International Journal of Biochemistry Research & Review
4(3): 243-252, 2014

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Multi-Detection by Target Mixed Loop-Mediated Isothermal Amplification

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Authors' contributions

This work was carried out in collaboration between all authors. Authors KN, TN and HK designed the study. Author KN wrote the protocol and the first draft of the manuscript. Authors KN, YK and JT managed the analyses of the study. Author TH managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

Received 8th November 2013
Accepted 1st February 2014
Published 13th February 2014

ABSTRACT

Loop-mediated isothermal amplification (LAMP), a nucleic acid amplification technology, has been characterized as having a short reaction time while generating a large amount of DNA product under isothermal conditions. Here, we found that LAMP is able to amplify more than one target simultaneously by using a mixture of human immunodeficiency virus-1, hepatitis B virus, and hepatitis C virus primers, a process we have named target mixed LAMP. Target mixed LAMP and detection can be performed in less than 1 h. In this study, we also used target mixed LAMP to successfully amplify GAPDH, gamma-actin, TATA box binding protein, and haptoglobin from pooled cDNA derived from mouse tissues. Furthermore, using eight plasmid vectors as templates, target mixed LAMP is able to amplify all targets simultaneously. These findings reveal the usefulness of LAMP for the diagnosis of infectious disease as well as for conducting gene expression analysis.

Keywords: LAMP; infectious disease; virus; diagnosis; magnesium pyrophosphate; DNA amplification.

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1. INTRODUCTION

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method that utilizes isothermal conditions to instantly amplify DNA or RNA [1,2]. The original LAMP reaction can be further accelerated by using additional primers, termed loop primers which, when used during clinical diagnostic testing, help decrease the time doctors and patients spend waiting for test results [3]. Furthermore, LAMP reactions generate extremely large amounts of DNA/RNA product in a short amount of time, leading to the simultaneous formation of a white magnesium pyrophosphate precipitate [4]. The appearance of this precipitate in the reaction mixture can be used to visually detect whether the DNA is being amplified or not. Since both amplification and detection can progress concurrently, LAMP is thought to be useful for a wide variety of applications, including basic research and clinical medicine. Moreover, single-stranded DNA can also be isolated from LAMP products for use in additional genetic analysis, such as DNA microarrays [5].

In clinical medicine, nucleic acid amplification technology is often used during genetic diagnosis of infectious diseases. Multiplex nucleic acid amplification is the most commonly used method during these diagnostic tests as it is able to amplify more than one target sequence in a single reaction through the use of multiple primer sets. During DNA amplification, this method utilizes multiplex polymerase chain reaction (PCR) and strand displacement amplification (SDA), whereas during RNA amplification, it uses nucleic acid sequence-based amplification (NASBA) [6-8]. Although the power to amplify multiple targets is extremely beneficial during infectious disease diagnosis [9], multiplex PCR requires more than one hour of reaction time and expensive machinery is necessary to run the experiments.

We have found that LAMP can be used to amplify multiple DNA targets simultaneously, a process named target mixed LAMP. In this paper, we report the use of target mixed LAMP for concurrent diagnosis of three diseases: human immunodeficiency virus-1 (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV). In addition, we also describe the methodology utilized for the amplification of four targets from an isolated pool of mouse cDNA.

2. MATERIALS AND METHODS

2.1 Template DNA

HIV, HBV, and HCV plasmid DNAs were purchased from the American Type Culture Collection (ATCC numbers 53069, 39629, and 40679, respectively).Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), gamma-actin (γ-actin), TATA box binding protein (TBP), and haptoglobin cDNAs were isolated from 17-day-embryonic mouse marathon-ready cDNA (Clontech; Palo Alto, CA) and then cloned into a pBluescript KS (-) vector.

Prior to use in our experiments, HBV-positive sera were confirmed by measuring the level of HBs antigen (HBsAg) present using Lumispot 'Eiken' HBs-Ag (Eiken; Tokyo, Japan). HBV-positive DNA testing using transcription-mediated amplification (TMA) methodology was entrusted to SRL, Inc., Tokyo, Japan. Further, for the LAMP and PCR reactions, sera were treated with an equal volume of 50 mM NaOH, incubated at 60ºC for 10 min, and then used as template material.

2.2 LAMP Reaction

LAMP reactions were performed using the Loopamp DNA Amplification kit (Eiken; Tokyo, Japan), which involves the addition of the specific primers, 0.25 µg/ml ethidium bromide (EtBr), and the target DNA. The primer sequences and concentrations used for LAMP amplification are listed in Table 1. To analyze the cDNA using target mixed LAMP, LAMP products were amplified from pooled embryonic mouse cDNA (Marathon-ready cDNA; Clontech). The LAMP mixtures were incubated at 65°C for one hour and analyzed in real time using the ABI PRISM 7700 sequence detection system (PE Applied Biosystems; Foster City, CA) [2]. We chose the reaction temperature that *Bst* DNA polymerase is most activated and the specific amplification occurred. We normalized the primer concentration for each set of primers so that the reaction time for each set was the same for a set quantity of template DNA.

For target mixed LAMP using HBsAg-positive serum, we used other HBV primers described previously [1,3]. A reaction mixture containing 1 µL of the serum was incubated at 62ºC for one hour. The primer concentrations were 1200 nM (HBVFIP and HBVBIP), 300 nM (HBVF3 and HBVB3), and 600 nM (loopF and loopB).

2.3 PCR Reaction

PCR was performed using the Expand long template kit (Roche; Mannheim, Germany). HBV DNA was amplified using the HBVF3 and HBVB3 primer pair described for the LAMP reaction above, with the following program: 94ºC for 3 min, followed by 35 cycles of 94°C for 30 s, 62ºC for 30 s, and 68ºC for 30 s, for a total run time of approximately 1.5 h.

2.4 Dot Blot Hybridization

The target mixed LAMP was performed using a 5′ DIG-labeled loop F primer instead of the non-modified primer. After the LAMP reaction was complete, products were purified with a PCR purification kit (QIAGEN K.K.; Tokyo, Japan). For the dot blot, 1 µL of each plasmid DNA (100 ng) was spotted on the Biodyne B nylon membrane (Pall; Port Washington, NY), which was then ultraviolet-cross linked. The empty pBluescript KS (-) vector was used as a negative control. The DIG-labeled LAMP products were then hybridized to the dot blot membrane to act as DNA probes. Hybridization was performed overnight in PerfectHyb buffer (TOYOBO; Osaka, Japan) at 60°C. Signals were detected using the DIG Detection kit (Roche; Mannheim, Germany) according to the manufacturer's protocol [5].

Table 1. Primer sequences and concentrations used in this study.

primer name		5'-primer sequence-3'	conc. (nM)
HBV	FIP	TCAGGGCATATTGACCACAGTTGACTGCCGATTGGTGGAG	1300
	BIP	CCGAATGCTCCCACTCCTACGGGACTTCAACCCCATCAAGGA	1300
	F3	GGAGAGATGGGAGTAGGCT	325
	B3	TTCGGAGCCCACTCAAAC	325
	loop F	GTCAACAATTCCTCCTCCTGC	650
	loop B	TGGTTGGCTGCTGGGCAGTGG	650
HCV	FIP	CCTTATAGCCCTGAGCTGCATACTCACCTCCATGCTCCCACA	1200
	BIP	TAGTACTCAACCCCTCTGTTGCATCCCATGAGCCTTGGACATG	1200
	F3	TCTCCACCAGTAGTGCCC	300
	B3	CCCGGTCCTGATGTTAGG	300
	loop F	CTTGGTGCTTTTGCCGCTGCC	600
	loop B	TGCAACACTGGGCTTTGGTGC	600
HIV	FIP	AGCCCTCAGATGCTGCATATAATGGTCTAACCAGAGAGACCCAG	800
	BIP	GGAAAGTCCCCAGCGGAAAGCTGCATCCGGAGTACTTCAAG	800
	F3	GAGCTCCCAGGCTCAAA	200
	B3	GCCTAGCATTTCATCACGT	200
	loop F	GCAGCTGCTTTTTGCCTGTA	400
	loop B	TTGTAGCAAGCTCGATGTCAGC	400
GAPDH	FIP	TGCATTGCTGACAATCTTGAGTGATGCCCCCATGTTTGTGAT	1600
	BIP	CTGCACCACCAACTGCTTAGCCCTTCCACAATGCCAAAGT	1600
	F3	AAACGGGTCATCATCTCCG	400
	B3	AGTGATGGCATGGACTGTG	400
	loop F	GTTGTCATATTTCTCGTGGTTC	800
	loop B	CTGGCCAAGGTCATCCAT	800
g-actin	FIP	TTGCCAATGGTGATCACCTGTGCTGCATCATCTTCCTCCTT	1200
	BIP	GCACTCTTCCAGCCTTCCTTGGAGTTGAAAGTGGTCTCATGG	1200
	F3	ATGTTGCCCTGGATTTTGAG	300
	B3	GCGGATATCCACATCACACT	300
	loop F	TCGGGCAGCTCGTAACTCT	600
	loop B	TGGGCATGGAGTCCTGTG	600
TBP	FIP	GCATTTCTTGCACGAAGTGCTCTACCGTGAATCTTGGCTGT	1600
	BIP	TAATCCCAAGCGATTTGCTGCAATCAACGCAGTTGTCCGTG	1600
	F3	AGAGCTCTGGAATTGTACCG	400
	B3	CCTGTGCACACCATTTTTCC	400
	loop F	GCAATGGTCTTTAGGTCAAGT	800
	loop B	CAGTCATCATGAGAATAAGAGAGC	800
Hapto-globin	FIP	TTCCTGGTACTTGGTGAGGCGGGGTCCAGCCTATCTTGAA	800
	BIP	GCAGTGCCTTTGCCATTCATTGTCAAAGCTCAGGATCCCA	800
	F3	TGCCCGAGAAGAAAAACTTG	200
	B3	CACACCATACTCAGCGACAG	200
	loop F	GCCAGCACAGAAGGTGTG	400
	loop B	GAGGAGGACACCTGGTACGC	400

3. RESULTS

3.1 Target Mixed LAMP to Detect HBV, HCV, and HIV

When three primer sets for HBV, HCV, and HIV were added to a reaction mixture, LAMP could efficiently amplify each target with levels reaching the detection limit in approximately 24 min (Fig. 1A). Next, we tried to amplify all three of the targets in a single reaction mixture.

This target mixed LAMP experiment revealed that the reaction rates for all three targets together was the same as when a single target was amplified (Fig. 1B). To determine whether all of the target DNA had been sufficiently amplified, we performed dot blot hybridization. LAMP products amplified with 5′ DIG-labeled primers were used as probes and hybridized on to a plasmid DNA-spotted nylon membrane. We found that the probes hybridized with each DNA spot, except for the negative control (Fig. 1B, inset), demonstrating that each of the three targets was simultaneously amplified in a single reaction tube. Thus, target mixed LAMP possesses the ability to quickly amplify large quantities of genetic material with a high specificity against multiple target nucleic acids at the same time.

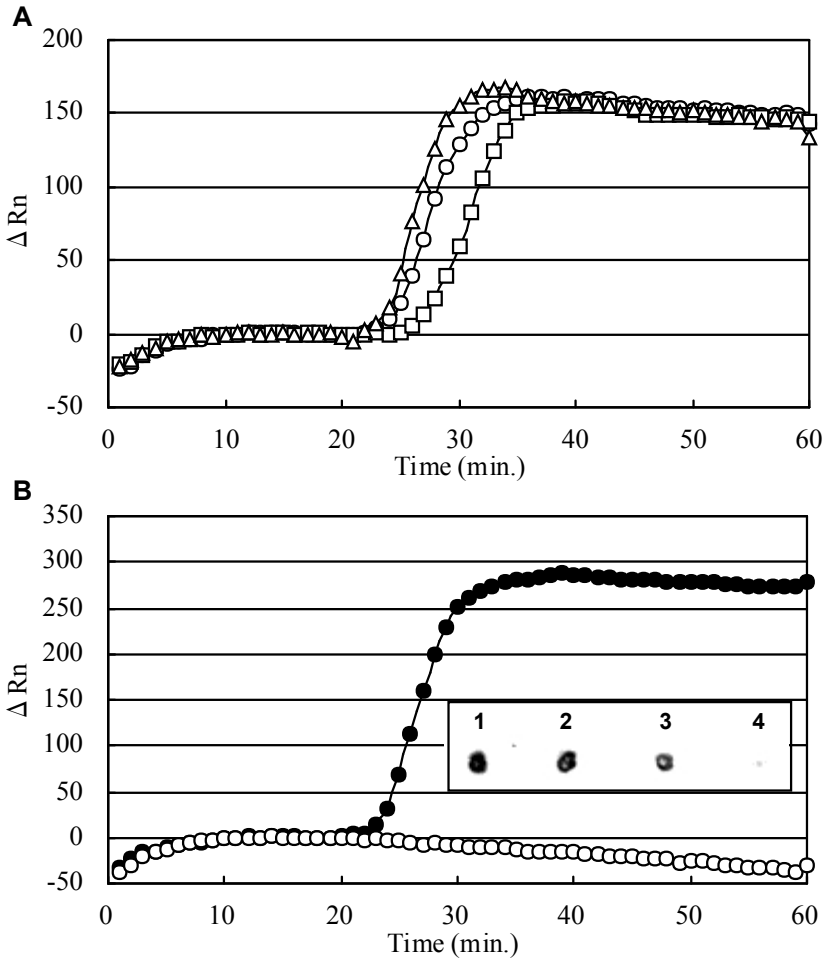

Fig. 1. Target mixed LAMP of HIV, HBV, and HCV.

A: Real-time monitoring of the LAMP amplification signals. The circle, triangle, and square show the amplification of HIV, HBV, and HCV, respectively. B: The closed circle shows the target mixed LAMP reaction using three mixed templates. The open circle represents the negative control. ∆Rn is the normalized emission at 615 nm. The inset shows the dot blot hybridization analysis. The DNA product synthesized from reactions using the 5' DIG-labeled primer was used as the hybridization probe. The numbers indicate the position of each plasmid DNA spotted on the nylon membrane. 1: HBV, 2: HCV, 3: HIV, and 4: empty pBluescript (negative control) plasmid DNA.

3.2 Detection of HBV DNA from Infectious Sera

We next examined the application of target mixed LAMP to detect HBV DNA found in infected sera. For this experiment, specific pairs of HBV primers, which recognize a conserved HB region, were used in order to detect various subtypes of the virus. Two of four sera samples could be amplified by LAMP (Table 2). In sample A, the positive and negative values for TMA on a logarithmic scale were 8.1 genome equivalents (LGE/mL) and less than 3.7 LGE/mL, the latter of which is lower than the detection limit [10]. In comparison with the results for sample A, amplification of sample B could not be detected by using TMA or PCR methods, but the target mixed LAMP was able to detect the low levels of HBV DNA indicating that the reaction efficiency of the LAMP reaction was higher than that for TMA and PCR. Moreover, the total reaction time for the PCR (1.5 h) was longer than that necessary for the LAMP reaction (1 h) in this experiment. In addition, using each HBV primer set in a uniplex LAMP reaction showed the same results as those obtained from the target mixed LAMP, suggesting that the primer mixture does not influence the individual LAMP reactions (data not shown). These results demonstrate that target mixed LAMP can detect multiple targets from infectious serum without hindering the efficiency or delaying the reaction time.

Table 2. HBV DNA amplification by TMA, PCR, or target mixed LAMP from HBsAg-positive serum

	sample A	sample B	sample C	sample D
TMA	+	-	-	-
PCR	+	-	-	-
LAMP	+	+	-	-

3.3 Target Mixed LAMP of Pooled Mouse cDNA

Four cDNAs, GAPDH, γ-actin, haptoglobin and TBP, were independently (using single primer sets) or simultaneously (using a mixture of multiple primer sets) amplified from pooled embryonic mouse cDNA (Fig. 2). When using a single primer set, amplification of GAPDH and γ-actin was detected at 20 min, while TBP and haptoglobin were detected at 25 min (Fig. 2A). The variable time of detection reflects the difference in cDNA expression levels for each gene, whereby GAPDH and γ-actin are more highly expressed than TBP and haptoglobin. These results were in agreement with the real-time PCR results (data not shown).

In the target mixed LAMP reaction using mixed primer sets, the signal was detected at 24 min (Fig. 2B). The specificity of the amplification products was confirmed by dot blot hybridization, which indicated that each primer pair efficiently amplified their target cDNA (Fig. 2B, inset). However, the final amplification levels for each cDNA could not be determined because target mixed LAMP products cannot be shown separately.

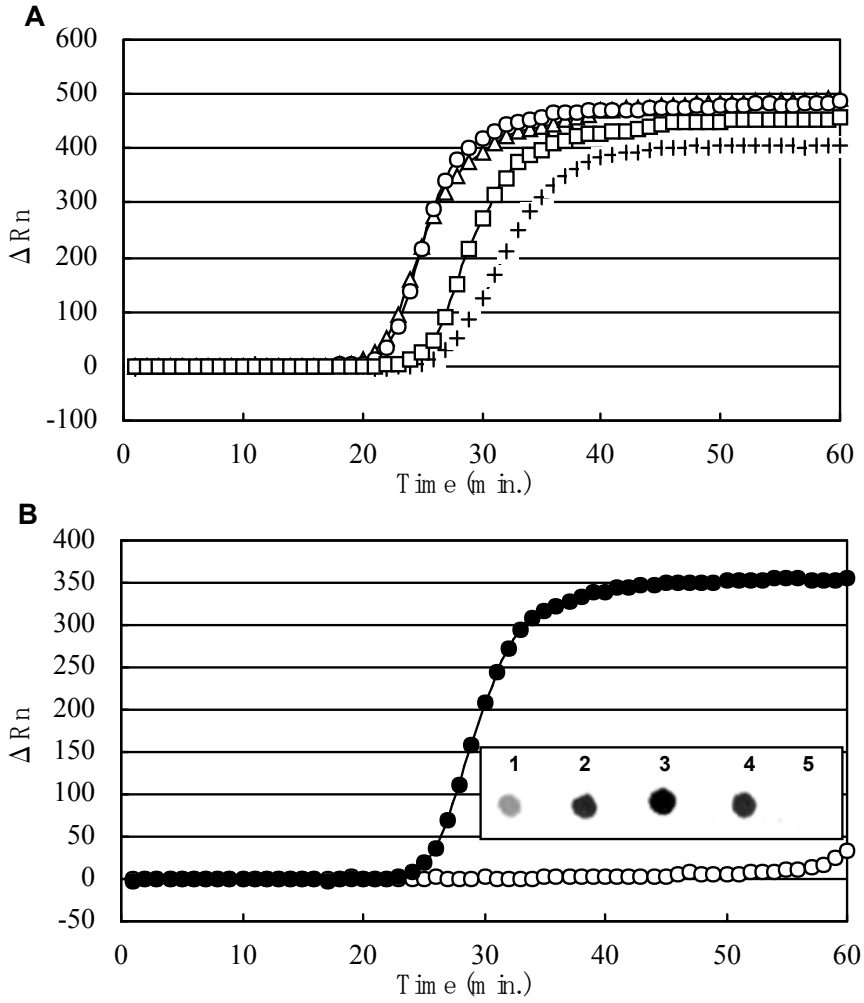

Fig. 2. Target mixed LAMP of GAPDH, γ-actin, haptoglobin, and TBP

A: Real-time monitoring of LAMP amplification signals. LAMP was performed using a single primer from pooled cDNA. The circle, triangle, plus symbol, and square show the amplification of γ-actin, GAPDH, TBP, and haptoglobin, respectively. B: Target mixed LAMP reaction. Target mixed LAMP was performed using mixed primer sets to amplify four genes from pooled cDNA. ΔRn is the normalized emission at 615 nm. The inset shows the dot blot hybridization analysis. 1: TBP, 2: GAPDH, 3: γ-actin, 4: haptoglobin, and 5: empty pBluescript (negative control) plasmid DNA.

4. DISCUSSION

In screening for HBV, HCV or HIV infections, it is important to detect whether the patients are infected with more than one of these viruses. In order to do so, it would be desirable for each set of primers to be designed for use under similar conditions. Although this synchronization of reaction rates is not absolutely necessary, doing so does help decrease the reaction time. We previously amplified HBV DNA prepared from infected sera [2]; however, amplification and detection of HCV and HIV from sera may prove to be more complicated as RNA is the primary means of detection for these viruses. Fortunately, the

LAMP reaction is also able to amplify RNA through the simple addition of reverse transcriptase [1]. In fact, reverse transcription LAMP has previously been used to detect viral RNA in clinical diagnostic testing [11-13]. Our findings suggest that target mixed LAMP can be performed using not only viral DNA but also RNA prepared from sera. In this study, there were two HBsAg-positive sera samples. Since the primer used for HBV DNA amplification from sera by LAMP can detect a minimum of six copies of DNA template [1], these two sera samples likely contained levels of HBV DNA below this detection limit. Although this study represents the first step toward using target mixed LAMP in diagnostic testing the limited number of clinical samples prevented a more thorough analysis, and we aim to increase the number of clinical samples in the future.

To perform target mixed LAMP from cDNA, the first step is to change the primer concentration in order to normalize the amplification efficiency. The LAMP reaction continues until the dNTP substrates are decayed. Divergences in sample reaction rates are thought to be caused by the preferential amplification of the more highly expressed DNA templates. Therefore, the reaction rates must be synchronized when the expression levels of multiple cDNA templates are analyzed. In our amplification procedures using cloned plasmid DNA as a template, we were able to control the plasmid copy number. Thus, 10^5 molecules of the target DNA could be amplified at the same reaction rate, resulting in all of the signals being detected at 22 min (data not shown). Our experimental data presented in Fig. 2 suggests that in 1 µL of pooled embryonic mouse cDNA, GAPDH and γ-actin were present in quantities greater than 10^5 molecules, while TBP and haptoglobin were present at levels less than 10^5 molecules. Due to the variability of gene expression in a normal cell, we question the suitability of this method for quantitative gene expression analysis, although further testing is necessary.

Iseki et al. reported the simultaneous detection of *Babesia bovis* and *Babesia bigemina* using two sets of LAMP primers [14]. Although the application of LAMP methodology has great potential, the number of primer sets seems to be a limitation of the method; in a LAMP reaction, six primers are used to amplify one target and the concentration of the primers in a single reaction tube may become too high. For use in a clinical setting, we recommend measuring at least four test items during genetic diagnosis (e.g., three test items and an internal control). Furthermore, Walker et al. reported successful multiplex SDA by amplifying a single pair of primers after appending them to the end of target fragments [7]. In relation to this, we also found that eight targets cloned into plasmids with common priming sequences could be amplified by a single pair of amplification primers in target mixed LAMP (Supplementary data). In order to succeed target mixed LAMP, it is necessary to be careful about interaction of primers, such as primer dimer, when primer sets are constructed.

The clinical detection of viruses and bacteria is currently being conducted through the use of multiplex PCR (or RT-PCR) [9]. Multiplex PCR (or RT-PCR) does, however, entail expensive machinery and the detection methods necessary also require expensive reagents, such as Taqman probes [15]. On the other hand, the LAMP methodology does not require an expensive machine as the reaction occurs at a constant temperature. Although we did not compare the sensitivity of multiplex PCR (or RT-PCR) to the LAMP method, the lower diagnostic costs associated with the LAMP method alone make this a more suitable diagnostic tool for various fields. Furthermore, the turn-around time for a diagnostic test is relatively long using traditional PCR from amplification until the result is obtained. LAMP is able to achieve a simple amplification in a short time under isothermal conditions and utilizes simple detection based on the turbidity of magnesium pyrophosphate [4,16].

Endpoint detection using a fluorescent metal indicator, such as calcein, has also been utilized in clinical practice [17].

5. CONCLUSION

In this study, we succeeded in amplifying multiple target DNA templates with more than one pair of primers using target mixed LAMP. We hypothesize that target mixed LAMP will be useful for genetic diagnosis, including the diagnosis of infectious diseases. Although we have successfully demonstrated the effectiveness of target mixed LAMP by real-time detection and dot blot hybridization, we intend to further develop the amplification detection methods to be used clinically, with particular emphasis on utilizing turbidity and calcein detections.

ACKNOWLEDGMENT

The authors thank K. Watanabe for technical support and S. Kojiya for help in preparing the manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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