Hepatitis Delta Virus Minimal Substrates Competent for Editing by ADAR1 and ADAR2

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A host-mediated RNA-editing event allows hepatitis delta virus (HDV) to express two essential proteins, the small delta antigen (HDAg-S) and the large delta antigen (HDAg-L), from a single open reading frame. One or several members of the ADAR (adenosine deaminases that act on RNA) family are thought to convert the adenosine to an inosine (I) within the HDAg-S amber codon in antigenomic RNA. As a consequence of replication, the UIG codon is converted to a UGG (tryptophan [W]) codon in the resulting HDAg-L message. Here, we used a novel reporter system to monitor the editing of the HDV amber/W site in the absence of replication. In cultured cells, we observed that both human ADAR1 (hADAR1) and hADAR2 were capable of editing the amber/W site with comparable efficiencies. We also defined the minimal HDV substrate required for hADAR1- and hADAR2-mediated editing. Only 24 nucleotides from the amber/W site were sufficient to enable efficient editing by hADAR1. Hence, the HDV amber/W site represents the smallest ADAR substrate yet identified. In contrast, the minimal substrate competent for hADAR2-mediated editing contained 66 nucleotides.

Hepatitis delta virus (HDV) is a human subviral pathogen that exists as a satellite of hepatitis B virus (24). Its genome consists of a single-stranded negative-sense circular RNA of approximately 1.7 kb that is 70% self-complementary and that can form a rod-like structure (11). HDV replicates via a rolling-circle mechanism in which a host-encoded RNA-directed RNA polymerase transcribes the circular genome to generate multimeric products (12). Within each monomeric unit of the multimer, a ribozyme self-cleaves, thereby releasing unit-length linear RNAs (12). The two termini of the linear monomer are joined by a host-encoded RNA ligase to create a circular replication intermediate referred to as the antigenome (23). Genome amplification occurs by analogous rolling-circle transcription of the antigenome, followed by self-cleave and ligation (12).

In addition to serving as template for the antigenome, the genome is also the template for an mRNA that encodes essential viral proteins (8). In this case, transcription is terminated after less than one-half of the genome is transcribed. A canonical AAUAAA polyadenylation signal mediates this event and is required for mRNA synthesis (8).

HDV is capable of producing its two viral proteins, the small delta antigen (HDAg-S) and the large delta antigen (HDAg-L), from a single open reading frame (29). These two proteins are identical in sequence except that the larger form contains 53 additional amino acids at its C terminus (11, 27); yet each protein has distinct and essential functions in the viral life cycle. Initially, following infection, only HDAg-S is expressed, and this protein is required for replication (10). Later, HDAg-L is also expressed and is required for virion assembly (4). The ultimate conversion of the HDAg-S UAG (amber) stop codon to a UGG (tryptophan [W]) codon enables the expression of HDAg-L (16), and this occurs as a result of RNA editing. Thus, amber/W editing is an essential step in the viral life cycle.

Editing occurs on rod-structured antigenomic RNA (3) and not on mRNA which lacks such a structure. It is not known whether nascent antigenomic RNA or the mature circular antigenome or both are substrates for editing. A host-encoded nuclear enzyme is thought to deaminate the adenosine of the stop codon, creating an inosine (I) at that position. During replication, the inosine is presumably recognized as a guanosine by the transcriptional machinery. Thus, the genome copied from the edited antigenome contains a 3′-ACC-5′ anticodon instead of 3′-AUC-5′. mRNA synthesized from this edited genome contains a UGG (tryptophan) codon and expresses HDAg-L (Fig. 1A).

A member of the ADAR family is thought to be responsible for editing the HDV antigenomic amber/W site. These enzymes have been well studied in the context of GluR-B and serotonin 2C receptor hnRNA editing (9). ADAR1 and ADAR2 are the only two human adenosine deaminases shown to be competent for mRNA editing (9). Each contains a domain in its carboxyl terminus that has homology with other deaminases, as well as a series of double-stranded RNA binding motifs in its amino terminus (9). ADARs target imperfect double-stranded RNA and edit specific adenosines in the substrate. The GluR-B hhnRNA is edited by ADARs at a few specific sites, and, as a consequence of editing, codon changes that alter the ion permeability and kinetics of the encoded ion channel result (7, 15). Similarly, the editing of HDV RNA is very specific and is almost completely restricted to the amber/W site (21).

Although the identity of the ADAR that edits HDV during human infection is still unknown, in vitro studies have shown...
that *Xenopus laevis* ADAR1 can efficiently and specifically edit the HDV amber/W site (20). In addition, HeLa nuclear extract and *Drosophila melanogaster* embryo nuclear extract are also competent for this process (3).

Studies of tissue culture have shown that HDV RNA can be edited in both the presence and absence of replication (3). In the latter case, approximately one-third of the antigenomic rod-like structure was deleted such that HDAg was not expressed. Thus, neither replication nor HDAg expression is required for editing (2, 3). Furthermore, expression of HDAg-S has been shown to reduce, but not abolish, editing at the amber/W site (21). Studies carried out in vitro and in vivo showed that point mutations near the editing site that disrupt base pairing decrease editing efficiency, while compensatory mutations that restore base pairing are found to complement the editing defect (3, 20). Thus, the secondary structure immediately around the edited adenosine plays a critical role in defining the substrate.

Protein kinase R (PKR), a double-stranded RNA (dsRNA) binding protein that shares homology in its dsRNA binding domain with ADAR1, binds to a particular region of genomic HDV RNA in vitro (6). Since a similar structure is thought to form in the analogous region of the antigenome, it can be speculated that such a region might be important for ADAR-mediated editing. However, this model has not been tested, and the minimal sequence and structure required for editing have not been determined. To that end, we created an editing-competent, nonreplicating reporter to assay editing in tissue culture. The reporter consists of an HDAg-S message in which the rod-like structure of antigenomic RNA is created. Editing is therefore detected by HDAg-L expression.

We found that the editing of the reporter was greatly enhanced upon coexpression of either human ADAR1 (hADAR1) or hADAR2, indicating that both enzymes are capable of editing the HDV amber/W site. Using deletion analysis, we found that the region on the antigenome complementary to the PKR binding site was not required for editing by either enzyme. In addition, a mere 24 nucleotides of HDV sequence were sufficient to enable editing by ADAR1.

### MATERIALS AND METHODS

**Construction of plasmids.** pKW42, the mammalian expression vector for HDAg-S, was constructed by subcloning cDNA of the antigenomic HDV sequence from position 1625 to 782 downstream of the cytomegalovirus (CMV) promoter on a pUC vector. Full-length, or wild-type, editing reporter pSS74 (U369) contains antigenomic HDV cDNA sequences from 1625 to 215, where the polyadenylation signal and the ribozyme were mutated by PCR using oligo 87 (5′-GAGTTGTCGACCCCAGTGAATCCCGGGTTTCCACTCAACAGGT3′) and oligo 63 (5′-ATCTCCTTAGATCCGGATAGAGAATCGAGAGAAAAGTGGCTCTCCCTTTACCATCCGAGTGGACCTGC3′). The PCR product was digested with SalI and XbaI to replace the same region on pKW42. The following plasmids were made identically to how pSS74 was made except for the downstream junction of the antigenomic HDV sequence noted: pSS72 (U101), from 1625 to 479; pSS98 (U48), to 529; pSS102 (U23), to 557; pSS131 (U4), to 596; pSS105 (U24), to 586; pSS143 (U0), to 580. pSS122 expresses the D89-145 reporter and was made by inserting the 149-bp EcoRI/XmaI fragment into the same sites in the HDAg open reading frame of pSS74. The M39 reporter is expressed from pSS140, where in a pSS74 backbone the mutations were introduced by PCR using oligo 397 (5′-CCCTCGAAGCTTAGTACTGAGGACTGCCGCCTCTAGCCGAGGGCGAGCCGGTCC3′) and oligo 398 (5′-TTCCGAGAATTCCTTTGATGTTCCCCAGCCAGGGATTTTCGTCCTCTTGTCTCCGG3′). The PCR product was digested with EcoRI and HindIII and used to replace the same region on pKW42. The following plasmids were made identical to how pSS74 was made except for the downstream junction of the antigenomic HDV sequence noted: pSS72 (U101), from 1625 to 479; pSS98 (U48), to 529; pSS102 (U23), to 557; pSS131 (U4), to 576; pSS105 (U24), to 586; pSS143 (U0), to 580; pSS122 expresses the D89-145 reporter and was made by inserting the 149-bp EcoRI/XmaI fragment into the same sites in the HDAg open reading frame of pSS74. The M39 reporter is expressed from pSS140, where in a pSS74 backbone the mutations were introduced by PCR using oligo 397 (5′-CCCTCGAAGCTTAGTACTGAGGACTGCCGCCTCTAGCCGAGGGCGAGCCGGTCC3′) and oligo 398 (5′-TTCCGAGAATTCCTTTGATGTTCCCCAGCCAGGGATTTTCGTCCTCTTGTCTCCGG3′). The PCR product was digested with EcoRI and HindIII and used to replace the same region on pKW42. All downstream deletions were derived from pSS74, where deletions were made in the following antigenomic sequences by PCR: pSS99 (D103), from 894 to 695; pSS106 (D54), from 954 to 634; pSS107 (D18), from 994 to 593; pSS127 (D5), from 1004 to 586. pSS130 (U23-D5), pSS128 (U13-D5), and pSS133 (U4-D5) were made by deleting antigenomic sequences from 1004 to 586 from

![Diagram of HDV replication and the nonreplicating HDV editing reporter.](attachment:diagram.png)
RESULTS

Experimental design. During HDV replication, HDAg-L expression is dependent on editing and its level of expression reflects the level of editing in antigenomic RNA (2, 31). Replication is also required for the production of HDAg-L, due to the fact that rod-structured antigenomic RNA is the substrate for editing. Since HDV replication can be sensitive to even small deletions, the minimal HDV amber/W site cannot be defined in the context of replication. Here, we used deletion analysis of a nonreplicating rod-structured HDAg-S message, shown in Fig. 1B, to define the minimal editing site. This editing reporter includes antigenomic sequences, not present in the natural message, which can form the rod-like structure. Two processing sites were utilized to prevent cleavage of the message in order to preserve its structure. The consensus sequence AAUAAA of the polyadenylation signal was mutated to AAUCCC, and a C-to-U substitution mutation at position 825 that was previously shown to inactivate the antigenomic ribozyme (26) was also introduced. A functional polyadenylation signal was then added to the 3′ terminus of the message to enable its export and translation. The level of expression of HDAg-L from the reporter should quantitatively reflect the level of RNA editing. However, several events must occur for HDAg-L to be made from this message. Following transient transfection of HEK293 cells with the reporter cDNA, the resulting message must be recognized as a substrate by an ADAR, and the amber/W site must then be edited. This should occur prior to export of the message from the nucleus, since ADARs preferentially localize to that organelle. In addition, ribosomes must be able to melt the rod-like secondary structure of the message so that it can be translated.

Unlike the natural HDV message, the HDAgr-L-expressing reporter would contain at least one inosine that should be recognized as a guanosine by the ribosome. In this sense, the editing reporter resembles the Glur-B message, which contains inosines that are translated as guanosines. Our initial reporter construct contained antigenomic sequences from position 1624 to 211. HDV sequences not present in this reporter were observed, demonstrating that both proteins can edit the HDV amber/W site.

**Effect of overexpression of tagged and untagged ADAR1 and ADAR2 on the editing of the reporter.** To study the ability of ADAR1 and ADAR2 to edit the HDV amber/W site, we transiently cotransfected HEK293 cells with vectors that express the editing reporter and either ADAR. To directly compare the levels of ADAR1 and ADAR2 expression, we also constructed expression vectors in which two HA epitope tags were included instead of the epitope tags used for pSS148 and pSS149. Additionally, 1.5 μg of ADAR1 vectors and 1.5 μg of ADAR2 vectors were used. Total sample DNA was adjusted to 3 μg with pSS43. At 3 days posttransfection, the supernatant was tested for secreted alkaline phosphatase activity by measuring enzymatic assay units (SEAP) activity using purified IgG as the primary antibody and then probing with protein A (1:1,000 dilution). All primary proteins and the non-HDV reporter containing the HDV minimal site were recognized as a guanosine by the ribosome. In this sense, the editing reporter resembles the Glur-B message, which contains inosines that are translated as guanosines. Our initial reporter construct contained antigenomic sequences from position 1624 to 211. HDV sequences not present in this reporter were observed, demonstrating that both proteins can edit the HDV amber/W site.

**Immunoblot analysis.** Protein samples were subjected to electrophoresis on 12% polyacrylamide gels for analyses of HDag and the non-HDV reporter containing the HDV minimal site and on 7% polyacrylamide gels for ADAR editing analyses. They were then electrophoretically transferred to nitrocellulose membranes. HDag was detected using rabbit polyclonal antiseraum (1:2,000 dilution) and secondary antibody (1:150,000 dilution) and labeled with alkaline phosphatase activity by measuring enzymatic assay units (SEAP) activity using purified IgG as the primary antibody and then probing with protein A (1:1,000 dilution). All primary antibodies, antiseraum, and secondary detection agents were diluted in 0.25% nonfat powdered milk in 1× phosphate-buffered saline. The blots were then exposed to a phosphor screen and quantified using a phosphorimager (Molecular Dynamics). Signals obtained for the ADAR proteins were normalized against aliquots of a single master sample of ADAR1-HA, which was run on all blots and given a value of 10. The experiments yielding results, shown in Fig. 2 through 8, were repeated at least three times.
We also probed the same samples with antibodies specific for either the HA tag, the C terminus of ADAR1, or the C terminus of ADAR2 (Fig. 2B to D, respectively). With both the HA and ADAR1 antibodies, we observed that the ADAR1 vectors express two forms of that protein, one whose mobility is consistent with that predicted for the full-length protein and another whose size is consistent with a protein initiated from the second methionine at position 296. Both of these forms had previously been observed in a number of different tissues and cell lines (18, 28). Visualized in lanes 6 and 7 of Fig. 2C are the two forms of ADAR1 endogenously expressed in HEK293 cells and in Huh7 cells, a human hepatoma cell line commonly used to study HDV replication. The ratio of the two forms of ADAR1 expressed by these cells was similar to that resulting from vector-driven overexpression.

By quantifying the signals from Fig. 2B to D, we were able to compare the levels of expression of both tagged and untagged forms of ADAR1 and ADAR2. We found that, for both ADAR1 and ADAR2, addition of the HA tag did not significantly alter the activity of the resulting protein. Since the levels of expression of hADAR1-HA and hADAR2-HA could be directly compared using a single blot, those two proteins were used in the subsequent assays.

**Effects of substitution mutations on the editing of the reporter.** The editing reporter contains a naturally contiguous self-complementary antigenomic sequence; however, it also contains mutations in two processing sites. The ribozyme mutation is very close to the region that is complementary to the PKR binding site in the genome; thus, if this region of the antigenome were involved in recognition by ADARs, then the mutation could potentially inhibit editing. We tested the effect of the two processing-site mutations on editing by using three constructs: a reporter with a wild-type polyadenylation signal and a wild-type ribozyme, one with a mutated polyadenylation signal and a wild-type ribozyme, and a third with both sites mutated.

Figure 3A shows the editing levels obtained with these reporters. The construct with a wild-type polyadenylation signal produced a large amount of HDAg-S but almost no HDAg-L, even when ADAR1 or ADAR2 were overexpressed, indicating that, as expected, little or no editing occurred with this reporter.
Others have shown that regions within the first 39 amino acids of HDAg possess RNA binding activity (5, 19). Furthermore, a coiled-coil domain resides in this region, and its deletion is expected to preclude stable multimeric assembly on RNA (33). In the second mutant, A89-145, amino acids 89 to 145 from HDAg were deleted (13). This reporter has a deletion of part of its rod-like structure and expresses a protein that lacks two critical arginine-rich motifs needed for RNA binding (14).

In three separate experiments, the editing of the A89-145 reporter was compared to that of the wild-type reporter. In the absence of transfected ADAR, a significant fourfold difference in editing of the two reporters was observed (approximately 5% for the wild type versus almost 20% for the mutant; data not shown). However, when ADARs were overexpressed, this difference was reduced to 1.5-fold (40 to 50% for the wild type versus 60 to 80% for the mutant; data not shown). The M39 mutant yielded results very similar to those obtained with A89-145. We conclude that, with the level of expression used in this study, HDAg has only a modest inhibitory effect on editing when ADARs are overexpressed.

**Deletion analysis.** We constructed variants of the reporter having progressive deletions that reduce the rod-like structure upstream of the editing site. This was accomplished by deleting HDV sequences in the 3' untranslated region, and the constructs are shown in Fig. 5A. The number after U (for upstream) designates the number of nucleotides of the HDV antigenomic sequence upstream of the editing site, where U0 indicates the cytidine at position 580 (C580) just opposite the adenosine that is deaminated. For example, U369 contains 369 nucleotides upstream of the editing site, where U369 indicates a 369-base pair fragment. U(−4) lacks base pairing at the editing site since all of the upstream base pairing was deleted as well as four nucleotides 5' of C580.

U369, U101, and U48 were edited at comparable levels by each enzyme (data not shown). Figure 5B shows editing levels of the other constructs. A decrease in editing by hADAR2-HA was noticeable in U13, and U4 was edited poorly by this enzyme. In contrast, hADAR1-HA was able to edit U4 efficiently. Expression of either enzyme did not enhance editing of U0 and U(−4). We conclude that HDV amber/W editing by ADAR1 and ADAR2 requires base pairing immediately upstream of
the editing site and that ADAR1 is more tolerant of upstream deletions than is ADAR2.

Reporters with progressive deletions of rod-like structure downstream of the editing site, shown in Fig. 6A, were made using the U369 construct as the starting vector. Similar to upstream deletion construct designations, the number after D (downstream) designates the number of HDV antigenomic nucleotides below position 580. The natural stop codon for HDAg-L was deleted in D18 and D5; hence translation of these edited messages is predicted to terminate at stop codons 35 and 29 codons downstream of the amber/W site, respectively. Consistent with these predictions, the larger proteins expressed upon editing D18 and D5 display retarded mobility compared to wild-type HDAg-L (Fig. 6B). Though there was a slight decrease in editing levels, both ADARs efficiently edited all three constructs. We conclude that, for sequences downstream of the editing site, only 14 nucleotides predicted to form a four-nucleotide stem and six-nucleotide loop are sufficient for editing by both ADAR1 and ADAR2.

To determine the minimal rod-like structure required in an
The natural HDAG-L stop codon was deleted in D5; thus translation of the edited message should terminate beyond the amber/W codon after an additional 23 codons for U23-D5, 16 codons for U13-D5, and 13 codons for U4-D5. Figure 7B shows that ADAR1-HA edited all three constructs with at least 20% efficiency, while ADAR2-HA could not edit U13-D5 or U4-D5 efficiently. We conclude here that ADAR1 only requires the predicted structure of 4 bp upstream and 4 bp downstream of the editing site, while ADAR2 requires a more rod-like structure upstream of the editing site.

Editing of the minimal ADAR1 substrate within a heterologous message. Since all of our reporters consist of a message encoding HDAG, we could not rule out the possibility that the HDV antigenomic sequence in the message itself played a role in substrate recognition by ADARs. To ascertain whether the 24 nucleotides in U4-D5 are the sole HDV sequence needed for editing by ADAR1, we created a non-HDV message reporter into which we inserted the 24-nucleotide amber/W substrate. As shown in Fig. 8A, this reporter carries part of the human placental alkaline phosphatase gene fused to a repeat of the HA epitope, followed by the U4-D5 structure where the amber codon is in frame with the upstream gene. The unedited
message is translated into a 121-amino-acid protein that can be detected using an anti-HA antibody, and when the stop codon in the HDV sequence is edited, a 168-amino-acid protein should be expressed.

We overexpressed untagged and HA-tagged hADAR1 to study their ability to edit this heterologous reporter in HEK293 cells. Lanes 3 and 4 in Fig. 8B show editing of the reporter by both versions of ADAR1. Though endogenous editing was almost undetectable in our assay, overexpression of ADAR1 dramatically increased editing levels of the reporter, shown by the presence of the larger species, which comigrates with the band expressed by the “preedited” UGG construct in lane 2. Thus, we conclude that the 24 HDV antigenomic nucleotides are both necessary and sufficient for editing by ADAR1 in vivo.

**DISCUSSION**

We observed that a nonreplicating reporter designed to mimic the structure of HDV antigenomic RNA was competent for amber/W editing. Furthermore, editing of this reporter was sensitive to the same mutations that inhibit HDV editing during replication (shown in Fig. 3). Therefore, in the region that includes the amber/W site, the reporter is likely to adopt a structure equivalent to that of the natural substrate.

Although HEK293 cells express relatively modest levels of ADAR1 and no detectable ADAR2 (Fig. 2, lanes 1 to 3), there was sufficient activity in these cells to edit roughly 5% of the reporter messages. This level of editing is comparable to that observed during HDV replication in a study by Polson et al. (21), when cells were harvested 5 days posttransfection. However, it is also known that much higher levels of edited RNA can be observed during HDV replication when cells are harvested at later times posttransfection: up to 25% at day 13 in one study (21) and exceeding 30% at day 18 in another (32). In contrast, using the nonreplicating reporter, we saw no significant change in the extent of editing when cells were harvested at later times posttransfection (data not shown). This result is not really surprising, however. When antigenomic RNA is edited during replication, the edited antigenome serves as a template for genome synthesis and the resulting mutation becomes fixed in the genome. This enables the mutation to accumulate over time. Thus the ratio of amber-encoded to W-encoded RNA observed at 13 days posttransfection represents the integration of all editing events that occurred in the preceding days. However, our reporter does not replicate; thus past editing events cannot be fixed, and, in this case, the extent of editing should not increase with time.

This was the first study to test the ability of hADAR1, or any form of ADAR2, to edit the HDV amber/W site. We found that both enzymes could edit this site with roughly comparable efficiencies. Though we have not determined the identity of the enzyme that edits the HDV amber/W site during replication, our results indicate that, in addition to ADAR1, ADAR2 should be considered a candidate. Recently, the sole ADAR expressed by *Drosophila* was cloned and was found to have greater homology with ADAR2 than with ADAR1 (17). Given that hADAR2 is able to edit the HDV amber/W site, perhaps it is not surprising that *Drosophila* nuclear extracts can also carry out this reaction. Even though we did not detect ADAR2 expression in two cell lines that support HDV replication, it remains possible that HDV replication might be required to induce ADAR2 expression. Of course, it is also formally possible that neither enzyme edits the HDV amber/W site during human infection. More direct evidence is needed to identify the enzyme that naturally edits the amber/W site during replication.

It was previously reported that the endogenous activity responsible for HDV editing is inhibited by HDAg expression. Here, we provide evidence consistent with that finding since reporters that expressed RNA-binding-deficient HDAg were edited at fourfold-higher levels by the endogenous activity than was the reporter that expressed wild-type HDAg. Interestingly, when either ADAR1 or ADAR2 was overexpressed, the difference in the editing of the two types of reporters was reduced to 1.5-fold. This observation is consistent with a model in which HDAg and ADARs compete for binding to the amber/W site on antigenomic RNA. Through overexpression, an ADAR might be able to compete more effectively, and hence inhibition by HDAg would be reduced.

Results from our extensive deletion analyses indicate that the region complementary to the PKR binding site is dispensable for editing by both ADAR1 and ADAR2. In addition, we observed that these two enzymes have different minimal substrate requirements. For efficient editing, ADAR2 required a 66-nucleotide substrate that included 21 predicted base pairs upstream from the site of editing. In contrast, ADAR1 was able to efficiently edit a 24-nucleotide substrate that contained only four upstream base pairs, and this represents the smallest ADAR substrate yet identified. Such a small substrate may be very useful for the biophysical study of ADAR1 and its interaction with RNA.

Like the HDV amber/W site, the GluR-B R/G site can be edited by both ADAR1 and ADAR2. In vitro, ADAR2 can edit a 57-nucleotide R/G substrate that has no base pairs upstream of the edited adenosine. We have converted the GluR-B R/G site into an amber/W so that we could assay editing by both ADARs in transfected cells as was done previously (30). We found that although ADAR2 was more efficient at editing the GluR-B site when it contained five upstream base pairs, it was still able to edit a substrate with no upstream base pairing (unpublished results). Similar results have been obtained in vitro with GluR-B R/G minimal substrates (25). In contrast, ADAR1 required five upstream base pairs and was completely unable to edit the substrate that lacked upstream base pairing (unpublished results). Thus, for the HDV amber/W site, ADAR1 required less upstream structure than did ADAR2, while for the GluR-B R/G site, the converse was true. We conclude that the minimal substrate requirements for ADAR1 and ADAR2 vary with each specific editing site and that there are differences in the manner in which these two enzymes recognize a given substrate.

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