Universal and mutation-resistant anti-enteroviral activity: potency of small interfering RNA complementary to the conserved cis-acting replication element within the enterovirus coding region

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The promising potential of RNA interference-based antiviral therapies has been well established. However, the antiviral efficacy is largely limited by genomic diversity and genetic instability of various viruses, including human enterovirus B (HEB). In this work, the first evidence supporting the anti-HEB activity of the small interfering RNA (siRNA) targeting the highly conserved cis-acting replication element (CRE) within virus coding region 2C is presented. HeLa cells pre-treated with siRNA complementary to the conserved sequence of the loop region of CRE(2C) were effectively rescued from the cytopathic effects of HEBs. Downregulation of virus replication and attenuation of cytotoxicity were consistently observed in various reference strains and clinical isolates. Cells treated with this siRNA were resistant to the emergence of viable escape mutants and showed sustained antiviral ability. Collectively, the data suggest that the siRNA based on the disordered structure within the highly conserved cis-acting coding region has potential as a universal, persistent anti-HEB agent. The same strategy can be successfully applied to the development of siRNA with consistent antiviral effects in other virus groups possessing similar RNA elements.

INTRODUCTION

Human enterovirus B (HEB) is a major pathogen involved in a wide spectrum of human diseases, such as aseptic meningitis, encephalitis, myocarditis, cardiomyopathy and diabetes (Pallansch & Roos, 2001). The lack of an available anti-HEB treatment has led to exploration of the possibility of HEB-targeting small interfering (siRNA). Many distinct viruses, such as coxsackievirus A9 (CVA9), CVB1–6, 28 echoviruses (Echo) and six enteroviruses (EV) are classified as strains of HEB. Indeed, HEB is one of the largest subgroups in the family Picornaviridae (Christian et al., 2005). The HEB genome consists of single-stranded RNA, surrounded by the structural virus proteins VP1–4 (Rueckert, 1996). Genomic RNA serves as the template for viral RNA transcription, and is translated into a single polypeptide that is subsequently cleaved by virus-encoded proteases to produce individual proteins.

RNA interference (RNAi) is a sequence-specific 'gene expression knock-down' process (Dykshoorn et al., 2006). siRNAs of approximately 19–23 nt, conjugated with an RNA-induced silencing complex (RISC), specifically trigger the catalytic degradation of complementary mRNAs (Elbashir et al., 2001; Zamore et al., 2000). RNAi has been examined as a novel technique for the discovery of powerful antiviral therapies. There has been a wide range of evidence supporting the potential of siRNAs for effectively combating infections by a variety of viruses, including human immunodeficiency virus, hepatitis viruses, poliovirus and
herpes simplex virus (Dave & Pomerantz, 2003; Gitlin et al., 2002; Johnson, 2006; Stevenson, 2003; Uprichard et al., 2005). Both chemically synthesized siRNAs and short hairpin RNAs (shRNAs) expressed from vectors can induce efficient antiviral activity in vivo as well as in vitro (Dykxhoorn et al., 2003; Leonard & Schaffer, 2006; Schubert et al., 2005a).

We have shown previously that cytopathic effects following CVB3 infection were abolished by CVB3-specific siRNA pretreatment in vitro (Ahn et al., 2005). It has also been demonstrated that five distinct siRNAs protected both HeLa cells and murine cardiomyocytes against CVB3 infection (Yuan et al., 2005). Among these, siRNA of the 2A viral protein was the most effective. Furthermore, the siRNA reduced virus titres in tissues and greatly promoted mouse survival in highly CVB3-susceptible type I interferon receptor-knockout mice (Merl et al., 2005).

There are two major obstacles that must be overcome for siRNA to become an effective anti-HEB agent. (i) The short duration of antiviral activity due to the emergence of escape mutants resistant to siRNA. As a result of the absence of a proof-reading function in virus RNA polymerase 3D, the mutation rate during HEB replication is approximately $1 \times 10^{-3}$–$10^{-4}$ nt (Cann, 2005). (ii) The difficulty in designing a specific siRNA that is simultaneously effective for multiple viruses, caused by the considerable variability of genomes between HEB strains. One of the practical strategies to address both issues at the same time is to design siRNA based on a highly conserved region of the virus genome. All picornaviruses, including HEB, have their most conserved sequences within the 5’ non-translated region (5’-NTR) of their genomes, which is crucial for both genome amplification and gene expression (Herold & Andino, 2000). Thus, theoretically it could be an ideal target region for siRNAs. However, studies with poliovirus, another member of the family Picornaviridae, demonstrated that siRNA residing in the 5’-NTR is less efficient than siRNA based on other regions at inducing antiviral activity (Gitlin et al., 2005; Saleh et al., 2004). The low level of antiviral potency seems to be due to the highly ordered structure of the 5’-NTR itself, as well as the formation of a protein–RNA complex in this region (Andino et al., 1993). Therefore, previous reports suggest that more systematic studies are needed to elucidate the proper conserved target sites of HEBs for siRNAs with universal and long-term antiviral effects.

Here, we show the universally persistent anti-HEB efficacy of an siRNA targeting the highly conserved cis-acting replication element (CRE) sequence within the 2C-coding region (Fig. 1) (van Ooij et al., 2006). The present study suggests that multi-HEB-CRE(2C) targeting siRNA efficiently attenuates cytotoxicity and virus replication of many different HEB species. The interfering effects were also maintained to a similar degree over time without the emergence of escape mutants at the target site for siRNA.

![Fig. 1. RNA secondary structure of CRE(2C) of CVB1 and the location of MET-2C siRNA.](image)
METODS

Cell culture and virus preparation. HeLa and Vero cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, l-glutamax (2 mM) and penicillin (100 IU ml\(^{-1}\)) or streptomycin (50 μg ml\(^{-1}\)) at 37°C in a 5% CO\(_2\) incubator. Reference-strain viruses (CVA9; VR-186, CVB1; VR-1032, CVB2; VR-29, CVB3; VR30, CVB4; VR-184, CVB5; VR-185, CVB6; VR-155, Echo6; VR-36, and Echo7; VR-37) were obtained from the ATCC. Wild-type viruses were acquired from patients with aseptic meningitis, as described previously (Joo et al., 2005). Viruses, including CVB3-GFP (H3/GFP), were propagated and titrated using a plaque assay (Ahn et al., 2003).

To quantify progeny virus production, cells were infected at an m.o.i. of 5 for 1 h with or without siRNA pre-treatment. After washing of virus inocula, cells were fed with medium for 12 h. Medium and cells were harvested, and plaque assays were performed by using the standard plaque assay. Virus stocks (500 μl per six-well plate), prepared by serial 10-fold dilutions in the medium, were used to inoculate monolayers of HeLa or Vero cells. After incubating the cells in 5% CO\(_2\) in an incubator for 2–3 days, plaques were visualized following neutral red staining and the titres were estimated as p.f.u. ml\(^{-1}\).

siRNA design and treatment. Multi-HEB targeting siRNA was designed using the in-house-developed siRNA design software CAPSID, which identifies conserved patterns among multiple sequences, and sequentially screens siRNA candidates. Using the software, we screened complete genomes of nine different HEB serotypes (CVA9, CVB1–6 and Echo6–7) and selected several multi-HEB-targeting siRNAs. From the potential siRNA candidates, we finally chose an siRNA referred to as MET-2C, which targets a site within the CRE located directly using an automated sequencer (ABI). The target site was completely conserved in all serotypes of the HEB cluster, including serotypes examined in this study. VP1-a was used as a control siRNA to specifically target a site in the VP1 region of CVB3 (nt 2857–2875) (Fig. 2a). The unrelated control siRNA was also described in a previous study (Ahn et al., 2005).

The 21 nt duplex siRNAs with dTdT 3’ overhangs were manufactured by Dharmacon, using the ‘ready-to-use’ option.

Cells were transfected with 100 nM siRNA complexes by using Oligofectamine reagent (Invitrogen) in OPTI-MEM medium (Invitrogen). Cells were recovered after 4 h, fresh growth medium containing 10% serum was added without removing the transfection mixture. After an additional 8 h, cells were infected with viruses at an m.o.i. of 0.1, 1 or 5.

Transmission electron microscopy (TEM). Cells were recovered and fixed overnight in 4% glutaraldehyde at 4°C. Cells were post-fixed with 2% osmium tetroxide, and stained en bloc with 0.5% uranyl acetate. After dehydration through a graded ethanol/acetone series, cells were embedded in Mollenhauer’s Epon/Araldite epoxy mixture No. 1. Ultra-thin sections were prepared and stained with 1% uranyl acetate and/or lead citrate. Images were obtained with a JEOL 100 CX transmission electron microscope.

Western blotting analysis. Cells in six-well plates were harvested and lysed in 100 μl lysis buffer (Intron). Denatured proteins were separated by using reducing SDS-PAGE (10% gel) and transferred to a PVDF membrane (Bio-Rad). The presence of VP1 was visualized using a VP1-specific antibody (Novocasera) and the ECL system (Pierce), along with an anti-actin antibody (Sigma).

RT-PCR for the siRNA target region and direct sequencing. Plaque-purified viruses were amplified in HeLa cells. Cells were infected with the virus and total RNA was extracted using Trizol solution (Gibco-BRL), according to the manufacturer’s instructions. Reverse transcription was performed with Moloney murine leukemia virus reverse transcriptase (SuperScript III; Invitrogen), 100 ng total RNA and 10 pmol anti-sense primer. CDNA (2 μl) was employed as a template for amplification with PCR SuperMix High Fidelity (Invitrogen). The primer sequences for the 378 bp fragment of the MET-2C region and 568 bp fragment of the VP1a region were: MET2C-sense, 5’-TGACCGCTCAAGATGGGACC-3’, MET2C-antisense, 5’-CATGACACACTACACGCTGC-3’, VP1a-sense, 5’-TGAGACTGTCGCTGCTCG-3’ and VP1a-antisense, 5’-CAGGTCACACGTCG-3’. The PCR product was resin-purified and sequenced directly using an automated sequencer (ABI).

RESULTS

MET-2C siRNA induces universal antiviral activity against various HEB species.

To produce antiviral effects on many different HEBs, we designed an siRNA targeting a site within the CRE located in the 2C protein coding region, referred to as MET-2C.
MET-2C, mainly residing in the loop structure of CRE(2C), is complementary to nt 4376–4394 of CVB1. The corresponding target sequences were completely conserved among nine distinct HEB strains (Fig. 2).

We then investigated various aspects of the potency of MET-2C as a multi-enteroviral-targeting siRNA. MET-2C rescued permissive HeLa cells from cytopathic disruption by all serotypes. HeLa cells were highly permissive for all the HEBs tested. In the absence of MET-2C, infected cells began to round up and were detached from the culture plates within 12 h post-infection (p.i.) (Fig. 3). In contrast, cells containing MET-2C maintained morphological integrity after virus challenge. Similarly, Hoechst 33342 staining data showed that MET-2C protected cells from nuclear destruction (data not shown). Nuclear aberration, characterized by heavily condensed Hoechst 33342-positive signals, disappeared following MET-2C treatment. As expected, pretreatment with non-specific unrelated control siRNA did not abolish the cytopathic effects of virus infection.

The effects of MET-2C on virus replication were quantified by comparing progeny virus production in the absence or presence of MET-2C at 12 h p.i. (Fig. 4). Regardless of the HEB serotype, virus generation in the ‘MET-2C’ sample was suppressed by 16±7.6-fold on average, compared with the ‘virus only’ sample (m.o.i. = 5). Ultrastructural observations with TEM supported the finding that the siRNA interfered with cytopathogenicity of HEB and virus production (Fig. 4b). Progeny virus particles were observed exclusively as honeycomb-shaped or lattice formations in the cytoplasm during CVB5 infection (lower box in middle panel). Collectively, the results suggest that MET-2C siRNA has universal antiviral capacity against HEB through inhibition of virus replication and attenuation of cytopathic effects.

**MET-2C targeting of the CRE(2C) region has long-term antiviral ability and retains the attenuation of cytopathic effects**

We investigated whether MET-2C from the conserved genome region produces a long-lasting virus silencing environment in infected cells. For comparison, VP1-a siRNA was employed (Fig. 2). VP1-a is one of the siRNAs specific for the CVB3 VP1 capsid protein region. The anti-CVB3 efficacy of VP1-a was similar to that of MET-2C until day 1 p.i. (Fig. 5). All distinct features caused by virus attack (cytotoxicity, VP1 protein production and progeny virus synthesis) were dramatically inhibited. However, the protective properties of VP1-a began to diminish after this time point. Morphological destruction, such as cell rounding, became evident in cells pretreated with VP1-a on day 3 p.i. (data not shown). On day 5 p.i., most cells floated on
In contrast, the infected cells treated with MET-2C did not show any morphological abnormality throughout the experimental period. The cells infected in the presence of VP1-a failed to persistently prohibit VP1 protein synthesis and a VP1 band was clearly detected on day 5 p.i. (Fig. 5b). In contrast, CVB3-infected cells transfected with MET-2C did not show any signs of cytotoxicity by day 5 p.i. Consistent with long-term anti-cytotoxic effects, neither VP1 synthesis nor virus production increased over time. No VP1 was detected in the presence of MET-2C by 5 days p.i. Upon MET-2C treatment, actin expression was constant over the entire experimental period. Fig. 5(c) shows that the decrease in viral amplification caused by MET-2C is stable over time. Non-specific control siRNA treatment did not interrupt virus replication at any time point (data not shown). Together with the observed morphological integrity of cells treated with MET-2C, the data confirm the minimal cytotoxicity of this siRNA.

Viable escape mutants emerge from infected cells treated with VP1-a, but not MET-2C

We analysed the possibility of the emergence of siRNA-resistant mutants by infecting cells with CVB3 expressing green fluorescent protein (GFP) (H3/GFP; m.o.i. = 1). H3/GFP
GFP virus encodes GFP, which generates a green colour in cells under a fluorescence microscope upon virus replication (Ahn et al., 2005). Following the infection of cells with H3/GFP, the green colour was visible from day 2 onwards in the presence of VP1-a, but not in the presence of MET-2C, until 5 days p.i. (data not shown). Genome analysis of viruses recovered on days 1 and 5 p.i. from VP1-a-treated samples revealed a silent mutation (A→G at position 13 of siRNA antisense) in the VP1-a target region on day 5 (Fig. 6a). Nine of 10 virus isolates retained this A:G mutation in the siRNA target region (mH3/GFP). Identical genetic analyses for MET-2C-treated samples revealed no mutations in the target region. In the mH3/GFP-infected cells treated with VP1-a siRNA, the unchanged percentage of GFP-positive cells supported the fact that pretreatment with VP1-a did not affect mutant virus replication. In contrast, virus replication and cytotoxicity were significantly reduced in the presence of MET-2C.

Virus pools recovered from HeLa cells infected with H3/GFP at an m.o.i. of 0.1 and treated with MET-2C were passaged sequentially to examine refractoriness to mutation. We designated the initial pool, which was recovered on day 5 p.i., as P1 and others were designated P2, P3 and P4 according to additional passage number. Direct sequence analysis of the entire virus pools failed to show any predominant mutant type within the MET-2C siRNA target sequence (Fig. 6b). Also, nucleotide sequence analysis of individual progeny viruses did not find mutations (no mutation per six viruses in each passage; data not shown). Altogether, the data imply that MET-2C is invulnerable to mutation, which may explain the sustained antiviral effect of MET-2C over time.

**MET-2C induces protective effects in HeLa cells infected with clinical isolates**

To determine the clinical potential of MET-2C siRNA, we challenged clinically isolated HEB serotypes with MET-2C (Fig. 7a). Only MET-2C siRNA efficiently protected cells from viruses identified as CVB1 (WT43-2, WT43-2S, WT57 and WT58), CVB3 (WT312) and Echo6 (WT14-2S) in a previous study (Joo et al., 2005). In the absence of MET-2C, progeny virus production in cells infected with CVB5 and WT312 was estimated to be $7.9 \pm 0.2$ and $8.1 \pm 0.2 \log_{10} \text{p.f.u. ml}^{-1}$, respectively. However, pretreatment with MET-2C reduced viral amplification to $6.3 \pm 1.0$ and $7.1 \pm 0.3 \log_{10} \text{p.f.u. ml}^{-1}$, respectively (Fig. 7b). Our findings demonstrate that MET-2C exhibits antiviral effects against a variety of HEB serotypes, including both reference and wild-type strains.

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**Fig. 6.** Characterization of escape mutants generated in infected cells. (a) Cells were challenged with CVB3-GFP (H3/GFP; m.o.i.=1) in the presence of VP1-a or MET-2C. The cells and media were harvested on days 1 and 5 p.i., and viral genotypic features in the VP-1a target region were analysed (mH3/GFP virus; viable escape mutant). (b) Population sequencing of the MET-2C target site during passage experiments. Cells were challenged with virus pools (P1) recovered by harvesting total cell lysates, including the culture medium from the cell culture, infected with H3/GFP at an m.o.i. of 0.1 in the presence of MET-2C on day 5 p.i. Additional passages were obtained on day 2 p.i. by carrying out similar experiments, which were designated P2, P3 and P4, respectively.
**DISCUSSION**

To achieve universally effective and sustained anti-HEB effects, we designed MET-2C siRNA targeting a highly conserved CRE located in the 2C coding region. CRE is an essential cis-element for virus replication (Gerber et al., 2001; Goodfellow et al., 2000; Paul et al., 2000; van Ooij et al., 2006). It acts as the primary template for VPgUpU synthesis through uridylylation of VPg, which is essential for initiating RNA genome synthesis. This region is characterized by a large stem–loop secondary structure (Fig. 1), and MET-2C was intentionally designed to be entirely complementary to the CRE loop with proper siRNA criteria. We then demonstrated that (i) multi-HEB targeting MET-2C siRNA displays universal and effective antiviral activity against various HEBs and (ii) MET-2C provides the additional advantage of prolonged interference effects by high resistance to the emergence of escape mutants.

The universal anti-HEB potency of MET-2C was relatively similar for the nine HEB reference strains examined. MET-2C also protected not only HeLa cells, but also Vero cells, from HEB infection (data not shown). Moreover, the siRNA induced efficient silencing effects on clinically isolated HEBs. MET-2C was similarly effective against all wild-type HEBs tested in this study, with the exception of WT14-2S. Previously, we determined that WT14-2S belongs to the Echo6 serotype with minimal sequence identity in the VP1 region (Joo et al., 2005). Due to the inability of MET-2C to inhibit WT14-2S replication, we sequenced the RT-PCR products that were amplified from target regions of the clinical isolates. The MET-2C target sequence was completely conserved in all wild-type viruses, including WT14-2S (data not shown), suggesting that the inability of MET-2C to suppress WT14-2S activity was not associated with target mismatch. It has been shown previously that resistant mutations of HIV can occur not only through nucleotide changes within the siRNA target sequence, but also through changes outside the target sequence that change the structure of the targeted region (Westerhout et al., 2005). We therefore suspect the possibility of a different local RNA structure for WT14-2S due to sequence dissimilarity outside the target site, resulting in the observed siRNA resistance of this strain. Further studies are required to elucidate the reasons for the ineffectiveness of MET-2C against WT14-2S.

The short-term antiviral effect of MET-2C was equivalent to those of other CVB3-specific siRNAs from the 2A viral protease region characterized previously (Merl et al., 2005; Yuan et al., 2005). A comparison of the morphological protection ability and the downregulation of VP1 expression revealed similarities in antiviral efficacy. As observed with VP1-a siRNA, the genome instability of enteroviruses enhances the chances of development of siRNA escape mutants. Another example is the case of poliovirus, which swiftly abolishes siRNA activity by generating point mutations within target regions, particularly the central region and 3′ end (Gitlin et al., 2002, 2005). However, MET-2C originating from well-conserved regions of various enterovirus serotypes may act against the emergence of viable mutants, leading to the persistent shutdown of virus replication.

RNAi efficiency may be influenced by the position of the mismatched bases formed by silent mutations and the nucleotide identity. Du et al. (2005) showed that G:U wobble base pairing is well tolerated for RNAi at most positions. On the other hand, Gitlin et al. (2005) reported that an A→G mutation, which produced a G:U mismatch, eliminated RNAi more efficiently than an A→C or

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**Fig. 7.** Antiviral capacity of MET-2C towards wild-type enteroviruses. HeLa cells were treated as described in the legend to Fig. 3, and the influence of MET-2C on cytopathic effects (a) and progeny virus production (b) was observed at 12 h p.i. Empty bars, virus only; shaded bars, control siRNA; filled bars, MET-2C. Values are means ± SD (n=3, except for control siRNA).
A→U substitution. In the presence of VP1-a, a viable A:G-switch escape mutant at position 12 was evident, which was consistent with these findings. Further studies should be carried out to elucidate the relationships between target mismatches and RNAi tolerance in detail.

Sequence alignment analysis of the CRE(2C) regions of human enterovirus genomes demonstrates that CRE(2C) elements of enteroviruses within the same subgroups are extremely conserved (Fig. 8). We found that MET-2C could not protect cells from attack by serotypes of other enterovirus clusters. For instance, CVA24 from human enterovirus C (HEC) replicated efficiently in HeLa cells treated with MET-2C, and triggered irreversible cell damage. However, an siRNA manufactured using the HEC CRE(2C) sequence, corresponding to the MET-2C target site, showed effective antiviral activity against CVA24 (data not shown). Thus the data suggest that the combination of siRNAs specific to the CRE(2C) sites of different human enterovirus clusters can be a powerful pan-enteroviral treatment.

There have been a few reports demonstrating that mutations can be accommodated in the terminal loop of the picornavirus CRE. Yang et al. (2002) found several mutation-acceptable positions in CRE by exhaustive mutagenesis of the HRV-14 CRE sequence. They then proposed a common motif for the loop segment of rhinovirus and enterovirus CRE, R1NNNAAR2NNNNNNR3. However, in that study, they investigated the degree of viral RNA replication using the DP1LucCRE replicon, which contains the luciferase gene in place of the P1 region. Thus, their data do not necessarily indicate the influence of various mutations on virus viability. In addition, HRV-14 CRE is located within the variable VP1 coding region, whereas the enterovirus CRE is in the highly conserved 2C-coding region (Argos et al., 1984). The 2C protein is deeply involved in virus RNA replication through at least two distinct mechanisms: cis- and trans-acting functions required for RNA initiation and elongation, respectively (Aldabe & Carrasco, 1995; Li & Baltimore, 1988; Mirzayan & Wimmer, 1992). Thus, mutations in the enterovirus CRE region, which induce amino acid changes in the 2C protein, might alter 2C function, possibly leading to the defect in RNA replication.

Many different substitutions were previously introduced into the poliovirus CRE (2C) (Goodfellow et al., 2000). The majority of these changes were in the third-base ‘wobble’ positions of codons, leaving the amino acid sequence of the 2C protein unaltered. Some of these mutations completely abolished genome replication, via extensive disruption of the 2C CRE structure. However, several single substitutions did not disrupt virus replication, including progeny virus production. Among them, mutations A26U, G27C, C34U and U40C are within the footprint of the MET-2C siRNA. Nevertheless, we could not observe the emergence of any predominant mutant, even in the extended passage experiment (Fig. 6).

The 2C protein functions in several steps of the virus life cycle: not only in virus RNA replication (Banerjee et al., 1997), but also in other events, such as the regulation of 3C protease (Banerjee et al., 2004), uncoating (Li & Baltimore, 1990), membrane binding (Teterina et al., 1997) and encapsidation (Vance et al., 1997). Therefore, nucleotide substitutions leading to the disruption of the CRE(2C) structure and amino acid alterations in the 2C protein could easily generate additional unexpected malfunctions. Thus, we propose that these double constraints may further reduce the appearance of the viable escape mutant in the MET-2C target region.
CRE(2C) has a hairpin structure containing a terminal loop of approximately 14 nt (Fig. 1). Recent studies have suggested that the efficacy of silencing depends on local secondary structures of target RNA, showing that a highly ordered target structure limits the applicability of siRNAs (Brown et al., 2005; Schubert et al., 2005b). MET-2C siRNA mainly targets the disordered loop region of the hairpin structure of CRE. Based on our data on the effective antiviral activity of MET-2C, the target accessibility of siRNA/RISC for this target site seems not to be hampered by a nearby double-stranded stem region. This study is the first report revealing that the siRNA targeting CRE located in the 2C coding region has promising potency as a universal and effective antiviral therapy with efficacy over long periods of time. Applying this approach to other viruses with RNA elements similar in size and shape to those of CRE(2C) may lead to similarly encouraging results.

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