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Detection of Hepatitis C Virus RNA by a Combined Reverse Transcription-Polymerase Chain Reaction Assay

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Amplification of RNA by the polymerase chain reaction (PCR) is normally a two-step process requiring separate enzymes and buffer conditions. We describe a combined reverse transcription-PCR (RT-PCR) assay for hepatitis C virus (HCV) RNA amplification in which a single enzyme and buffer condition are used. In this assay, both the RT and PCR steps are carried out with the thermoactive DNA polymerase of *Thermus thermophilus*. A transcription vector containing HCV sequences has also been constructed to generate quantifiable HCV RNA templates that can be used to optimize reaction conditions and to assess the efficiency of amplification. Amplification from ≤100 copies of RNA was detected reproducibly by gel electrophoresis. The assay sensitivity was increased to 10 RNA copies by hybridization to a probe. The patterns of viremia in three individuals infected with HCV were examined by amplification of HCV RNA from plasma samples collected serially over a period of 1 year. These results were correlated with the times of seroconversion and the onset of rise in levels of alanine aminotransferase in serum. In all three subjects, HCV RNA was detected prior to seroconversion and the initial rise in levels of alanine aminotransferase in serum. Upon seroconversion, HCV RNA fell to a level below the detection limit of the assay. This pattern of transient viremia appears to be characteristic of acute, resolving HCV infections. The combined RT-PCR assay is a sensitive method which circumvents the problems associated with PCR amplification of RNA. Using this assay, we demonstrated that three donors infected by the same index case all have similar patterns of viremia.

Hepatitis C virus (HCV) is the major causative agent for parenterally transmitted non-A, non-B hepatitis (5). Detection of HCV infection has been facilitated by the development of antibody detection assays. However, antibody detection methods are of restricted use because there is a mean window period of 22 weeks between infection and seroconversion (1). Loss of antibody in some persistently infected individuals has also been documented (4). Furthermore, data on antibody status do not provide a means to differentiate between current, active infection; chronic infection; and past, resolved infection. Methods which permit the direct detection of virus can augment information from antibody testing. Amplification of viral nucleic acids by the polymerase chain reaction (PCR) has been shown to be an effective means for the direct detection of HCV (8, 18, 23).

The genome of HCV is composed of a single-stranded RNA molecule (5). As such, amplification by PCR must be preceded by a step to generate a cDNA copy. The synthesis of the cDNA copy generally employs a retroviral reverse transcriptase. Amplification of the cDNA is typically performed by a thermostable and thermoactive DNA polymerase from *Thermus aquaticus*. Previously, the requirement for different enzymes in the two steps necessitated the addition of new enzyme and change of buffer conditions between the reverse transcription (RT) and amplification steps. This is cumbersome and increases the likelihood of contamination.

We report here the development of an HCV RNA amplification assay in which both the RT and the amplification steps are carried out with one enzyme under a single set of buffer conditions. This combined RT-PCR assay takes advantage of the fact that the DNA polymerase of the thermophilic bacterium *Thermus thermophilus* possesses enhanced reverse transcriptase activity in the presence of manganese (17). Although optimal conditions for DNA and RNA templates are different (17), a set of buffer conditions which supports both RT and PCR has been determined. The use of a single enzyme and buffer condition obviates the need to open reaction tubes between the RT and DNA amplification steps and thus minimizes the possibility of contamination. Coupled RT-PCR systems in which retroviral reverse transcriptase-mediated cDNA synthesis and *Taq* polymerase-mediated amplification are carried out in the presence of both enzymes under one buffer condition have been described (6, 10). However, the lower temperature necessary for retroviral reverse transcriptase activity may reduce the efficiency with which cDNA can be synthesized through stable secondary RNA structures (2, 14). The elevated reaction temperature required for the thermoactive *T. thermophilus* DNA polymerase used in the current assay would destabilize some of the secondary structure present in the RNA template and in addition increase the specificity of primer extension.

The development of assays for the amplification of HCV RNA has been hampered by the lack of well-defined templates to optimize amplification parameters and to determine the analytical sensitivity of the assay. To generate such defined templates, we have cloned the relevant portion of the HCV genome into a transcription vector. The concentration of the HCV RNA transcribed in vitro was measured, and known amounts were used as templates in amplification reactions. This is the first description of an HCV RNA amplification assay in which the amplification conditions and analytical sensitivity of the assay are completely characterized.

The combined RT-PCR assay was used to document the natural history of HCV infection in three individuals who

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were infected by contaminated blood products from the same index case. Plasma samples collected serially at different time points postinfection were examined to determine the pattern of viremia over time in each individual.

MATERIALS AND METHODS

Oligonucleotide primers and probe. The combined RT-PCR was performed with a primer pair selected from the highly conserved 5′ terminus of the HCV genome (11, 12, 19). The pair consisted of upstream primer KY80, 5′-GCA GAAAGCGTCTAGCCATGGCGT (nucleotides [nt] 56 to 79 [12]), and downstream primer KY78, 5′-CTCGCAAGCAC CCTATCAGGCAGT (nt 276 to 299). (KY78 contains a biotin residue at the 5′ terminus to facilitate nonradioactive detection of amplification products.) KY88, 5′-GTTGGGTCGCG AAAGGCCTTGTGGT (nt 251 to 275), served as the hybridization probe.

RNA template production. To construct an HCV RNA transcription vector, HCV sequences from nt 50 to 599 (12) were cloned into the pSP64(poly A) vector (Promega, Madison, Wis.). The identity of the final construct (pHCV1.1A) was confirmed by DNA sequence analysis.

HCV template RNA was transcribed in vitro from pHCV1.1A according to the manufacturer's instructions. Briefly, 5 μg of plasmid DNA was linearized and then incubated with 40 U of SP6 RNA polymerase for 90 min at 37°C in the presence of 500 μM (each) ribonucleoside triphosphates (GTP, ATP, UTP, and CTP), 100 U of RNasin, 10 mM dithiothreitol, 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, and 10 mM NaCl in a total reaction volume of 100 μl. After the transcription reaction, the DNA template was degraded by two rounds of digestion with RNase-free DNase (Promega) for 15 min at 37°C with 5 and 10 U of enzyme, respectively. The RNA was diluted with 100 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA and adjusted to 240 mM sodium acetate (pH 4.0) to facilitate partitioning of residual DNA into the organic phase during subsequent extraction with phenol (13). The HCV RNA transcripts, which contained a poly(A) tail, were further purified on an oligo(dT)-cellulose column (21). The RNA concentration was determined spectrophotometrically by UV A_{260} (22). An aliquot was analyzed by agarose gel electrophoresis to assess its integrity. A typical yield was between 10 and 15 μg of HCV RNA transcript. Serial dilutions of the RNA templates were made in the presence of *Escherichia coli* rRNA (Sigma, St. Louis, Mo.) as the carrier at 10 ng/μl.

Combined RT-PCR and detection of amplification products. The combined RT-PCR amplifications were carried out with 20-μl reaction mixtures containing 2 μl of template, 200 μM (each) deoxyribonucleoside triphosphates (dNTPs) (dATP, dCTP, dGTP, and dTTP), 0.85 mM $MnCl_2$, 150 nM (each) primers KY78 and KY80, 5 U of *T. thermophilus* DNA polymerase (Perkin-Elmer, Norwalk, Conn.), and 1× RT-PCR buffer (10× RT-PCR buffer consists of 100 mM Tris-HCl [pH 8.3] and 900 mM KCl). The reactions were performed in a GeneAmp PCR system 9600 thermocycler utilizing thin-walled MicroAmp reaction tubes (Perkin-Elmer) without a mineral oil overlay. The thermocycler was preheated to 70°C. RT was allowed to proceed for 15 min at 70°C and was followed by a 1-min incubation at 95°C to facilitate denaturation of RNA-DNA heteroduplexes. PCR amplification proceeded with 2 cycles at 95°C for 15 s and 60°C for 20 s followed by 38 cycles at 90°C for 15 s and 60°C for 20 s and a final extension step of 4 min at 60°C. The reaction mixtures were then held at 15°C. The total elapsed time for the RT and subsequent 40-cycle DNA amplification was less than 90 min. Omission of the 70°C RT step, which effectively prevents cDNA synthesis without compromising amplification of DNA, was used to distinguish amplification of DNA from RNA.

Upon completion of the amplification reaction, 4 μl of each reaction was analyzed by electrophoresis through a 1.7% agarose gel in Tris-borate-EDTA buffer (pH 8.0) and ethidium bromide staining. DNA was transferred from the gels onto BioTrans nylon membranes (ICN, Irvine, Calif.) by alkaline transfer (20). The transferred DNA was cross-linked by UV light at the autocross-link setting of a Stratalinker (Stratagene, San Diego, Calif.). The blots were prehybridized with a solution containing 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 5× Denhardt's solution, 100-μg/ml sheared denatured herring sperm DNA, and 0.1% sodium dodecyl sulfate (SDS) at 50°C for 1 h and then were hybridized at the same temperature for 1 h with probe KY88 labelled with ^{32}P by using polynucleotide kinase (Boehringer Mannheim; Indianapolis, Ind.). The blots were washed twice at room temperature with 2× SSPE–0.1% SDS and then were washed twice for 15 min at 65°C with the same buffer. The blots were autoradiographed without an intensifying screen with Kodak XAR X-ray film at 25°C for 3 h. Alternatively, amplification products can be hybridized in solution to probes immobilized in wells of microtiter plates and detected colorimetrically (16).

Clinical samples and sample preparation. Seroconversion panels from three HCV-infected donors were obtained from Serologicals, Inc. (Clarkston, Ga.). The panels are composed of plasma samples collected serially over a period of 1 year. All three donors were infected through the injection of packed erythrocytes from the same index case. (The erythrocyte injections were administered to generate antibody against Rh factor.) Patient 1 received a single injection. Patient 2 received two injections 25 days apart. Patient 3 received three injections; the second and third injections were given 1 and 3 days, respectively, after the initial injection. The volume of cells injected each time was 2 ml or less. Plasma from the index case, collected at the same time as the erythrocytes, initially tested negative for HCV antibody by a prototype test but was subsequently determined to be positive by a Food and Drug Administration-approved test (Ortho Diagnostics, Rahway, N.J.). Plasma samples obtained from the index case on later dates also were positive for HCV antibody. The index case plasma exhibited no elevation in serum alanine aminotransferase (ALT) level at the time of original donation or thereafter. Information on the size and nature of inocula, antibody status, and levels of ALT in serum was provided by Serologicals, Inc. It should be noted that the plasma samples as supplied by Serologicals, Inc., had been stored under less-than-ideal conditions. All specimens were subjected to at least two rounds of freeze-thaw prior to testing, and some specimens were stored at 4°C for extended periods.

Total nucleic acids were isolated from 100-μl aliquots of each serial plasma sample by using the IsoQuick nucleic acid extraction kit (Microprobe Corporation, Bothell, Wash.). Briefly, virus was lysed with a solution containing guanidium isothiocyanate, and the lysate was incubated at 65°C for 10 min. Proteins were then extracted twice with an extraction buffer and an organic extraction matrix slurry. Nucleic acids were recovered from the aqueous phase by precipitation with isopropanol, washed sequentially with 70 and 95%

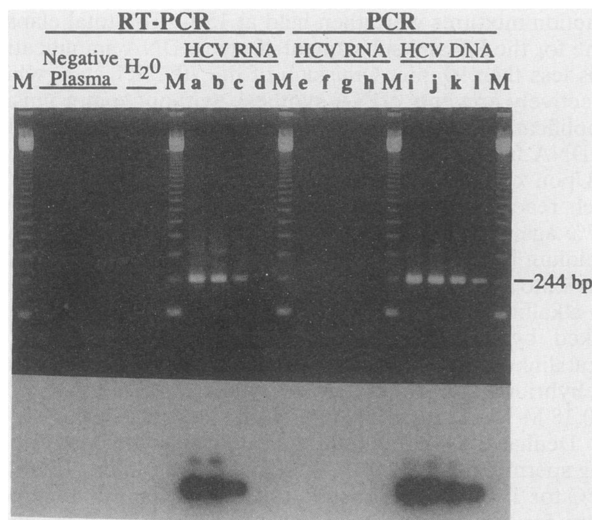

FIG. 1. Analytical sensitivity of HCV RNA amplification by the combined RT-PCR assay. A 10-fold dilution series of HCV RNAs transcribed in vitro from the recombinant vector pHCV1.1A was amplified by combined RT-PCR to assess the analytical sensitivity of the assay. As a comparison, a similar dilution series of pHCV1.1A DNAs was amplified. Control reactions in which RNA templates were amplified in the absence of an RT step were included to demonstrate that amplification was from RNA templates and not residual plasmid DNA. Lanes: a through d, amplification of 10^4, 10^3, 10^2, and 10^1 copies of HCV RNA transcripts, respectively, by combined RT-PCR; e through h, amplification of HCV RNA transcripts shown in lanes a through d in reactions in which the RT step was omitted; i through l, amplification of 10^4, 10^3, 10^2, and 10^1 copies of pHCV1.1A DNA, respectively; M, 123-bp ladder size markers. The bottom panel is a Southern blot of the gel in the top panel.

ice-cold ethanol, and then air dried. The nucleic acids were resuspended in 20 μl of water treated with diethyl pyrocarbonate and stored at −70°C. Two microliters of each sample (equivalent to the nucleic acids isolated from 10 μl of plasma) was amplified in the combined RT-PCR assay in duplicate. The samples were not blinded in this study. However, at least two separate nucleic acid extractions and RT-PCR analyses were performed for each specimen.

Appropriate precautions were taken to avoid sample-to-sample carryover and contamination by cloned DNA or PCR products during both the sample preparation and amplification setup steps (15). Negative control plasma samples were randomly interspersed with clinical samples during sample preparation and carried through amplification. In addition, no-nucleic-acid controls were included with each set of amplification reactions.

RESULTS

Analytical sensitivity of combined RT-PCR assay. In order to assess the analytical sensitivity of amplification, a 550-nt fragment from the 5′ end of the HCV genome was cloned into a transcription vector under the control of an SP6 promoter. The concentration of HCV RNA transcribed in vitro from the plasmid pHCV1.1A was determined, and known amounts of RNA transcript were used as templates in amplification reactions. Amplification signals from 100 RNA molecules were seen consistently by gel electrophoresis (Fig. 1). Occasionally, weak signals from 10 RNA copies were also seen (data not shown). Hybridization to probes immobilized on microtiter plates allowed the consistent detection of 10 RNA copies (data not shown). Amplification of nucleic acids from a serum whose viral titer was determined by infection of chimpanzees (kindly provided by A. Prince) yielded a titer that is 10-fold higher than the titer determined previously (data not shown). This level of sensitivity is comparable to those achieved by nested PCR systems reported by others (7).

The efficiency of DNA amplification was determined by parallel amplification with the pHCV1.1A DNA as template. The signal from 10 copies of DNA was roughly equivalent to that from 100 copies of RNA (Fig. 1). This suggests that cDNA copies are generated from only a fraction of the RNA templates during the RT step. To ensure that products from RT-PCR resulted from amplification of RNA rather than amplification of residual DNA present in the RNA preparation, amplification reactions in which the RT step was omitted were performed. As shown in Fig. 1, lanes e through h, no products were generated from as many as 10^6 RNA copies when the RT step was omitted. At input RNA levels of 10^8 or higher, amplification products were seen in the absence of RT (data not shown), suggesting the presence of residual DNA or a very low level of RT activity during DNA amplification.

Pattern of RNA detection in HCV-infected patients. Nucleic acids were extracted from plasma samples collected serially over a period of 1 year from three individuals infected with HCV. The nucleic acid from the equivalent of 10 μl of plasma was amplified by the combined RT-PCR assay. Figure 2 summarizes the results from ALT determination and antibody and RNA testing for all three individuals. All three subjects shared similar patterns of viremia, ALT elevation, and seroconversion. HCV RNA was detected within 10 to 14 days of exposure to HCV. RNA remained positive until seroconversion. At about that time, the viral RNA level fell to below the 100-copy limit of detection by gel electrophoresis and Southern blot hybridization in the 10 μl of plasma tested, a titer of $<10^4$/ml. These samples were not analyzed in microtiter plates. Elevated ALT levels were first evident 38 to 42 days postinfection. ALT levels returned to normal 114 to 140 days postinfection and remained normal for the remainder of the follow-up. Antibody to HCV was first detected 46 to 113 days after exposure, and the samples remained positive thereafter. Representative results of RNA amplification by combined RT-PCR from patient 1 are shown in Fig. 3. A band of higher molecular weight is consistently seen both in these clinical samples and in the cloned templates (Fig. 1). The origin of this band is not known, but the band is HCV-specific, given its ability to hybridize to the HCV probe.

DISCUSSION

We have developed a combined RT-PCR assay in which HCV RNA can be reverse transcribed into cDNA and then amplified by PCR under a single set of conditions. This assay is superior to conventional methods for the amplification of RNA by PCR in that the need to change or add reagents between the RT and PCR steps is eliminated. This obviates the need to open reaction tubes after initial setup, minimizes the possibility of contamination, and reduces the hands-on work required. The use of a thermoactive enzyme also permits RT to be carried out at elevated temperatures, which increases the specificity of priming and increases efficiency by destabilizing secondary structures in the RNA template. In addition, the high enzyme concentration utilized for

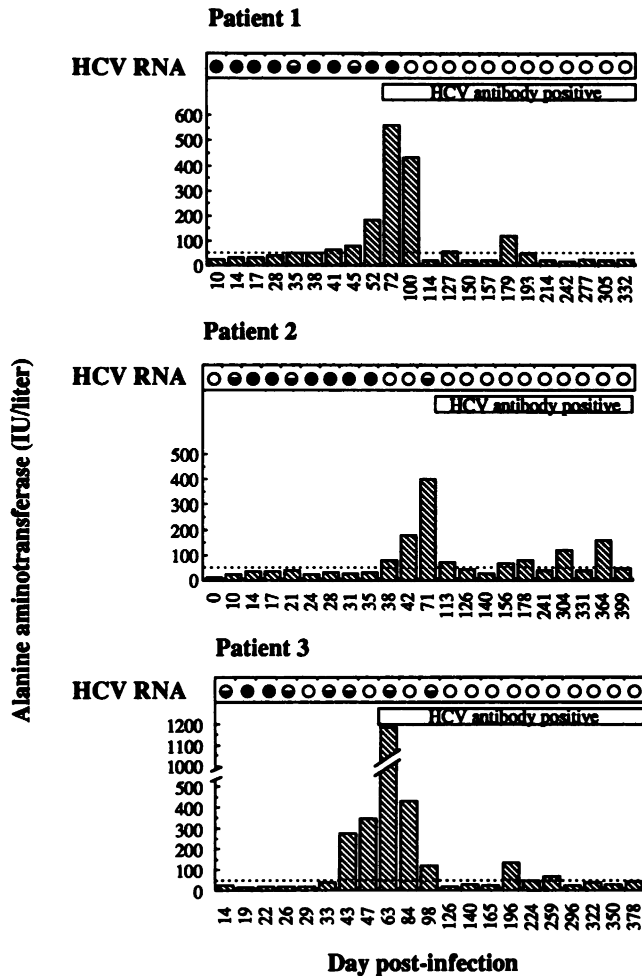

FIG. 2. Patterns of HCV RNA, serum ALT level, and HCV antibody in the three study subjects. Solid circles indicate that HCV RNA was detected consistently, half-filled circles indicate that specimens were positive for HCV RNA in only some of the replicate analyses, and open circles indicate that HCV RNA was consistently not detected at the limit of 100 copies in the 10 μl of plasma tested.

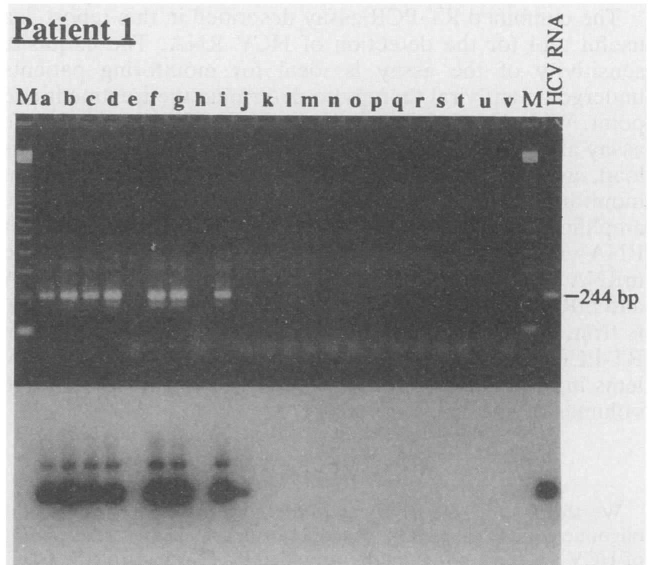

FIG. 3. Representative results of combined RT-PCR amplification of plasma samples from patient 1. Lanes a through v correspond to days postinfection as in Fig. 2, patient 1. Lanes M, 123-bp ladder size markers. The bottom panel is a Southern blot of the gel in the top panel.

efficient RT permits short extension times to be used during DNA amplification, greatly reducing the total time required for the assay.

Determination of the efficiency of RT-PCR assays as applied to the detection of HCV RNA has been hampered by the lack of a method for growing large quantities of virus. Chimpanzee infectious dose titered virus stocks are not useful for this purpose because only infectious virus is measured. While measurement of infectious virus may be clinically relevant, it does not necessarily correlate with virus particle count, as demonstrated by us and others (9). To obtain a direct measure of the analytical sensitivity of our combined RT-PCR assay, we have constructed a transcription vector from which HCV RNA is transcribed in vitro and the RNA concentration is determined photometrically by UV A_{260}. By using such well-characterized templates, the analytical sensitivity of the combined RT-PCR assay was found to be ≤100 copies of RNA templates when analyzed by gel electrophoresis. Hybridization to the probe improves the analytical sensitivity to 10 RNA copies. This level of sensitivity was achieved without a need for the nested amplification reported by others, in which two pairs of primers were used in sequential amplification reactions (8, 18). Nested PCR did not increase the detection limit of the combined RT-PCR assay (data not shown). The need for nested PCR in most systems reported to date points to the inefficiency of these assays and highlights the need for well-characterized RNA templates to optimize amplification conditions. Furthermore, the use of two separate primer pairs in sequential amplification reactions during nested PCR requires the transfer of reagents during amplification. This is labor intensive and increases the likelihood of contamination.

To demonstrate the clinical utility of the combined RT-PCR, we examined the time course of viremia in three individuals. Plasma samples collected serially over a period of 1 year were examined for the presence of HCV RNA. Similar patterns of transient viremia early in infection followed by a decline to levels below 100 molecules in 10 μl of plasma at about the time of seroconversion were seen in all three subjects. This pattern of transient viremia has been reported in cases of acute, resolving HCV infections in humans and chimpanzees (7, 8). The fact that all three individuals in this study were infected by the same index case and showed similar patterns of viremia is consistent with the hypothesis that the infecting virus is an important factor in determining the natural history of HCV infection (7). However, interpretation of the clinical significance of these results must be made in light of the small sample size (three subjects) and less-than-ideal storage conditions of the specimens (3). Curiously, HCV RNA was not detected in the source plasma from the index case (data not shown). This suggests that the concentration of virus may be below 100 RNA copies in the 10 μl of plasma tested (the equivalent of 10^4 copies per ml). This implies that the infectious dose of HCV may be low, because each subject was exposed to no more than 2 ml of packed erythrocytes. Subsequent samples from the index case taken at 3, 5, and 7 months after the initial donation were positive for HCV RNA.

The combined RT-PCR assay described in this report is a useful tool for the detection of HCV RNA. The exquisite sensitivity of the assay is ideal for monitoring patients undergoing antiviral therapy to determine the treatment end point. With the addition of appropriate internal controls, the assay also has the potential for quantitative analysis of virus load, useful in determining treatment regimen and patient monitoring. In addition, the assay can be applied to the amplification of any RNA template. These include other RNA viruses (such as human immunodeficiency virus) and mRNA. In situations involving detection of retroviral RNA and mRNA, care must be taken to ensure that amplification is from RNA templates. In an assay with the sensitivity of RT-PCR, amplification of residual DNA can lead to problems in the interpretation of results, especially where large volumes of specimen are tested.

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