. Trinklein, Y. Fu, H. Xi, R.M. Myers, Z. Weng, Transcription factor binding and modified histones in human bidirectional promoters, Genome Res. 17 (2007) 818–827.
[9] E. Zanotto, A. Hakkinen, G. Teku, B. Shen, A.S. Ribeiro, H.T. Jacobs, NF-Y influences directionality of transcription from the bidirectional Mrps12/Sarsm promoter in both mouse and human cells, Biochim. Biophys. Acta 1789 (2009) 432–442.
[10] F. Authier, B.I. Posner, J.J. Bergeron, Insulin-degrading enzyme, Clin. Invest. Med. 19 (1996) 149–160.
[11] I.V. Kurochkin, S. Goto, Alzheimers beta-amyloid peptide specifically interacts with and is degraded by insulin degrading enzyme, FEBS Lett. 345 (1994) 33–37.
[12] J.R. McDermott, A.M. Gibson, Degradation of Alzheimer's beta-amyloid protein by human and rat brain peptidases: involvement of insulin-degrading enzyme, Neurochem. Res. 22 (1997) 49–56.
[13] W.Q. Qiu, D.M. Walsh, Z. Ye, K. Vekrellis, J.M. Zhang, M.B. Podlisny, M.R. Rosner, A. Safavi, L.B. Hersh, D.J. Selkoe, Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation, J. Biol. Chem. 273 (1998) 32730–32738.
[14] J. Hardy, D.J. Selkoe, Medicine – the amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics, Science 297 (2002) 353–356.
[15] W. Farris, S. Mansourian, Y. Chang, L. Lindsley, E.A. Eckman, M.P. Frosch, C.B. Eckman, R.E. Tanzi, D.J. Selkoe, S. Guenette, Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid

precursor protein intracellular domain in vivo, Proc. Nat. Acad. Sci. USA 100 (2003) 4162–4167.
[16] S.O. Abdul-Hay, D. Kang, M. McBride, L. Li, J. Zhao, M.A. Leissring, Deletion of insulin-degrading enzyme elicits antipodal, age-dependent effects on glucose and insulin tolerance, PLoS One 6 (2011) e20818.
[17] A. Bonnefond, P. Froguel, M. Vaxillaire, The emerging genetics of type 2 diabetes, Trends Mol. Med. 16 (2010) 407–416.
[18] A. Serretti, P. Olgiati, D. De Ronchi, Genetics of Alzheimer's disease. A rapidly evolving field, J. Alzheimers Dis. 12 (2007) 73–92.
[19] L. Zhang, Q. Ding, Z. Wang, Nuclear respiratory factor 1 mediates the transcription initiation of Insulin-degrading enzyme in a TATA box-binding protein-independent manner, PLoS One 7 (2012) e42035.
[20] M. Tiana, D. Villar, E. Perez-Guijarro, L. Gomez-Maldonado, E. Molto, A. Fernandez-Minan, J.L. Gomez-Skarmeta, L. Montoliu, L. del Peso, A role for insulator elements in the regulation of gene expression response to hypoxia, Nucleic Acids Res. 40 (2012) 1916–1927.
[21] M.Q. Yang, L.L. Elnitski, Diversity of core promoter elements comprising human bidirectional promoters, BMC Genomics 9 (Suppl 2) (2008) S3.