Internal Ribosome Entry Site within Hepatitis C Virus RNA

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The mechanism of initiation of translation on hepatitis C virus (HCV) RNA was investigated in vitro. HCV RNA was transcribed from the cDNA that corresponded to nucleotide positions 9 to 1772 of the genome by using phage T7 RNA polymerase. Both capped and uncapped RNAs thus transcribed were active as mRNAs in a cell-free protein synthesis system with lysates prepared from HeLa S3 cells or rabbit reticulocytes, and the translation products were detected by anti-gp35 antibodies. The data indicate that protein synthesis starts at the fourth AUG, which was the initiator AUG at position 333 of the HCV RNA used in this study. Efficiency of translation of the capped methylated RNA appeared to be similar to that of the capped unmethylated RNA. However, a capped methylated RNA showed a much higher activity as mRNA than did the capped unmethylated RNA in rabbit reticulocyte lysates when the RNA lacked a nucleotide sequence upstream of position 267. The results strongly suggest that HCV RNA carries an internal ribosome entry site (IRES). Artificial mono- and dicistronic mRNAs were prepared and used to identify the region that carried the IRES. The results indicate that the sequence between nucleotide positions 101 and 332 in the 5′ untranslated region of HCV RNA plays an important role in efficient translation. Our data suggest that the IRES resides in this region of the RNA. Furthermore, an IRES in the group II HCV RNA was found to be more efficient than that in the group I HCV RNA.

1The nucleotide sequence of almost the entire genome of hepatitis C virus (HCV), the main causative agent of chronic non-A, non-B hepatitis (NANBH), has been elucidated (3, 15, 23, 28). The genome of HCV is a single-stranded RNA with positive polarity and consists of approximately 10,000 nucleotides. This RNA carries only one long open reading frame for the synthesis of a large single polyprotein of 3,010 amino acids. Analysis of the amino acid sequences deduced from the nucleotide sequences of different HCV isolates (3, 15, 28) revealed that the HCV genomes have a gene organization similar to those of flaviviruses and their related viruses (29). Sequence analysis also revealed the existence of a fairly long 5′ untranslated region (UTR) that harbors three (15, 28) or four (6) AUG sequences. This feature of the 5′ UTR resembles that of picornaviruses rather than that of flaviviruses.

Picornavirus RNAs are uncapped messengers and have unusually long 5′ UTRs (ranging from 610 to 1,200 residues, depending on the virus species) which contain many silent AUG sequences. These features seem incompatible with efficient translation by the scanning ribosome mechanism, which is the initiation mechanism of most eukaryotic cellular and viral mRNAs (16, 18). The rule of the scanning model is that ribosomes initially bind to the 5′ end of mRNAs and scan the RNAs to reach the authentic initiator AUGs, which are, in many cases, the first AUG sequences. Furthermore, the cap structures at the 5′ ends of mRNAs are generally believed to play an important role in the initial ribosome entry or binding step. Thus, the initiation of translation on picornavirus RNAs has been considered to take place by an unusual mechanism. Recently, it was proved that translation initiation on picornavirus RNAs occurs by binding of ribosomes to an internal sequence within the 5′ UTR (10, 11, 26). An internal entry site for ribosomes has been called the IRES (internal ribosome entry site) (10, 11). IRES function requires a specific segment of the 5′ UTR of approximately 400 nucleotides which does not include the extreme 5′-proximal nucleotide sequences.

Structural similarities of the 5′ UTR of HCV RNA to those of picornavirus RNAs provide the possibility that IRES function resides in the 5′ UTR of HCV RNA, although the 5′ UTR of HCV RNA is shorter than a segment of picornavirus RNAs required for its IRES function, and it is not known whether the 5′ end of HCV RNA is capped.

Here we demonstrate, by using cell-free translation systems, that the translation initiation on HCV RNAs occurs by entry of ribosomes to the internal sequence within the 5′ UTR and that IRES function requires a segment of nucleotide positions 101 to 332 at most, which is the shortest sequence thus far identified as an IRES. We also show that the function of the IRES of group II HCV RNA is more efficient than that of the IRES of group I HCV RNA in a cell-free protein synthesis system from HeLa S3 cells.

MATERIALS AND METHODS

DNA procedure. Restriction endonucleases, DNA polymerase I (Klenow fragment), and T4 DNA ligase were purchased from Takara Shuzo Co. (Kyoto, Japan); calf intestinal alkaline phosphatase was from Boehringer (Mannheim, Germany); KS vector was from Stratagene (La Jolla, Calif.). These enzymes and compounds were used according to the instructions of the manufacturers. DNA primers were made by a DNA synthesizer (Applied Biosystems).

Isolation of HCV cDNAs. HCV cDNA that corresponds to nucleotide positions 9 to 791 (28) was isolated from pooled plasma of NANBH patients by a method involving polymerase chain reaction (PCR) as reported by Tsukiyama-Kohara et al. (31), using a sense primer, 5′-GGCCGACACTCCACCATAGATC-3′, and an antisense primer, 5′-ATGCGGC
AGGGCCCTGGCGACG-3'. The cDNA thus synthesized was inserted into the SmaI site of cloning vector pUC119. Nucleotide sequence analysis of 10 clones by using the 7-deaza Sequenase kit (United States Biochemical) revealed that 7 of the 10 clones had the same nucleotide sequence. A cDNA clone, named clone 2-1, was chosen from these seven clones as an HCV cDNA clone and used for further plasmid construction. A cDNA clone, EN2-3, that corresponds to nucleotide positions 676 to 1772 was isolated by immunoscreening from a AgtI1 cDNA library prepared from sera of chronic NANBH patients as previously described (31).

A BamHI-ClAI cDNA fragment (corresponding positions 9 to 700) of clone 2-1 and a Clal-HindIII cDNA fragment (positions 700 to 1772) of clone EN2-3 were ligated and inserted into the KS vector that had been digested with BamHI and HindIII. The plasmid thus obtained contains an HCV cDNA that corresponds to nucleotide positions 9 to 1772 downstream of the phospho T7 RNA polymerase and was designated pKIV (Fig. 1).

Construction of deletion mutants. Mutants with deletions of the 5' UTR (pK15, pK16, pK17, pK18, pK19, pK10, pK11, and pK16) were constructed by the PCR method, using synthetic primers. Nucleotide sequences of sense DNA primers were designed to give an XbaI site to the corresponding 5' end of the PCR products. A XbaI-AatII cDNA fragment of plasmid pKIV was replaced by the corresponding cDNA fragments of the PCR products. In the case of pK16, a segment corresponding to nucleotide positions 333 to 434 was synthesized by the PCR method, digested with AatII, and inserted into pKIV that had been digested with StuI and AatII. To exclude the influence of a sequence existing between the T7 promoter and HCV cDNA, the XbaI and HindIII cDNA fragments of pKIV and its deletion plasmids were filled in with Klenow fragment and subcloned with correct orientation into the StuI site of plasmid pNar3, which had been derived from pUC119 (Fig. 1); the resulting constructs were designated pNV, pN5, pN6, pN7, pN8, pN9, pN10, pN11, and pN16 (see Fig. 6A). The StuI-HindIII cDNA fragment of pKIV was also subcloned into the StuI site of pNar3, and the resulting plasmid was designated pNSt (Fig. 1).

Construction of cDNA clones that carry CAT cDNA. The chloramphenicol acetyltransferase (CAT) cDNA carrying SacI and XbaI sites at the corresponding 5' and 3' ends, respectively, was introduced into the SacI and XbaI sites of pKIV, pK16, pK17, pK18, pK19, pK10, pK11, and pK16. These cDNA clones encoding dicistronic mRNAs were designated pCV, pC6, pC7, pC8, pC9, pC10, pC11, and pC16, respectively (see Fig. 6A). To construct plasmid 5HeCAT, an XbaI-BspHI fragment of pKIV, which carries a segment of untranslated region of HCV, and the CAT cDNA that had BspHI and SalI sites at the corresponding 5' and 3' ends, respectively, were ligated and inserted into KS vector that had been digested with XbaI and SalI (Fig. 1). A StuI-Sall fragment of 5HeCAT, after treatment with Klenow fragment, was inserted with correct orientation into the StuI site of pNar3. The plasmid thus obtained was designated 5HSICAT (Fig. 1).

Construction of cDNA that carries the 5' UTR sequence of group II HCV RNA. A segment of group II HCV cDNA (corresponding to nucleotide positions 49 to 408) was prepared from plasma of a patient infected with group II HCV alone by the PCR method, using 5'-CTGTCTTCACGCA GAAACGC-3' and 5'-CCGAAAATCTGATCTCGT3'-3' as primers, and subcloned into the SmaI site of pUC19; the nucleotide sequence was then determined as described above. An XbaI-AatII cDNA fragment of pKIV was replaced by the XbaI-AatII cDNA fragment containing the 5' UTR sequence of group II HCV. An XbaI-HindIII cDNA fragment (1,724 bp) of the plasmid thus obtained was subcloned into the StuI site of pNar3, and the resulting plasmid was designated pNII5.

RNA transcription. Plasmids were linearized by digestion with SalI (for 5HeCAT, pCV, pC6, pC7, pC8, pC9, pC10, pC11, and pC16), XbaI (for 5HSICAT), or EcoRI (for pNV, pN5, pN6, pN7, pN8, pN9, pN10, pN11, pN16, pNII5, and

FIG. 1. Strategy for construction of the expression vector. *, restriction site introduced by the PCR method.
pNST) and transcribed into RNA in vitro with T7 RNA polymerase (Takara Shuzo Co.). In most cases, cap structure, m'GpppG or GpppG (Pharmacia LKB), was incorporated at the 5' ends of RNA transcripts as described previously (14).

In vitro translation. Suspension-cultured HeLa S3 cells were infected with coxsackievirus B1 (CVB1) as previously described (8). Cyttoplasmic extracts (S10) of mock- or CVB1-infected cells were prepared 3 h after the infection and were treated with micrococcal nuclease as previously described (9). Conditions for the translation reaction (12.5 µl) were as described by Rose et al. (27). After incubation at 37°C for 1 h in the presence of [35S]methionine (ICN Radiochemicals), 5 µl of each of the translation mixtures was mixed with an equal volume of sodium dodecyl sulfate (SDS) sample buffer, boiled for 1 min, and analyzed by electrophoresis on a 12.5% polyacrylamide gel in Laemmli's buffer system (21). In some cases, translation products were cotranslationally processed in the presence of canine microsomal membrane (Amersham). The processed translation products were mixed with rabbit anti-gp35 antibodies and then with Affi-Gel protein A (Bio-Rad). The precipitates were analyzed by electrophoresis as described above. Rabbit reticulocyte lysate (RRL; Amersham N150) was also used as in vitro translation system. Translation reactions in RRL were performed at 30°C for 1 h. In some cases, translation reactions were carried out in the presence of 20 µM S-adenosylhomocysteine (SAH) to prevent methylation of GpppG-ended RNA during the reaction (2, 5).

Preparation of rabbit anti-gp35 antibodies. A cDNA clone, C10-E12, that corresponds to HCV RNA, including a sequence encoding gp35, was isolated by immunoscreening from the λgt11 cDNA library prepared from sera of NANBH patients (31). This cDNA clone was inserted into the hemagglutinin gene downstream of the P7.5 promoter of the Lister strain of vaccinia virus (30). Rabbits were immunized by intradermal inoculation of the recombinant vaccinia virus. The specificity of the rabbit serum was verified by its specific reaction with two kinds of products directed by the HCV genome region encoding gp35; that is, materials produced by using a baculovirus expression vector system and an in vitro translation system (15a).

Secondary structure of the HCV 5' UTR. A possible secondary structure of HCV RNA was predicted for nucleotide positions 1 to 600 by using the Fold program (Biosequence Analysis System of the Institute of Medical Science, the University of Tokyo) on a VAX-11/750 computer.

RESULTS

HCV protein produced in vitro. Hiijkata et al. (7) have demonstrated that capped HCV RNAs with a short 5' UTR can be translated into the correct HCV proteins in the RRL cell-free translation system. The RNA used by Hiijkata et al. (7) did not have AUG sequences upstream of the initiator AUG. To determine the effect of the upstream AUG sequences on translation, an RNA with a free 5' end synthesized from linearized pNV was examined for mRNA activity in a cell-free translation system prepared from HeLa S3 cells (Fig. 2). Efficient translation occurred, and a polypeptide with the expected size (approximately 52 kDa) was observed on a gel (Fig. 2, lane 2). A small amount of product that migrated slightly faster than the 52-kDa polypeptide was also observed (lane 2). This material may have resulted from partially degraded RNA template. In any event, the data strongly suggest that the translation of uncapped HCV RNA starts at the fourth AUG, the authentic initiator AUG, from the 5' end of the RNA. Thus, the upstream AUG seems not to have a deleterious effect on the translation initiation on HCV RNA in this in vitro translation system.

To confirm that the translation products contain the correct HCV polypeptides, the translation reaction was performed in the presence of canine microsomal membrane (Fig. 2, lane 3). Two major proteins with approximate molecular masses of 22 and 35 kDa were observed in addition to the unprocessed products. This observation is compatible with that reported previously (7) and therefore indicates that these products are p22 and gp35, respectively. A faint band at approximately 11 kDa may be the product of the region encoding a part of gp70. An immunoprecipitation experiment involving rabbit anti-gp35 serum was performed as described in Materials and Methods. The precipitates with the rabbit serum were separated by the SDS-polyacrylamide gel electrophoresis (Fig. 2, lane 4). The rabbit serum reacted with both gp35 and the unprocessed products. These results indicate that the in vitro translation products contain the correct HCV polypeptide. It is therefore possible that ribosomes bind to an internal sequence within the 5' UTR like the translation initiation on picornavirus RNAs.

Effect of cap on translation. Since it is known that cap structure linked to the 5' end of picornavirus RNAs has little effect on the efficiency of translation initiation (25), mRNA activities of HCV RNAs carrying m'GpppG (capped methylated) or GpppG (capped unmethylated) at the 5' end were compared with each other in the absence (Fig. 3a, lanes 2 to 7) or presence (Fig. 3b, lanes 2 to 7) of SAH. mRNA activity of capped methylated HCV RNA derived from pNV appears to be similar to that of capped unmethylated HCV RNA from...
FIG. 3. Effect of cap on in vitro translation. In vitro translation reactions were carried out in the absence (a) or presence (b) of 20 μM SAH. Capped methylated (m'GpppG-linked) (lanes 3, 5, 7, 10, 12, and 14) or capped unmethylated (GpppG-linked) (lanes 2, 4, 6, 9, 11, and 13) RNAs derived from pNV (lanes 2, 3, 4, and 5), pNST (lanes 6 and 7), 5HCAT (lanes 9, 10, 11, and 12), and 5HStCAT (lanes 13 and 14), or no RNA (lanes 1 and 8) were translated in HeLa cell lysates (lanes 1, 2, 3, 9, and 10) or in RRL (lanes 4 to 8 and 11 to 14), and the translation products were analyzed as described in the legend to Fig. 2. Positions of products from translated regions of HCV and CAT RNAs are indicated at the right; positions of molecular weight markers are indicated at the left.

pNV in cell-free translation systems prepared from HeLa S3 cells (Fig. 3a and b, lanes 2 and 3) and rabbit reticulocytes (Fig. 3a and b, lanes 4 and 5). In the latter system, however, capped methylated RNA seems to be slightly more efficient than capped unmethylated RNA. In the case of HCV RNA derived from pNST, which lacks a nucleotide sequence upstream of position 270, capped methylated RNA is much more effective as mRNA than is capped unmethylated RNA in RRL (Fig. 3a and b, lanes 6 and 7). These data strongly suggest that a segment within the 5' UTR of HCV RNA has the ability to initiate protein synthesis independently of cap functions. The relative efficiency of the translation was not affected by the presence of SAH, a competitive inhibitor for an RNA (guanine-7-)methyltransferase activity existing in HeLa S10 and RRL (2, 5). This finding suggested that differences in translation efficiencies observed between capped methylated RNAs and capped unmethylated RNAs (Fig. 3a and b, lanes 4 to 7) were not underestimates.

To exclude the possibility that the translated region of HCV RNA is involved in the cap-independent initiation of translation, the translated region of HCV RNAs was replaced by CAT mRNA as described in Materials and Methods. Translation experiments similar to those shown in Fig. 3a and b, lanes 2 to 7, were carried out on these recombinant RNAs (Fig. 3a and b, lanes 9 to 14). The data are very similar to those obtained in the experiment involving parental HCV RNAs. These results indicate that a segment required for the cap-independent translation initiation resides within the 5' UTR of HCV RNA.

Existence of IRES within the 5' UTR of HCV RNA. To prove the existence of IRES within the 5' UTR of HCV RNA, a capped methylated (m'GpppG-linked) dicistronic mRNA consisting of CAT mRNA as the second cistron and HCV RNA as the second cistron was synthesized from linearized pCV. The structure of pCV is shown in Fig. 1. The translation experiments were performed on this dicistronic mRNA by using RRL (Fig. 4, lane 2) and lysates of CVB1-infected HeLa S3 cells (Fig. 4, lane 3). A protein synthesis system prepared from CVB1-infected HeLa S3 cells has the ability to suppress cap-dependent initiation of translation (unpublished results) as observed in that from poliovirus-infected HeLa cells (4, 22). Both CAT mRNA and HCV RNA appear to be translated in RRL (Fig. 4, lane 2). However, only HCV RNA appears to be translated in lysate of CVB1-infected HeLa S3 cells (Fig. 4, lane 3). Since a dicistronic mRNA on polysomes was shown to be intact in HeLa cell extracts (26), the data strongly suggest that the second cistron is translated even when ribosome entry does not occur at the 5' end of the dicistronic mRNA. Therefore, it is very likely that an IRES exists within the 5' UTR of HCV RNA.

Possible secondary structure of the 5' UTR of HCV RNA. A possible secondary structure was predicted as described in Materials and Methods and is shown in Fig. 5. Six possible stem-loop structures observed in the 5' UTR were designated A to F, respectively, from the 5' end of RNA. To confirm the validity of the secondary structure, RNA sequences of the 5' UTR of group 1 HCV and group II HCV RNAs (31) were compared with each other. Nucleotides of group II HCV that are different from those of group I HCV are indicated by arrows in Fig. 5. Many different nucleotides...
FIG. 5. Possible secondary structure of the 5' UTR of group I HCV RNA predicted by computer analysis. Different nucleotides within the 5' UTR of group II HCV RNA are indicated by arrows and bold letters. Major stem-loop structures are designated A to F. The initiator AUG is indicated by bold letters.

were observed in stem-loop structure E. However, most of the nucleotide differences in stem-loop structure E occurred without destruction of possible base pairing. This observation confirms the validity of the possible secondary structure shown in Fig. 5, especially with respect to stem-loop structure E.

Identification of an IRES. To identify the IRES of HCV RNA, artificial mono- and dicistronic mRNAs were constructed, taking positions of upstream AUG sequences and possible stem-loop structures into consideration as described in Materials and Methods (Fig. 6A). Capped unmethylated (GpppG-linked) RNAs derived from pNV, pN6, pN7, pN8, pN9, pN10, pN11, and pN16 and capped methylated (m'GpppG-linked) RNAs from pCV, pC6, pC7, pC8, pC9, pC10, pC11, and pC16 were used as mRNAs in RRL (Fig. 6B and C). RNAs derived from pNV, pN6, and pN7 were active mRNAs in RRL, and an RNA derived from pN8 was a very inefficient mRNA (Fig. 6B). Similar results were obtained from experiments involving dicistronic mRNAs derived from pCV, pC6, pC7, and pC8 (Fig. 6C). These results indicate that an IRES appears to reside downstream of nucleotide position 101. RNAs from pC9, pC10, and pN11, in which the first AUG is the initiator AUG, restored mRNA activities (Fig. 6B, lanes 6 and 7). This phenomenon may result from the fact that eukaryotic ribosomes have a tendency to recognize and bind to the end of RNA (17). Indeed, the translation product of the HCV RNAs derived from dicistronic cDNAs, pC10 and pC11, was not observed (Fig. 6C, lanes 7 and 8). This observation may support the finding that translated dicistronic mRNA is intact during the translation reaction (26). To identify the 3' end of the IRES, a deletion RNA derived from pN16 was synthesized and tested for mRNA activity. This deletion RNA was not an active mRNA (Fig. 6B, lane 8). A similar observation was made when a dicistronic mRNA derived from pC16 was used as mRNA in RRL (Fig. 6C, lane 9). These results indicate that the IRES of the 5' UTR of HCV RNA resides between nucleotide positions 101 and 332.

Efficiency of group II HCV RNA. Since nucleotide differences were observed in IRESs within the 5' UTRs between group I and group II HCV RNAs, IRES functions of group I and group II HCV RNAs were compared in a cell-free protein synthesis system of HeLa S3 cells. Capped unmethylated (GpppG-linked) HCV RNAs (nucleotide positions 49 to 1772) containing the 5' UTRs of group I and group II HCV RNAs were prepared from pN5 and pNII5, respectively, as described in Materials and Methods and used as mRNAs. As shown in Fig. 7, translation initiation from the 5' UTR of group II HCV RNA is more efficient than that of group I HCV RNA. Similar results were obtained with three pairs of RNA preparations (data not shown). Furthermore, amounts of radioactivity incorporated into the product increased proportionally with an RNA dose of up to 2 μg per reaction mixture. These results may indicate that regions harboring different nucleotides between group I and group II HCV RNAs play an important role(s) in IRES function.

DISCUSSION

In this study, we demonstrated that initiation of HCV protein synthesis occurs in a cap-independent manner like that of picornavirus-specific protein synthesis and that an IRES within the 5' UTR of HCV RNA resides between nucleotide positions 101 and 332. Previous studies of picornavirus RNA have shown that IRES function requires a specific segment of approximately 400 nucleotides (11, 26). Thus, the IRES of HCV RNA is the shortest among IRESs so far discovered. Mutational analysis of picornavirus RNAs indicated that 21- and 7-base sequences conserved in the 5' UTR of human enterovirus and rhinovirus RNAs are important for translation initiation (8, 9, 19, 25). These sequences were not observed in the 5' UTR of HCV RNA. Therefore, HCV and picornaviruses may have different mechanisms for the entry of ribosomes to the IRES. An oligopyrimidine tract before the AUG at position 206, however, was observed within the 5' UTR of HCV RNA. An oligopyrimidine tract before an AUG is conserved in picornavirus RNA and is considered to be important for translation initiation on the viral RNAs (1, 19, 24). This AUG is the initiator AUG (encephalomyocarditis virus and foot-and-mouth disease virus) or most 3'-proximal AUG (rhinovirus and poliovirus) in the 5' UTR (12, 13, 20). The existence of this RNA sequence in both HCV and picornavirus RNAs suggests that the same initiation factor(s) is involved in the translation initiation on both viral RNAs.

Like the IRES of encephalomyocarditis virus, the IRES of HCV is very effective in both HeLa cell lysates and RRL. Indeed, the HCV IRES is much more effective than the CVB1 IRES both in mock- and CVB1-infected HeLa cell lysates (unpublished result). Capped methylated and capped unmethylated HCV RNAs did not show any difference in the
efficiency of translation in HeLa S3 lysates (Fig. 3a and b, lanes 2 and 3). However, capped methylated HCV RNA is more efficient than capped unmethylated HCV RNA in RRL (Fig. 3a and b, lanes 4 and 5). It is therefore possible that HCV RNA requires a cap structure to be a fully active mRNA in RRL. This phenomenon is difficult to explain at present. It is of interest that the IRES of group II HCV is more effective than the IRES of group I HCV in HeLa cell lysates (Fig. 7), although group I HCV appears to be the major group of HCV (31). In any event, different nucleotides observed in the IRESs of group I and group II HCV RNAs must influence IRES function, which may provide some insight into the mechanism of the IRES function of HCV. Mutational analysis of HCV RNA with respect to IRES function is currently under way.

Although the nucleotide sequence of almost the entire genome of HCV has been elucidated (3, 15, 28), the 5' and 3' structures of the genome are not clear at present. Therefore, it is not known whether HCV RNA is capped. However, elucidation of cap-independent translation of HCV indicates that HCV has a mechanism for translation initiation totally different from that of a member of the family Flaviridae;
FIG. 7. Translation efficiency from the 5' UTRs of group I and group II HCV RNAs. No RNA (lane 1) and capped unmethylated (GpppG-linked) HCV RNAs that carried the 5' UTRs of group II HCV (lane 2) and group I HCV (lane 3) were used as mRNAs in a cell-free protein synthesis system prepared from HeLa S3 cells. Both HCV RNAs correspond to nucleotide positions 49 to 1772, as described in Materials and Methods. The translation products were analyzed as described in the legend to Fig. 2. Positions of molecular weight markers are indicated at the left.

therefore, creation of new virus family may be desirable for classification of HCV.

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