Proc. Nat. Acad. Sci. USA
Vol. 72, No. 4, pp. 1226–1230, April 1975

Genetic Evidence for an Additional Function of Phage T4 Gene 32 Protein: Interaction with Ligase

(DNA replication/"joint" molecules/"recombinant" molecules/sedimentation/Cs_2SO_4 gradients)

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Communicated by A. D. Hershey, December 11, 1974

ABSTRACT **Gene 32 of bacteriophage T4 is essential for DNA replication, recombination, and repair. In an attempt to clarify the role of the corresponding gene product, we have looked for mutations that specifically inactivate one but not all of its functions and for compensating suppressor mutations in other genes. Here we describe a gene 32 *ts* mutant that does not produce progeny, but in contrast to an *am* mutant investigated by others, is capable of some primary and secondary DNA replication and of forming "joint" recombinational intermediates after infection of *Escherichia coli* B at the restrictive temperature. However, parental and progeny DNA strands are not ligated to covalently linked "recombinant" molecules, and single strands of vegetative DNA do not exceed unit length. Progeny production as well as capacity for covalent linkage in this gene 32 *ts* mutant are partially restored by additional *r*II mutations. Suppression by *r*II depends on functioning host ligase [EC 6.5.1.2; poly(deoxyribonucleotide):poly(deoxyribonucleotide) ligase (AMP-forming, NMN-forming)]. This gene 32 *ts* mutation (unlike some others) in turn suppresses the characteristic plaque morphology of *r*II mutants. We conclude that gene 32 protein, in addition to its role in DNA replication and in the formation of "joint" recombinational intermediates, interacts with T4 ligase [EC 6.5.1.1; poly(deoxyribonucleotide):poly(deoxyribonucleotide) ligase (AMP-forming)] when recombining DNA strands are covalently linked. The protein of the mutant that we describe here is mainly defective in this interaction, thus inactivating T4 ligase in recombination. Suppressing *r*II mutations facilitate substitution of host ligase. There is suggestive evidence that these interactions occur at the membrane.**

It is evident that proteins involved in DNA metabolism function as complexes (1). Direct analysis of functions and interactions of proteins in such complexes is often difficult, especially when a protein by itself has no enzymatic activity and when it participates in several complexes performing different functions. One approach to defining biological interactions is to look for mutations in a gene that inactivate some but not all functions of the gene product and, in addition, to search for compensating suppressor mutations in other genes, thereby determining which other gene products are involved. In general, one expects such suppression to be mutation-specific if the corresponding gene products interact directly, but to be gene-specific when the gene products influence each other indirectly.

We have used this genetic approach to investigate different functions of the gene 32 protein of phage T4. This protein is essential for DNA replication, recombination, and repair (1–8). It binds cooperatively to single-stranded DNA; it affects denaturation and renaturation of DNA; and it binds to and stimulates T4 DNA polymerase (6, 9–11). It is produced in large amounts after T4 infection and is limiting for phage production (12). This could mean that (*i*) DNA replication, recombination, and repair are interdependent, and although gene 32 protein is directly involved in only one of these processes, it indirectly affects the other two; (*ii*) the protein is directly involved in all three processes and serves the same function in each; or (*iii*) the protein serves a direct but different function in each of the three processes.

We have found that different gene 32 *ts* mutants have quite different phenotypes, and that at least three, L171 being one of them, are partially suppressed by *r*II mutations (ref. 13; Mosig, Bock, and Berquist, manuscript in preparation). Here we show that the L171 mutation affects the function of gene 32 protein mainly in ligation of recombining DNA molecules, but little in early DNA replication. Suppressing *r*II mutations allow ligation of recombining DNA strands (and consequently progeny production) by facilitating the use of host ligase without significantly affecting DNA replication. Thus, we conclude that gene 32 protein participates in a different manner in the complexes involved in DNA replication and in covalently linking recombinational intermediates.

MATERIALS AND METHODS

Bacteria. Escherichia coli B, S/6, the nonlysogenic K strain DG 75 (14), obtained from J. Wechsler (permissive for *r*II mutants, restrictive for *am* mutants), CR 63 (permissive for *am* and *r*II mutants), and K(λh) (restrictive for *r*II mutants) have been maintained in this laboratory. The ligase-deficient host N 2668 *lig ts* 7 (15) was obtained from M. Gellert.

Phage. All gene 32 mutants (from R. S. Edgar) and *r*II point mutants have been described (13). The *r*II deletion *r*1272 in the genetic background of T4D (16) came from T. Homyk. Multiple mutants were constructed by us. Phage were grown and assayed by standard procedures (17).

Density Shift Experiments were done as described (18, 19). Sedimentation was measured in neutral or alkaline 5–20% sucrose gradients (20, 21).

RESULTS

Different *r*II A or *r*II B point mutations partially restored viable progeny production of L171 to a similar extent. The deletion *r* 1272 was a slightly stronger suppressor, but since it lacks D1 and part of *den* B (endonuclease IV), in addition to the *r*II region (22), we describe below mainly the experiments involving the *r*II A point mutation *r*71.

Suppressed and Unsuppressed L171 Mutants Show Similar Replication Patterns. We compared DNA replication and

TABLE 1. *Phage yields and DNA replication at 42°*

Phage	Single infection: Average burst size[a]	Single infection: *E. coli* B Parental DNA replicated (%)	Single infection: Relative ^3H incorporation[b]	Multiple infection: Average burst size[a]	Multiple infection: *E. coli* B Parental DNA replicated (%)	Multiple infection: Relative ^3H incorporation[b]	*lig*⁻ Average burst size[a]
L171	<0.1	30	1.0	0.3	16 min:32	1.0	<0.1
					30 min:44	2.0	
L171*r*71	1.5	42	1.1	7	16 min:57	1.1	0.2
					30 min:65	1.9	
*r*71	154	64	14.8	134	16 min:61	3.0	680
					30 min:60	11.3	

[a] No. of progeny per infected bacterium (4 repeated experiments).

[b] To correct for potential differences in the number of parental DNA templates and for losses during sample preparations, we normalized the ^3H incorporation into phage DNA to the amount of parental [^{32}P]DNA that had replicated. (Parental chromosomes of the three phage strains and infected bacteria were labeled with the same specific activities.) We set this ratio in L171 as unity, and expressed all other ratios as multiples of it. Unity represents 48,000 ^3H counts/1600 ^{32}P counts in single infections 30 min after infection and 134,000 ^3H counts/7000 ^{32}P counts in multiple infections 16 min after infection. We compared relative ^3H incorporation separately for single and multiple infections since, in our experience, primary DNA replication, particularly after single infection, uses mostly precursors derived from bacterial DNA.

At permissive temperature (25°) L171 replicated 54%, L171*r*71 55%, and *r*71 65% of parental [^{32}P]DNA after single infection of *E. coli* B.

molecular recombination between parental and progeny strands of L171, L171*r*71, and *r*71 phage after multiple infection of *E. coli* B bacteria. Light bacteria were separately but simultaneously infected with ^{13}C,^{15}N,^{32}P-labeled particles of each of the three strains, incubated at 42° in the presence of [^3H]thymidine, and lysed at different times after infection to monitor the fate of parental and progeny DNA.

In all three phage strains, a large proportion of the parental DNA became fast sedimenting (Fig. 1), as previously shown for T4 DNA (24–27).

We measured the proportion of total parental DNA that had replicated as the proportion of parental ^{32}P that banded at lighter than parental density in neutral Cs_2SO_4 gradients. The DNA was sheared to separate replicated chromosomal segments from unreplicated segments. Fig. 2A–C shows representative gradients. The arrows indicate the positions (in order of decreasing densities) of (*a*) heavy parental phage DNA (labeled with ^{32}P), (*b*) hybrid phage DNA (containing ^{32}P and ^3H), (*c*) light phage DNA (labeled with ^3H), and (*d*) bacterial DNA (labeled with ^3H). The last of these can be used as a reference density marker. Some [^{32}P]DNA banded at lighter than hybrid density (because of recombination, discussed below), but this did not interfere with our analyses.

To determine whether the parental chromosomes had replicated partially or completely, we compared the banding of sheared and unsheared DNA of the same lysates in neutral Cs_2SO_4 gradients. If some chromosomes were only partially replicated and unbroken, shearing the DNA should decrease the proportion of parental ^{32}P found at lighter than parental density. Such a comparison indicated that most chromosomes that initiated must have replicated their entire length, because the proportions of shifted ^{32}P in sheared and unsheared 30-min lysates were similar. (In the L171 lysates there may have been a slight difference, but it did not exceed 6% of the total ^{32}P.) Table 1 summarizes the proportion of parental DNA that had replicated (column 6) and the ^3H incorporation into progeny DNA (column 7) at different times after infection (measured in density gradients). Clearly, both the

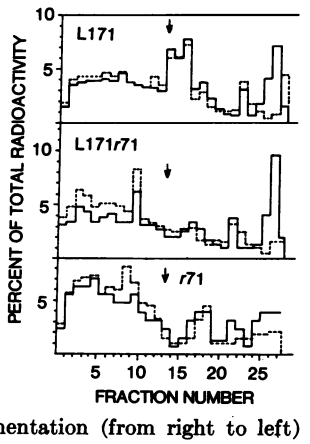

FIG. 1. Sedimentation (from right to left) through neutral sucrose gradients of DNA from multiply infected *E. coli* B bacteria. The position of ^3H-labeled reference DNA from normal T4 particles (*arrows*) was determined in a different tube of the same centrifugation. *Solid curve*, ^{32}P; *broken curve*, ^3H.

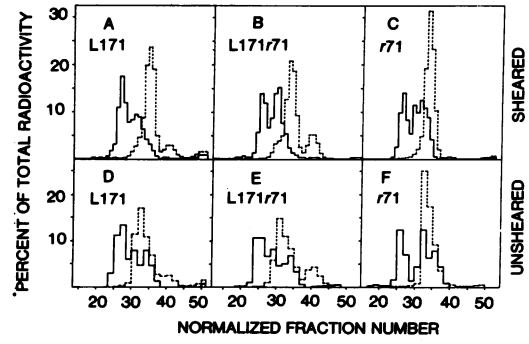

FIG. 2. Density distribution in neutral Cs_2SO_4 gradients of sheared (A–C) and unsheared (D–F) L171, L171*r*71, or *r*71 DNA, extracted from multiply infected (6, 4, or 7 particles per bacterium, respectively) *E. coli* B, incubated at 42° in the presence of 20 μCi of [*methyl*-^3H]thymidine (Schwarz) per ml. We confirmed by DNA·DNA hybridization (30) that the major ^3H band in panels A and B contained only phage DNA and the (minor) lightest band contained only bacterial DNA. *Solid curve*, ^{32}P; *broken curve*, ^3H.

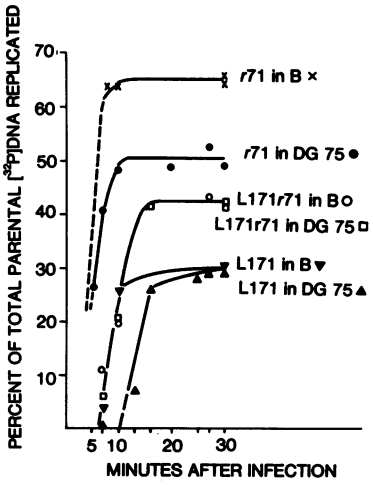

FIG. 3. Time course of primary replication of parental DNA after single infection at 42°. Each point was determined from a Cs_2SO_4 gradient of a sheared lysate by measuring the proportion of ^{32}P that had shifted its density. The points for each mutant in each host came from two independent experiments in which ^3H label was given as thymine (25 μCi/ml) or thymidine (33 μCi/ml).

suppressed and unsuppressed L171 mutants showed extensive primary and some secondary DNA replication. The extent of secondary replication was, in fact, similar in both strains (although it was considerably lower than in *r*71). It should be recalled that suppressed L171 mutants produced progeny but the unsuppressed mutant produced few or none (Table 1). By contrast, only a slightly larger proportion of the parental chromosomes replicated in the suppressed than in the unsuppressed mutant; this alone could not account for the difference in viable phage production.

Suppressed and Unsuppressed Mutants Are Somewhat Deficient in Initiation. We also measured density shift of parental DNA of each of the three strains after single infection in the presence of [^3H]thymidine or [^3H]thymine. Fig. 3 (time course of primary replication determined from Cs_2SO_4 gradients of sheared lysates) and Table 1, column 4, show that again, as after multiple infection, suppressed and unsuppressed L171 mutants produced similar, limited amounts of progeny DNA per replicated template. Surprisingly, however, they replicated less of their parental DNA and gave smaller burst sizes than after multiple infection (compare columns 3 with 6 and 2 with 5 in Table 1). This is in contrast to *r*71 as well as wild-type T4 (unpublished), which after single infection replicated larger proportions of their parental DNA than after multiple infection and gave similar burst sizes.

Suppressed and Unsuppressed Mutants Can Form "Joint" Molecules. There are three lines of evidence that both in the suppressed and in the unsuppressed L171 mutants, some parental DNA associated with progeny DNA to form "joint" recombinational intermediates.

(*i*) Much of the parental ^{32}P label was associated with light progeny DNA after multiple infection (Fig. 2), but little (L171*r*71) or none (L171) after single infection (data in ref. 13, Fig. 3).

(*ii*) Both suppressed and unsuppressed L171 replicated a larger proportion of their parental DNA after multiple than after single infection (Table 1). Enhanced primary replication after multiple infection is most readily explained if both L171 and L171*r*71 were partially deficient in initiation and were

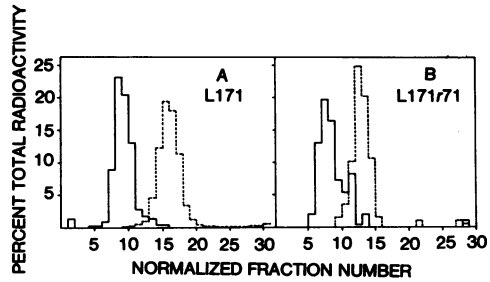

FIG. 4. Separation in alkaline Cs_2SO_4 gradients of replicated phage DNA into ^{13}C,^{15}N,^{32}P-labeled parental (*solid curve*) and light ^3H-labeled progeny strands (*broken curve*). (A) L171 (pooled fractions 30–39 from gradient D, Fig. 2); (B) L171*r*71 (pooled fractions 29–39 from gradient E, Fig. 2). Pooled fractions from the 16-min lysate of L171, from the 30-min lysate of L171*r*71, or from DG75 infections gave similar profiles to those shown here.

capable, after multiple but not after single infection, of attaching nonreplicating chromosomes to replicating ones, thus facilitating their replication.

(*iii*) The sedimentation patterns of suppressed and unsuppressed L171 and of *r*71 vegetative DNA were similar (Fig. 1), but in both cases the DNA sedimented faster after multiple than after single infection (data for the latter not shown).

Suppressed Mutant Forms Covalently Linked "Recombinant" Molecules But Unsuppressed Mutant Does Not. To determine the nature of the association between parental and progeny DNA strands, we pooled the replicated [^{32}P]DNA from gradients of unsheared lysates and recentrifuged them in alkaline Cs_2SO_4 gradients. Such gradients (Fig. 4 shows a representative example) revealed very little covalent linkage between parental and progeny strands in L171 DNA, but considerable covalent linkage in L171*r*71 DNA. We conclude that the association between parental and progeny strands seen in neutral gradients represents "joint molecules" in L171 DNA, but at least some "recombinant" molecules in L171*r*71 DNA. It appears that this difference in the potential for ligation of parental and progeny strands can account for most of the difference in phage production between the two strains. This interpretation is further strengthened by the results of alkaline sucrose gradients (Fig. 5). Some progeny DNA strands of the *r*II-suppressed L171 mutant became slightly longer than unit length, while strands of the unsuppressed L171 mutant remained shorter. Note that the parental strands of all strains showed similar sedimentation profiles, indicating that the suppressing *r*II mutations did not significantly affect nicking.

Suppression Depends on the Host Ligase. The results described above suggested that the gene 32 mutant L171 is unable to ligate recombining DNA strands. L171 does not appear to contain an additional ligase mutation nor to critically decrease the production of T4 ligase [EC 6.5.1.1; poly(deoxyribonucleotide):poly(deoxyribonucleotide) ligase (AMP-forming)]: it complements three different ligase mutants and gives the expected fraction of wild-type recombinants. When B bacteria were simultaneously infected with L171 and either of the three ligase mutants, burst sizes at 42° ranged from 116–155 with 13–15% wild-type recombinants. By comparison, a different gene 32 *ts* mutant complemented a ligase mutant to give a burst size of 103 with 13% wild-type recombinants. L171 does not complement the gene 32 *am*

mutant A453 and maps between other gene 32 mutants (Mosig, Bock, and Berquist, manuscript in preparation; compare also burst sizes in Table 1).

Thus we considered the possibility that in L171, T4 ligase is partially inactive and that rII mutations suppress by facilitating use of host ligase [EC 6.5.1.2; poly(deoxynucleotide):poly(deoxynucleotide) ligase (AMP-forming, NMN-forming)]. If this were the case, rII mutations should not suppress in a ligase-defective host. As expected, we found little or no suppression of L171 by rII mutations in a *lig ts7* host (Table 1, column 8; Mosig, Bock, and Berquist, manuscript in preparation). Single strands of L171 and L171rII progeny DNA grown in this host were shorter than unit length but longer than 0.3 unit length, i.e., much longer than Okazaki pieces (data not shown). In this host, parental strands were nicked, except in strains containing r1272 in which the D1 and *den* B regions are deleted (22).

The L171 Mutation Suppresses the rII-Plaque Morphology. At 37°, when suppressed and unsuppressed L171 had nearly normal burst sizes, L171 (unlike some other gene 32 mutants) suppressed the characteristic r-plaque morphology in *E. coli* B (without restoring growth in λ-lysogens). Similarly, some, but not all, T4 ligase mutations suppressed the r-plaque morphology. Since r-plaque morphology (23) on B bacteria results from a membrane change in rII-infected cells that releases lysis inhibition, its suppression by gene 32 or 30 (lig^-) mutations suggests that gene 32 protein and T4 ligase are directly involved in the rII-induced membrane change.

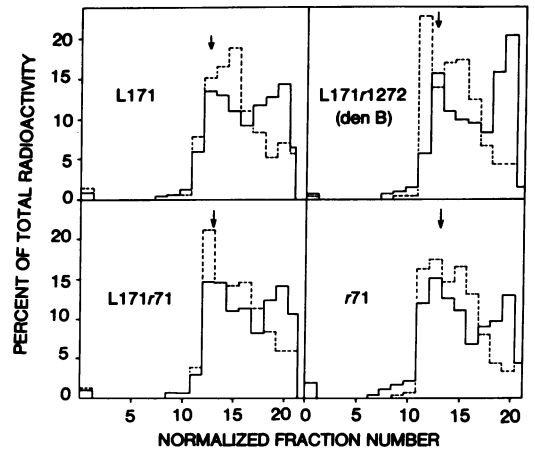

FIG. 5. Sedimentation through alkaline sucrose gradients of DNA extracted 25 min after multiple infection of *E. coli* with different phage strains. In DNA extracted at earlier times, progeny strands sedimented more slowly; at later times, unit length strands accumulated due to maturation (except in L171). *Solid curve*, parental ^{32}P-labeled strands; *broken line*, progeny ^{3}H-labeled strands. *Arrows* indicate the position of reference DNA (in a separate tube of the same centrifugation). DNA was prepared as in the density shift experiments, except that parental DNA was not density-labeled. When samples were lysed on top of the gradient, none of the DNA sedimented faster than shown here.

DISCUSSION

It has been shown that a gene 32 *am* mutant (A453) is defective in secondary DNA replication, although it can replicate much of its parental DNA once (4). This mutant also cannot form "joint" or "branched" recombinational intermediates (3, 4, 28). Since "joint" molecules are precursors of "recombinant" molecules, an additional role of gene 32 protein in ligation could not be detected in this *am* mutant.

The gene 32 *ts* mutant that we have investigated here has a different phenotype. Under restrictive conditions, when no viable progeny is produced, this mutant shows measurable albeit limited, primary *and* secondary DNA replication. Some parental and progeny DNA strands appear to be associated as "joint" recombinational intermediates, but they are not converted into covalently linked "recombinant" molecules. Additional rII mutations partially suppress the latter defect in the presence, but not in the absence, of functioning host ligase, without significantly enhancing DNA replication. Facilitated ligation of recombining strands in the suppressed mutant correlates well with enhanced progeny production. Conversely, this gene 32 *ts* mutation suppresses the characteristic plaque morphology of rII mutants.

We suggest the following model to explain our results:

(*i*) Gene 32 protein is required not only for formation of "joint" molecules, but also for ligation of the recombining strands. In the latter process (but not in the former or during DNA replication), it must somehow interact with phage ligase. The L171 mutant protein is mainly defective in its interaction with T4 ligase. It is, however, partially functional in its interaction with the replication complex. (We consider it most likely that the gene 32 protein-ligase complex involves other proteins, but for reasons of simplicity we shall not discuss them here.)

(*ii*) Suppressing rII mutations facilitate ligation by the host ligase, which in B bacteria is inactivated after T4 infection. (Our experiments cannot distinguish whether the L171 protein or an analogous host protein interacts with the host ligase.)

(*iii*) Only DNA molecules that are longer than unit length (concatemers) can complete head-filling (29). In T4, concatemerization requires recombination (24, 25, 31–33). Concatemers containing "joint" or "branched" intermediates are not packaged into viable particles.

Since the rII proteins are bound to the membrane (34–36) and to DNA (37), the rII-suppression of L171 implies that the conversion of "joint" into "recombinant" molecules occurs at the membrane. This implication is strengthened by the suppression of the rII-plaque morphology by some gene 32 (and ligase) mutations. We suggest that the T4 ligase-gene 32 protein complex binds to and changes the membranes of infected cells so that normal lysis inhibition is prevented. Wild-type rII proteins restore lysis inhibition.

We consider several other explanations for our results to be unsatisfactory:

(*i*) The possibility that some mutant rII proteins themselves would change the residual function of the mutant L171 proteins by inducing conformational changes is unlikely, since rII deletions suppress as well as or better than any point mutant (six rII A and three rII B) that we have tested.

(*ii*) The hypothesis that the rII suppression is caused by general physiological differences in membrane permeability or ion content between rII and wild-type infected cells (38–42) does not account for the requirement for host ligase, nor for the fact that only some gene 32 mutations are suppressed, nor for the suppression of the rII plaque morphology by L171. In fact, as discussed above, the rII-dependent membrane change depends on functional gene 32 protein (as well as on T4 ligase) and does not occur when the L171 protein is inactive.

(*iii*) It is unlikely that the *r*II suppression is similar to the *sud* suppression of gene 32 *am* mutations in hosts containing weak suppressors (43), because *r*II *am* A453 double mutants do not grow in a suppressing strain in which *sud am* A453 grows (A. Rodriguez, personal communication).

(*iv*) It is also unlikely that the *r*II suppression results from decreased nuclease activity in *r*II as compared with r^+ infected cells (44, 45), since L171, L171*r*71, or *r*71 parental DNA strands showed similar sedimentation profiles in alkaline sucrose gradients (Fig. 5).

(*v*) *r*II B (but not *r*II A) mutations affect translational control (49). Since *r*II A as well as *r*II B mutations suppress L171, altered translational control cannot be the only reason for suppression.

Our model assumes that the *r*II proteins exert their different functions by direct or indirect interactions with other proteins, similar to those that we have described here for gene 32 protein. This is consistent with all previous observations on *r*II mutants, particularly with the *r*II suppression of T4 ligase mutations (45–48). We assume, however, that in the gene 32 mutant L171, host ligase is mainly needed to convert "joint" into "recombinant" molecules but not to join Okazaki pieces. Colowick and Colowick (personal communication) have shown that *r*II mutations affect the ATP level in T4-infected K(λ) bacteria. It is intriguing to note that the phage ligase requires ATP, while the host ligase does not.

Our experiments cannot yet distinguish whether the premature cessation of DNA replication in the unsuppressed and suppressed L171 mutants is a direct or indirect consequence of their defective recombination and whether the interactions of gene 32 protein during ligation of DNA and during initiation of DNA replication involve the same or different sets of phage and/or host proteins. Our results show clearly, however, that mutations can specifically affect one of the several functions of gene 32 protein. This implies caution in assigning a unique function in a biochemical pathway or reaction to a gene product when the phenotype of only a few mutants is known.

We thank Susan Bock for excellent technical assistance, R. S. Edgar, T. Homyk, M. Gellert, and J. Wechsler for phage and bacterial strains, J. Weil, T. Homyk, and D. Henderson for stimulating discussions, J. Cairns and F. Stahl for constructive criticism of the manuscript, and NIH (Research Grant GM 13221) for support.

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