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Genetics

cis-acting mutations that affect rop protein control of plasmid copy number

(regulation of DNA replication/temperature-sensitive mutant plasmid/small plasmid-encoded protein)

DAVID R. MOSER, DOREEN MA, CATHERINE D. MOSER, AND JUDITH L. CAMPBELL

Department of Chemistry, California Institute of Technology, Pasadena, CA 91125

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ABSTRACT **A number of pMB1 derivatives provide a *trans*-acting function that can suppress lethal runaway replication of a temperature-sensitive copy-number mutant of NTP1. Deletion analysis indicates that the region of the pMB1 genome that contains the *rop* gene is required for this suppression. Mutant derivatives of the temperature-sensitive copy-number mutant plasmid whose conditional lethal phenotype is not suppressed in *trans* by the region encoding the *rop* gene have been isolated. These *rop*-insensitive derivatives contain single nucleotide changes within the RNA I coding region.**

Initiation of replication of ColE1-type plasmids is regulated by two *trans*-acting negative control elements (Fig. 1) (1–5). One of these elements, RNA I, acts as a replicon-specific inhibitor of plasmid replication and is responsible for plasmid incompatibility (2, 7, 8). Purified RNA I inhibits the processing of a second plasmid transcript, RNA II, *in vitro*. This processing is necessary to form the primer for initiation of DNA synthesis. The target of RNA I inhibition is believed to lie within a region of RNA II that is complementary to RNA I (7, 9, 10). Both RNA I and its complementary region on RNA II may adopt a secondary structure with three stem-loop structures. Genetic studies indicate that the single-strand loops are involved in the inhibitory activity of RNA I, which occurs by base-pairing with the complementary sequence of RNA II (7, 10). The hybridization of the two transcripts prevents the formation of an RNA·DNA hybrid structure between RNA II and its template near the replication origin (2). The RNA·DNA hybrid is a substrate for ribonuclease H, which cleaves the transcript at the origin of replication to create the 3′-OH end of the primer (11).

The second element in the copy control system is the product of a gene located 500 base pairs (bp) downstream from the replication origin of ColE1 and pMB1 (3–5). Twigg and Sherratt (3) observed that deletions of this region of ColE1 and pMB1 derivatives caused an increase in the plasmid copy numbers. They also showed that the elevated copy numbers could be reduced to wild-type levels by providing the deleted function in *trans* from a second compatible plasmid in the cell. Cesareni *et al.* (4) reported that *in vivo* expression of the *lacZ* gene directed by the primer promoter was reduced in the presence of a plasmid carrying this "repressor." They proposed that the repressor is a 63 amino acid polypeptide that is conserved in both ColE1 and pMB1. They speculated that this repressor, which they designated rop (for repressor of primer), acted independently of RNA I to regulate plasmid replication by limiting transcription initiation of RNA II, the primer precursor.

We have observed that a ts runaway replication mutant plasmid fails to express its conditional lethal phenotype in the presence of certain compatible plasmids. Genetic evidence suggests that this suppression is mediated by the *rop* gene encoded on the second plasmid. We have taken advantage of this activity of rop to select mutant derivatives of the ts plasmid that are insensitive to rop suppression. DNA sequencing of a number of these rop-insensitive derivatives provides information about the mechanism of rop-mediated regulation of plasmid replication.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. All of the transformation and plasmid studies were carried out in *Escherichia coli* HB101 *pro gal hsdR hsdM recA1*. Plasmid pJN75 is a 5.4-kilobase ampicillin-resistant derivative of NTP1 (12, 13). pJN75 exhibits a ts mutant copy-number phenotype. The presence of the plasmid is lethal to the host cell at the restrictive temperature, 37°C. The lethality is a result of runaway plasmid replication. A single nucleotide difference from the wild-type NTP1 sequence within the region required for replication appears to be responsible for the ts mutant phenotype (12). This mutation is a G·C to A·T transition located 398 bp upstream of the origin of DNA synthesis. Plasmid pMB9 is a tetracycline-resistant derivative of pMB1. Other plasmids are described in Tables 1 and 2.

Isolation of pJN75 Mutants Whose ts Runaway Replication Phenotype Is Not Suppressed by the *rop* Gene Product. Twenty cultures of *E. coli* HB101 containing pJN75 were grown at 30°C to an A_{590} of 0.3 in 5 ml of L broth. Chloramphenicol (100 μg/ml) and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (20 μg/ml) were added and incubation was continued overnight. Plasmid DNA was isolated from each of the cultures used to transform individual cultures of *E. coli* HB101 containing pMB9 by the procedure of Dagert and Ehrlich (17). Cells were spread onto L plates containing tetracycline (15 μg/ml) and a high concentration of ampicillin (4 mg/ml) and incubated at 34°C overnight. Under these conditions, cells containing pJN75, which is at high copy number, survive. However, cells containing both pJN75 and pMB9 do not form colonies under these conditions, presumably because *rop* suppression of pJN75 replication reduces the copy number of pJN75 to a level below what is needed to confer resistance to 4 mg of ampicillin per ml. Mutants of pJN75 that are insensitive to the *rop* suppression are able to grow in the presence of pMB9 at 34°C on 4 mg of ampicillin per ml. Several colonies grew up from each of the 20 transformations carried out with mutagenized DNA, whereas no colonies were obtained when nonmutagenized DNA was used. Colonies from each of the 20 plates were then tested for expression of the ts lethal phenotype by replica-plating colonies onto two sets of plates containing tetracycline (15 μg/ml) and a low concentration of ampicillin (50 μg/ml) and growing one set at 30°C and the other set at 42°C overnight. Approximately 25% of the colonies grew at 30°C but did not grow at the higher temperature. Plasmid DNA was purified from seven ts colonies, each derived from a separate mutagenized DNA to insure independence of mutants examined.

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Abbreviations: ts, temperature sensitive; bp, base pair(s).

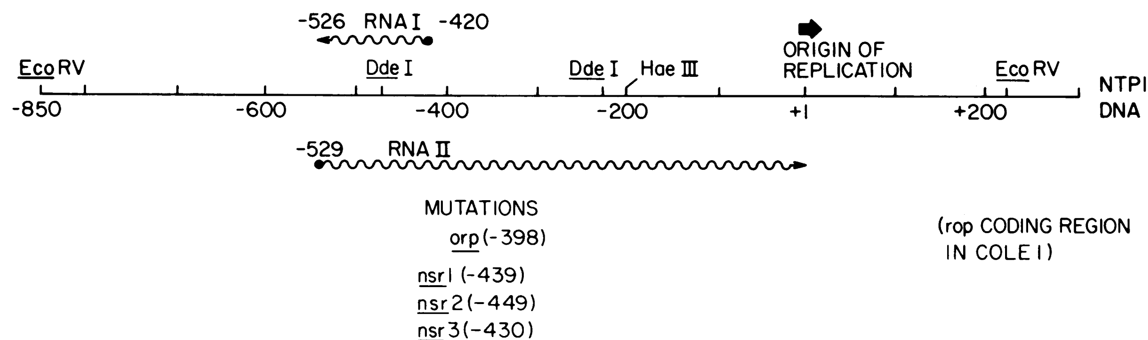

FIG. 1. DNA replication origin of plasmid NTP1 (RSF1030). The origin region of NTP1 has a sequence identical to that of RSF1030 (6). The locations of the coding region for RNA I and RNA II are shown relative to the site of initiation of DNA synthesis. This plasmid does not contain a *rop* gene. The original temperature-sensitive (ts) mutation (*orp*) maps at −398, within the sequence for RNA II, whereas the mutations identified in this study map at −430, −439, and −449.

DNA Sequence Analysis. The nucleotide sequence surrounding the replication origin of pJN75 has been reported (13, 14). The sequence of the origin region of seven pJN75 mutants was determined by the chemical degradation method of Maxam and Gilbert (21). Approximately 800 nucleotides were sequenced, encompassing a region extending from 700 nucleotides upstream of the origin of DNA synthesis to 100 nucleotides downstream of the origin. Some of the sites used for sequencing are indicated in Fig. 1.

RESULTS

Suppression of pJN75 Runaway Replication by the *rop* Gene Product. Plasmid pJN75 is a ts copy-number mutant derivative of plasmid NTP1. (12, 13). [Although isolated independently, NTP1 is apparently identical to plasmid RSF1030 (6, 14, 15).] Bacteria containing pJN75 grow normally at the permissive temperature (30°C) but cease growth and die one or two generations after a shift to the nonpermissive temperature (37°C). Loss of viability appears to be a consequence of runaway replication, which results in an increase in plasmid synthesis from about 8% of chromosomal DNA synthesis to 150% in the first 10 min after the temperature shift (13).

We have previously identified the mutation in pJN75 responsible for the ts mutant replication phenotype (12). This mutation is a single base pair change, a G·C to A·T transition, located 398 bp upstream of the origin of DNA synthesis (see Fig. 1). The temperature-induced over-replication phenotype is most likely the consequence of thermolability of a secondary structure within the primer precursor that is normally essential for regulation, though this has not been shown conclusively (12, 22). The inability of this structure to form at the restrictive temperature may render replication of the plasmid insensitive to normal regulation through RNA I inhibition.

We have observed that runaway replication of pJN75 at the nonpermissive temperature is suppressed in the presence of certain compatible plasmids. This suppression was first observed in double transformants of pJN75 and pMB9, a tetracycline-resistant derivative of plasmid pMB1. The double transformants grew normally at 42°C as well as at 30°C in the presence of both ampicillin and tetracycline. Plasmid DNA levels were analyzed from lysates of cells grown in liquid cultures before and several hours after a shift to the restrictive temperature. Although this analysis was somewhat complicated by the similarity in molecular weights of pMB9 and pJN75, we were still able to observe that with pMB9 present in the cell, there was very little temperature-induced amplification of pJN75 DNA (data not shown). Suppression of the conditional lethal phenotype of pJN75 was also observed with the pMB1 derivatives, pBR322 and pBR325 (Table 2). The electrophoretogram shown in Fig. 2 illustrates the effect of a plasmid derived from pBR325 on the temperature-induced amplification of pJN75 DNA.

NTP1 is compatible with pMB1-derived plasmids—i.e., NTP1 and pMB1 derivatives can stably coexist in the same cell. This is apparently a consequence of the fact that the RNA I species encoded by NTP1 is not identical to the pMB1 RNA I species. *In vitro* experiments described by Tomizawa and Itoh (7) indicate that purified NTP1 (RSF1030) RNA I does not inhibit primer formation of a ColE1 template

Table 1. Major plasmids used in these studies

Plasmid	Characteristic	Compatibility	Drug resistance	Refs.
pJN70	Wild type	RSF1030	Ampicillin	6, 14, 15
pJN75	ts	RSF1030	Ampicillin	12, 13
pMB9	rop$^+$	pMB1	Tetracycline	16

Other plasmids used are found in Table 2. *E. coli* HB101 *pro gal hsdR hsdM recA1* (1) was used for all of the transformation and plasmid studies.

Table 2. *trans*-suppression of pJN75 runaway replication by various compatible plasmids

Plasmid	Incompatibility class	Presence of *rop* gene	Suppression of ts lethal phenotype of pJN75-containing cells
pMB9	pMB1	+	+
pBR322	pMB1	+	+
pBR325	pMB1	+	+
pAT153	pMB1	−	−
pKO1	pMB1	−	−
pUC8	pMB1	−	−
pACYC184	p15A	(−)?	−
pDM254	p15A, pMB1	(−)?	−

E. coli HB101 cells containing pJN75 were transformed with the indicated plasmids following the transformation procedure of Dagert and Ehrlich (17). Cells were spread onto L plates containing ampicillin (50 μg/ml), which were then incubated overnight at 42°C. Plasmids that produced temperature-resistant colonies with a high transformation efficiency (>10^6 per μg of plasmid DNA) are indicated by a + sign. A − sign indicates those plasmids that did not give temperature-resistant colonies. Plasmids pMB9, pBR322, and pBR325 are all pMB1 derivatives that contain the region of the plasmid genome encoding the *rop* gene. Plasmid pAT153 (3) is identical to pBR322 except for deletion of a 622-bp *Hae* II restriction fragment that contains the entire *rop* gene (4). Plasmids pKO1 (18) and pUC8 (19) are also derived from pBR322 and contain only the portion of the *rop* gene that codes for the last 9 amino acids of the 63 amino acid polypeptide. Plasmid pACYC184 contains the replication origin from p15A (20). There is no evidence of a *rop* gene encoded on pACYC184 or p15A. Plasmid pDM254 (8) is a pACYC184 derivative with four copies of the pMB1-RNA I gene cloned in tandem at the *Bam*HI site.

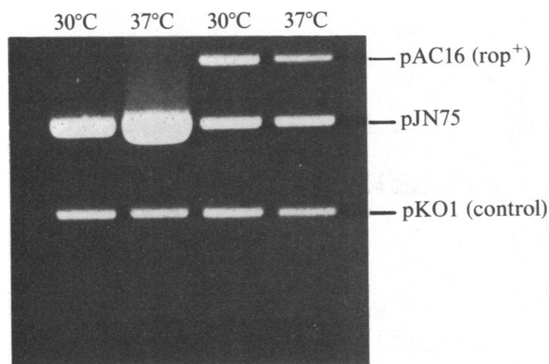

FIG. 2. Suppression of temperature-induced amplification of pJN75 DNA by a pMB1 derivative carrying the *rop* gene. HB101 cells carrying either pJN75 (ampicillin resistant) or pJN75 and plasmid pAC16 (tetracycline resistant, chloramphenicol resistant) were grown in L broth containing the appropriate antibiotics at 30°C to an A_{590} of 0.15. Half of each culture was then transferred to 37°C and all cultures were incubated for an additional 3 hr. A portion of each culture was then mixed with a portion of another cell culture containing an equal number of HB101 cells carrying plasmid pKO1 (18). The pKO1-containing cells were added immediately prior to cell harvest to provide a control plasmid for standardizing plasmid DNA recoveries. After harvesting by centrifugation, the cells were lysed and total plasmid DNA was isolated as described (8). The covalently closed circular plasmids were analyzed by electrophoresis through a 1.0% agarose gel. Plasmid pAC16 (23) is a pBR325 derivative that contains a 700-bp rat actin cDNA insert cloned into the *Pst* I site and therefore does not confer ampicillin resistance to its host.

and that ColE1 RNA I does not inhibit NTP1 primer formation. Since there is apparently no cross-inhibition of plasmid replication by the two wild-type RNA I species, we speculated that a negative control element other than RNA I was responsible for the suppression of pJN75 replication by the pMB1 derivatives. The inability of plasmid pDM254 (8), which contains multiple pMB1 RNA I genes, to suppress pJN75 runaway replication supports this notion (Table 2). Since the pMB1 derivatives that provide suppressor activity also encode the *rop* gene described by Cesareni *et al.* (4), the *rop* gene product seemed a likely candidate for the *trans*-acting suppressor. Therefore, we looked at the effect of cocultivation of pJN75 with several pMB1-derived plasmids that contained deletions of all or part of the *rop* gene (Table 2). No suppression was observed with the pBR322 derivative pAT153, pKO1, or pUC8. Plasmid pAT153 differs from pBR322 by a deletion of the 622-bp *Hae* II restriction fragment that encodes the entire *rop* gene. Plasmids pKO1 and pUC8 contain a deletion of the 3′ end of the *rop* gene. These results imply that the *rop* gene product is responsible for the suppression of the runaway replication phenotype of pJN75.

Isolation of Mutant Derivatives of pJN75 Insensitive to *rop* Suppression. These observations suggested a way to identify the site where *rop* interacts in suppressing pJN75 runaway replication—namely, by isolating mutants of pJN75 that are insensitive to *rop*-mediated suppression. The rationale for selecting such mutants depends on the idea that, at temperatures intermediate between permissive (30°C) and nonpermissive (37°C), the copy number of the mutant plasmid should be elevated but not lethal to the cells. To test this idea, cells carrying pJN70 or pJN75 were plated at 34°C on L agar plates containing a high concentration of ampicillin (4 mg/ml). As expected, cells carrying pJN75 were able to grow because the copy number was high, whereas cells carrying the wild-type plasmid did not produce enough β-lactamase to survive. Furthermore, cells carrying pJN75 plus pMB9 did not form colonies under these conditions, because *rop*-mediated suppression of pJN75 replication reduces the copy number to a level below what is required to confer resistance to ampicillin at 4 mg/ml (Table 2). To isolate a *rop*-insensitive plasmid, mutagenized pJN75 DNA was used to transform cells that contained pMB9 at 34°C. Only pJN75 derivatives insensitive to replication inhibition by *rop* function were expected to grow. Several such colonies were isolated and shown to retain the ts lethal phenotype at 42°C, indicating that the original mutation in pJN75 was still present. Seven of the colonies able to grow at 34°C but not at 42°C were selected for further characterization. Analysis of the DNA content of these cells revealed that both plasmids (pMB9 and the pJN75 derivative) were present as monomers, suggesting the phenotype was due to a mutation of the pJN75 genome. The plasmids exhibiting the non-suppressed phenotype in the presence of *rop* were designated pJN75*nsr* for nonsuppressible by *rop*.

Mapping of the *nsr* Mutations. The nucleotide sequence surrounding the replication origin of the *nsr* derivatives of pJN75 was determined following the procedure of Maxam and Gilbert (21). Each of the mutant plasmids was found to contain the original pJN75 alteration and a single additional base pair change within the 800-bp region extending from 100 bp downstream of the origin of DNA synthesis to 700 bp upstream of the origin. One of the mutants contained a G·C to A·T transition at position −439 (*nsr1*). (Position +1 is defined as the site of incorporation of the first deoxynucleotide during the initiation of plasmid DNA replication.) Five other mutants contained a G·C to A·T transition 10 bp away at position −449 (*nsr2*). An additional mutant appeared at −430 (*nsr3*). All of these mutations cause disruption of base-pairing within the stem of loop-structure III in the primer transcript and loop-structure III′ of RNA I (Fig. 3). This result is consistent with the *galK* fusion studies of Som and Tomizawa (5), which indicate that sequences important for rop function do not fall in the promoter for RNA II, but instead lie further downstream. The mutations we have identified define this region to be within the coding sequnce for both RNA I and RNA II, near the 5′ terminus of RNA I.

DISCUSSION

In this paper we have shown that the *rop* gene provides a *trans*-acting function that can suppress runaway replication of the NTP1 ts copy-number mutant pJN75. This conclusion is based on the observation that plasmids with deletions of part or all of the *rop* gene lack the suppressor activity. This interpretation is also supported by previous reports that the *rop* gene acts in *trans* to lower plasmid copy number (3, 5). We have used the ability of the *rop* gene product to suppress the ts lethal phenotype of the runaway replication mutant pJN75 to further investigate the general mechanism by which *rop* modulates plasmid copy number. Although the molecular details of this inhibition are not yet understood, one model has been that the *rop* gene product may be inhibiting transcription of RNA II and therefore controlling replication by limiting the amount of precursor RNA available for primer formation. This idea is supported by the observations of Cesareni *et al.* (4) and Som and Tomizawa (5) that the *rop* gene product inhibits production of β-galactosidase or galactokinase when the *lacZ* or *galK* genes are fused to the primer downstream of the primer promoter.

To investigate how such inhibition might occur, we looked for mutants resistant to inhibition by the *rop* gene product as described above. We have identified mutants of pJN75 designated *nsr*, which express the lethal runaway replication phenotype at 42°C even in the presence of the *rop* gene encoded on a second plasmid in the cell. The nucleotide sequence of seven *nsr* plasmids revealed that none of the mutations falls within the promoter for RNA II, as might have been expected from the model just described. The mutations disrupt base pairs within stem-loop structure III (see Fig. 3)

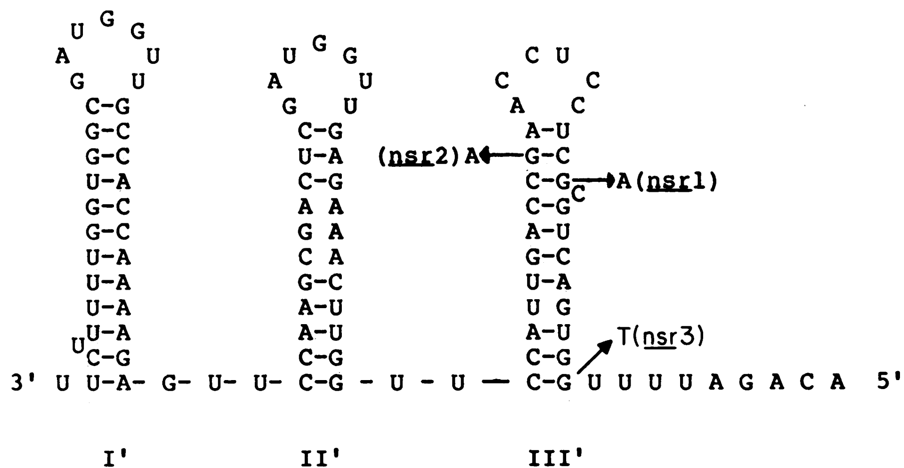

FIG. 3. RNA I transcript from plasmid pJN75. Mutations *nsr1*, *nsr2*, and *nrs3* are those identified within the mutant pJN75 derivatives that were selected by their ts phenotypes in the presence of pMB9. The RNA I secondary structure shown is that proposed by Som and Tomizawa (6) based on the secondary structure of ColE1 RNA I proposed by Morita and Oka (24).

present in both RNA I and RNA II. It is perhaps worth noting that the two nucleotide pairs affected by the mutations *nsr1* and *nsr2* are conserved in the RNA I species from ColE1, p15A, RSF1030, and CloDF13 (25). In addition, on the same stem structure immediately adjacent to these paired bases is an unpaired nucleotide that is also conserved in these plasmids. It is imaginable that this "spur" on the stem could be a part of a recognition site for the binding of a small protein. Our findings, along with those of Som and Tomizawa (5), that a region within the coding sequence of RNA II, rather than in the primer promoter, was necessary for inhibition suggests that the rop protein does not act by binding to an operator site near or within the promoter and competing with RNA polymerase binding. This is also consistent with the fact that the rop protein from pMB1 can inhibit replication of plasmids such as NTP1, which have virtually no sequence homology to pMB1 in the DNA immediately 5′ to the primer RNA, in the primer promoter.

Som and Tomizawa (5) have recently suggested that the rop protein enhances hybrid formation between RNA I and its complementary region of RNA II. Our results are consistent with, though by no means prove, this interpretation, in that all of the *nsr* mutations fall in the region of RNA I and RNA II overlap. Thus, one possibility is that the rop protein may recognize one or more of the stem-loop structures on either RNA I or RNA II and bind them. Binding of the rop protein might then favor the interaction of RNA I and its target region of RNA II.

However, there is one important caveat in interpreting the *nsr* mutations as identifying a rop–nucleic acid interaction site. Analysis of the *nsr* mutations is complicated by the fact that the *rop*-insensitive mutations described here may also alter the structure of RNA I or RNA II such that the inherent ability of these RNAs to interact with each other is diminished. In other words, an altered copy-number phenotype associated with a mutation in the RNA I coding region can be a consequence of either a defective (unstable) RNA I inhibitor, an altered RNA I target site within the primer transcript, increased RNA II transcription, or the inability of the rop protein to interact with either of the mutant transcripts. It is quite likely that all of these functions could be affected by a single point mutation. The mutation *nsr1* that we have identified affects a nucleotide at the same position in pJN75 RNA I as the *svir19* mutation of pMB1 described by Lacatena and Cesareni (10). The latter mutation was obtained by using a "phasmid" selection designed to isolate mutants exhibiting reduced sensitivity to the wild-type replication inhibitor RNA I. When released from the λ chromosome, the mutant plasmid containing the *svir19* mutation was lethal for the host cell. We have also found that *nsr1* and *nsr2* mutants are lethal to the host cell in the absence of pMB9 or other compatible rop$^+$ plasmids (data not shown). This lethality is independent of temperature and is assumed to result from uncontrolled or runaway plasmid replication, although this has not been demonstrated either for *svir19* or for the *nsr* mutants. Thus, *nsr1* and *nsr2* appear to retain at least some sensitivity to rop protein. These results are consistent with the possibility that the mutations *nsr1* and *nsr2* in NTP1 and *svir19* in pMB1 affect both the RNA I–RNA II interaction and the rop protein function. The rop protein may enhance the hybridization of the two RNAs as proposed by Som and Tomizawa (5). However, the possibility that rop may affect transcription or stability of RNA II or stability of RNA I is not ruled out by our data.

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