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Cis-Limited Action of the Gene-A Product of Bacteriophage φX174 and the Essential Bacterial Site

(*E. coli*/electron microscopy/*cis*-acting protein/specifically-nicked RF)

BERTOLD FRANCKE* AND DAN S. RAY

Molecular Biology Institute and Department of Zoology, University of California, Los Angeles, Calif. 90024

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ABSTRACT **Parental replicative-form (RF*) DNA of bacteriophage φX174 in a replication-deficient host cell (rep_3^-) exhibits two characteristic features that correlate the function of viral gene A with the initiation of viral DNA replication: a specific discontinuity in the viral strand of a constant number of RF molecules and elongation of the viral strand to yield replicative-intermediate DNA forms with single-stranded tails. At high multiplicities of infection, these initiation events are limited to an average of four specifically nicked RFII molecules per cell. The limiting factor from the host cell may be related (or identical) to the essential bacterial sites known to limit the participation of parental genomes in RF replication. Double-infection experiments with wild-type phage and phage carrying an amber mutation in gene A show that the formation of gene A-specific RFII and RI is *cis*-limited to only the wild-type DNA. These results provide a basis at the DNA level for the known asymmetric complementation of gene A.**

Of the eight known genes of bacteriophage φX174, gene A plays a key role in the replication of the viral DNA. After infection under nonpermissive conditions with phages carrying a mutation in gene A, the infecting, single-stranded, circular DNA is converted into the double-stranded, parental, replicative form (RF) (1, 2), but no further virus-specific DNA synthesis takes place. Thus, the initiation of all events leading to the production of progeny RF- and single-stranded DNA depends on this gene. In addition to the viral function, a number of host-cell functions are required to replicate RF (3, 4). Although the nature of the impaired functions in replication-deficient host cells is poorly understood, one of these mutants, rep_3^-, (5) has proven useful in elucidation of the function of the viral gene A. Since no RF replication occurs, even though all viral proteins are made in rep_3^- cells, the effect of gene A on the parental RF could be studied (6). After infection of this mutant, the parental RF has several distinguishing features. (*a*) A specific RFII with a discontinuity in only the viral strand is found at a fairly constant number per cell that is independent of the multiplicity of infection. Excess parental RF remains in the supercoiled RFI form with both strands covalently closed. (*b*) Some elongation of the viral strand on a closed-circular complementary strand occurs. Both these features of the parental RF in rep_3^- cells depend upon an active gene A. In the absence of active gene A product [i.e., gene A amber mutants or high concentrations of chloramphenicol (1)], the majority of the parental RF exists as RFI, with a low percentage of nonspecific RFII, and no strand elongation is seen.

Gene A is unique among φX174 genes in that in mixed infections it may complement mutants in late functions, but cannot itself be rescued by any other mutant or by wild type (2). In this communication, biophysical studies on double infections of rep_3^- bacteria bearing on the molecular nature of the *cis*-limited action of gene A and its asymmetric complementation behavior are presented. The results are discussed in terms of the possible components of an initiation complex of viral DNA replication.

MATERIALS AND METHODS

Media, buffers, growth conditions, infection of cells, labeling of DNA, and lysis have been described (6, 7), as have the sources of the bacterial and most of the viral strains.

Neutral and alkaline sucrose gradient centrifugations and determination of radioactivity were described (7). Equilibrium centrifugation in CsCl density gradients in the presence of ethidium bromide was performed as follows: 1.845 g CsCl was dissolved in 1.73 ml of DNA solution [in 0.05 M NaCl–0.01 M Tris–1 mM EDTA (pH 8.0)] and 0.27 ml of a 0.1% ethidium bromide solution and overlayered with mineral oil. Centrifugation was in a SW56 rotor of a Spinco L2-65 centrifuge at 43,000 rpm for 36 hr at 20°C. 38 ± 2 fractions were collected from the bottom of the tube.

For electron microscopy, the fractions of interest from a CsCl-ethidium bromide gradient were dialyzed at 4°C in the dark for 2 days against four changes of 0.05 M NaCl–0.01 M Tris–1 mM EDTA (pH 8.0). Samples were prepared for electron microscopy by the formamide spreading technique described by Davis, Simon, and Davidson (8).

RESULTS

We have already described the properties of parental RF DNA found after infection of rep_3^- cells with φXam3 (a lysis-defective mutant in gene E, used as a representative wild type with respect to viral DNA replication) (6). The experimental approach used a double-label technique: ^{32}P in the parental viral strand, and ^{3}H in the complementary strand from [^{3}H]dT added to the medium during infection. The viral DNA from such an infection was separated into two peaks

Abbreviations: RFI, replicative form DNA with both strands covalently closed; RFII, replicative form DNA with one or more discontinuities in either strand; RI, replicative intermediate DNA.

* Present address: The Salk Institute, P.O. Box 1809, San Diego, California 92112.

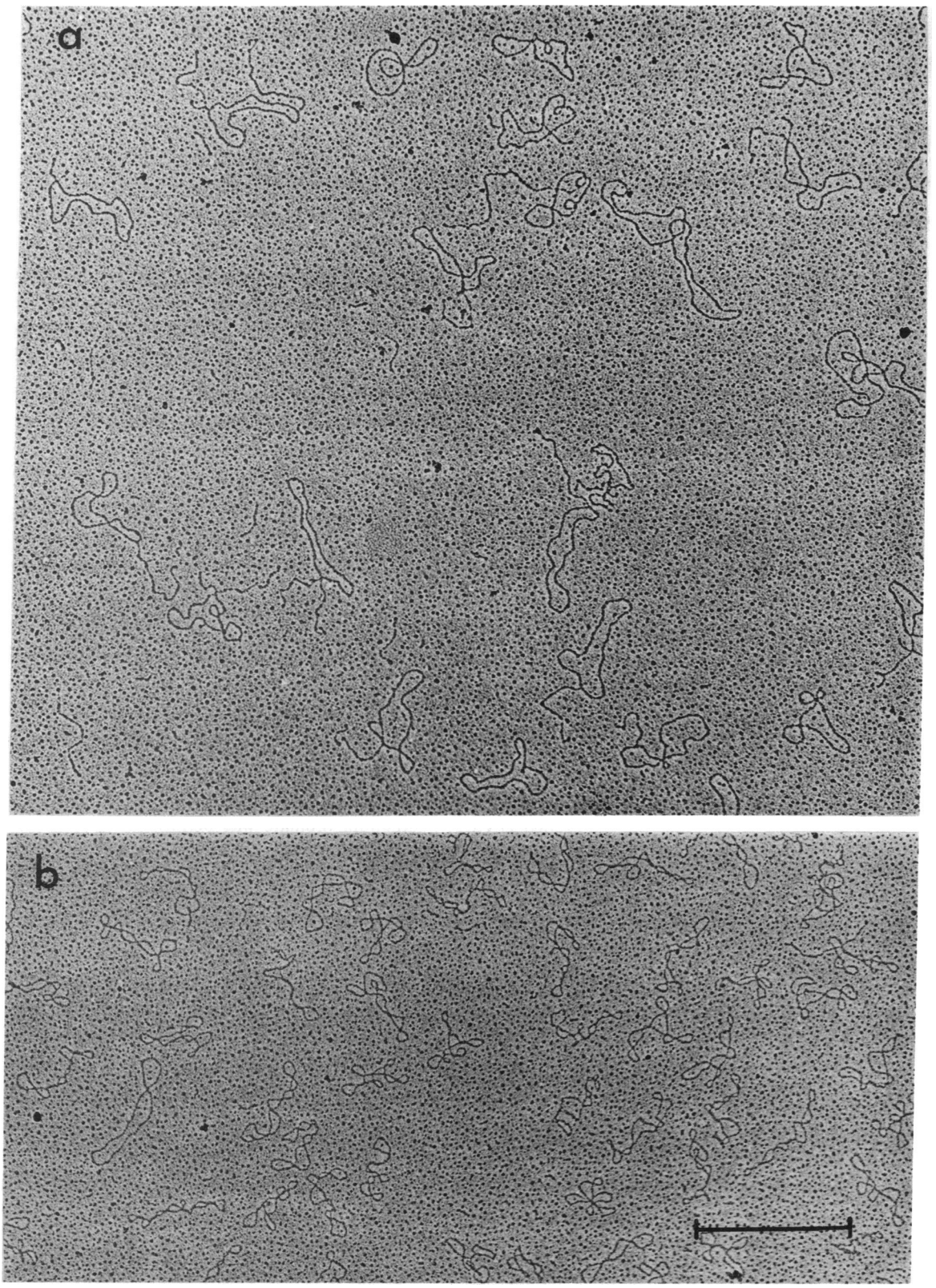

PLATE 1. Electron micrographs of RFI and RI from rep_3^- cells infected with ϕXam3. (*a*) Shows DNA from the light band and (*b*) from the dense band of the CsCl equilibrium gradient that contained ethidium bromide shown in Fig. 1*b*. Samples were prepared and spread by the Kleinschmidt procedure in the presence of formamide. The bar indicates 1 μm.

by neutral sucrose gradient sedimentation (Fig. 1a). The action of gene A could be monitored by two criteria: the slower-sedimenting peak consisted of RFII specifically nicked in the viral strand (6), and the faster sedimenting RFI-region contained, in addition to supercoiled RFI, denaturable DNA composed of circular complementary strands and elongated viral strands (RI). In order to obtain the RI molecules in their native state, the viral DNA from the RFI-region was centrifuged to equilibrium in a CsCl density gradient in the presence of ethidium bromide (Fig. 1b). The two peaks from this gradient were further characterized by both velocity and equilibrium sedimentation under neutral and alkaline conditions (not shown here) and by formamide spreading and electron microscopy. While the material from the dense peak consisted only of RFI (see Plate 1b), the light peak contained RI molecules with the same characteristics as described for the denaturable material derived from the RFI-region by alkaline sucrose gradient sedimentation (6). The electron micrograph of native RI molecules (Plate 1a) confirms the secondary structure proposed previously, namely, double-stranded DNA circles with tails. The tails are identified as single-stranded DNA on the basis of their contours and grain distributions. Under these spreading conditions, double strands show a smoother contour and a more uniform grain distribution. RNA cannot be visualized in an extended form by this procedure.

A striking feature of the micrographs in Plate 1 (a) is the presence of rings having two tails extending from a single point on the double-stranded ring. These structures most likely represent molecules in which branch migration (9) has occurred. Rings having two tails extending from different points on the ring are very rarely seen, though two such molecules are found in the upper half of this particular micrograph. Their significance is not understood. The frequency of occurrence of various structures in this preparation is given in Table 1.

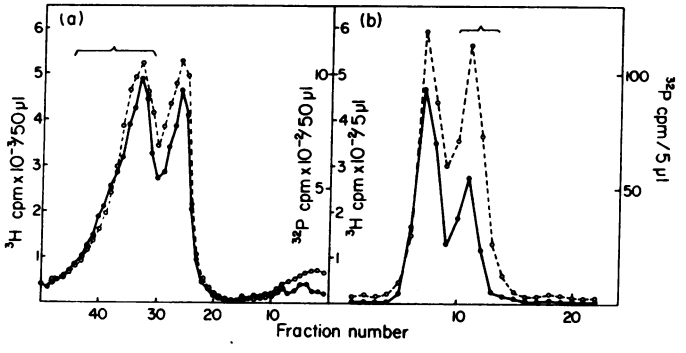

FIG. 1. Parental RF from rep_3^- cells infected with φXam3. 50 ml of medium with 2 × 10^8 rep_3^- Su$^-$ cells/ml were infected with 10 ^{32}P-labeled φXam3/cell for 30 min at 37°C. [^{3}H]dT was present throughout the infection. The cells were washed three times with cold 0.05 M tetrasodium borate in 6 mM EDTA and lysed in 3 ml of the same buffer with lysozyme, sodium dodecyl sulfate, and Pronase (7). (a) Neutral sucrose gradient sedimentation of the total lysate (sedimentation was from *right* to *left*); (b) CsCl gradient equilibrium centrifugation in the presence of ethidium bromide of the RFI-region, as indicated by the *bracket* in (a) (the density increased from *right* to *left*; 22 of the 37 fractions collected are shown). ●——●, ^{32}P cpm (parental label); O——O, ^{3}H cpm (after infection label).

TABLE 1. *Frequency of occurrence of structures observed in Plate 1 (a)*

Open rings	Rings with a single tail	Rings with two tails extendng from the same point
124	83	62

Rings with two or more tails extending from separate points	Supercoiled rings	Ambiguous structures
8	3	16

Thus, the parental label from the RFI-region that bands at the position of nonsupercoiled DNA in CsCl–ethidium bromide gradients can be used as a measure of the proportion of parental viral DNA molecules undergoing strand elongation on a circular complementary template. In Table 2, three experiments of this type at different multiplicities of infection are summarized. While at low multiplicities most of the parental DNA was in either RFII or RI molecules, at high multiplicities of infection an excess of RFI was found. Similar data were obtained when the amount of RI was calculated as denaturable, slowly-sedimenting DNA in alkaline sucrose gradients of RFI-region DNA. These results extend our observation (6) on the limitation of the number of gene A-specific RFII molecules per cell at high multiplicities of infection to the number of parental RF molecules that can undergo strand elongation.

φXam8 is a mutant in gene A. If φXam8 infects rep_3^- cells, no gene A-specific RFII is formed (i.e., the small amount of RFII found is open in either strand) and no RI molecules are seen (6) (also compare Table 3, line 2). Fig. 2 shows a double-infection experiment, in which rep_3^- cells were infected simultaneously with ^{3}H-labeled φXam3 and ^{32}P-

TABLE 2. *Proportion of parental viral DNA molecules in RFII, RFI, and RI*

Multiplicity of infection		Number of parental DNA molecules per cell (single-stranded-DNA equivalents)		
			In RFI-region	
Input (plaque-forming units)	Recovered (single-stranded-DNA equivalents)	In RFII	Supercoiled molecules	Non-supercoiled molecules
2	1.5	0.6	0.4	0.5
15	12.2	3.2	4.9	4.1
100	82.0	7.1	56.4	18.5

Experimental conditions were as described in Fig. 1, except that no [^{3}H]dT was added during infection. ^{3}H-labeled, purified RFI DNA was added to the CsCl–ethidium bromide gradients to identify the density of supercoiled molecules. Single-stranded-DNA equivalents were calculated from the specific radioactivity of the phage preparation, the recovered multiplicity of infection from the total cpm in the sucrose gradient, RFII as the area under the slower-sedimenting peak in neutral sucrose, and supercoiled and nonsupercoiled molecules as the areas under the dense and light peaks, respectively, after CsCl–ethidium bromide equilibrium centrifugation.

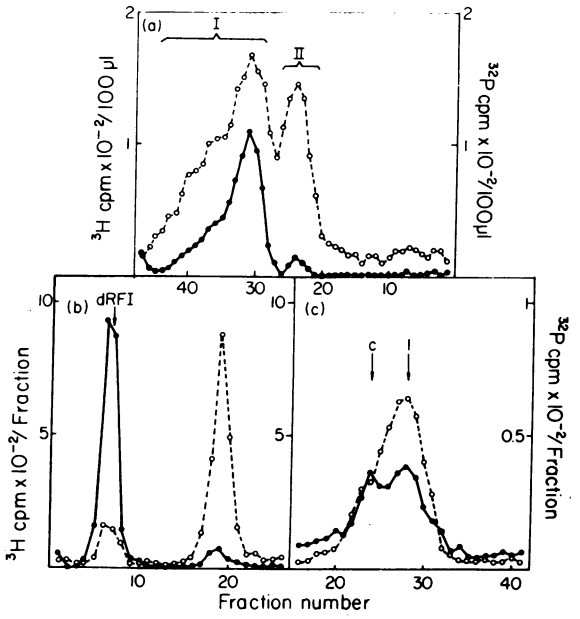

FIG. 2. Parental RF from rep_3^- cells doubly infected with φXam3 and φXam8. 20 ml of medium with 2 × 10^8 rep_3^- cells/ml were simultaneously infected with 5 ^{3}H-labeled φXam3 and 5 ^{32}P-labeled φXam8/cell. Experimental details were as described in the legend to Fig. 1, except that no [^{3}H]dT was added. (*a*) Neutral sucrose gradient sedimentation; (*b*) alkaline sucrose gradient sedimentation for 90 min of RFI-region [as indicated by I in (*a*)]; (*c*) alkaline sucrose gradient sedimentation for 6 hr of RFII [as indicated by II in (*a*)]. Centrifugation conditions were described (7). Sedimentation was from *right* to *left*. Of the alkaline gradient in (*c*), fractions 16–41 of a total of 75 fractions collected are shown. The sedimentation positions of denatured supercoils (dRFI), single-stranded circles (*c*), and unit-length linear molecules (l) are indicated by *arrows*. ●——●, ^{32}P cpm (am8 parental label); O——O, ^{3}H cpm (am3 parental label).

labeled φXam8, both at a multiplicity of 5. At this multiplicity, it is likely that a significant proportion of the cells was infected by more than one mutant phage. There also was no disadvantage for the infecting φXam8 DNA regarding its penetration and RF formation, since more than 80% of both parental labels was recovered in the two RF peaks of the neutral sucrose gradient (Fig. 2*a*). The lack of complementation between the intact gene A of φXam3 and the mutated gene of φXam8 was clearly demonstrated by three observations in Fig. 2: (*a*) The amount of RFII (Fig. 2*a*) that contained parental am8 DNA was much lower than for am3; (*b*) the RFII formed by the am3 DNA (Fig. 2*c*) contained the parental strand mostly in the linear form, while the parental strands in the am8 RFII had a higher proportion of circular molecules (for clarity, the scale for ^{32}P cpm in Fig. 2*c* is enlarged 10-fold); and (*c*) there was no evidence for am8 RI molecules (Fig. 2*b*), whereas the am3 DNA in the RFI-region was mainly in the denaturable form. The small amount of ^{32}P label that sediments slowly in Fig. 2*b* consists of single-stranded DNA resulting from incomplete removal of adsorbed, but unpenetrated, phage (6). Only amounts of 15% or more were considered significant for strand elongation. Table 3 summarizes a series of single- and double-infection experiments with φXam3 and φXam8. The apparent lack of action of the am3 gene A product on am8 RFI was also seen when an excess of φXam3 over φXam8 was used (line 4) or when both mutants infected at equal, but higher, multiplicities (line 5).

DISCUSSION

The first detectable effect of gene A on the parental φX174 RF is a nick in the viral strand (6). Since in gene A mutants no RF replication takes place, any model for RF replication in a permissive host cell will have to take into account this nick as an initial event. The significance of the gene A-dependent DNA synthesis beyond the parental RF in rep_3^- cells

TABLE 3. *Single- and double-infection experiments with amber mutants*

			% Labeled parental DNA					
			Of total recovered DNA		Of RFII		Of RFI-region	
	Mutant	Multiplicity*	In RFII	In RFI-region	In circular molecules	In linear molecules	In supercoiled molecules	In denaturable molecules
Single infections	am 3	20	22	78	28	72	43	57
	am 8	20	6	94	47	53	86	15
Double infections	am 3	4	30	70	21	79	17	83
	am 8	4	9	91	53	47	85	15
	am 3	20	27	73	15	85	60	40
	am 8	4	7	93	48	52	88	12
	am 3	10	30	70	16	84	34	66
	am 8	10	9	91	50	50	86	14

Experimental details as in Fig. 2. The individual components were identified as follows: RFII (slow-sedimenting) and RFI-region (fast-sedimenting) from the neutral sucrose gradient, circular (fast-sedimenting) and linear (slower-sedimenting) molecules from the alkaline sucrose gradient of the recovered RFII, supercoiled (rapidly-sedimenting) and denaturable (slow-sedimenting) molecules from the alkaline sucrose gradient of the recovered RFI-region. Areas under the respective peaks were calculated and are expressed as percent of the total cpm in the gradient (recovery of cpm from the gradients was more than 85% in all cases).

* Multiplicity of infection (plaque-forming units).

is still uncertain. These RI molecules contain elongated viral strands (6), and by electron microscopy (Plate 1*a*) show single-stranded tails on circular duplex molecules. This structure supports the rolling-circle model proposed by Gilbert and Dressler (11), in which the complementary strand remains closed while the viral strand is open and elongated. In this case, the defect in rep_3^- cells might affect the synthesis of complementary strands on the elongated viral strands. On the other hand, RF replication might be by some other mechanism, which could be completely blocked in rep_3^- cells, and the observed structures might have arisen from an attempt at single-strand synthesis.

Neither the generation of RFII nor the initiation of strand elongation increases proportionately with the input multiplicity (Table 2). Therefore, some cellular function must limit the number of initiation events (7.1 in Table 2 was the highest value seen). A similar conclusion was reached by Yarus and Sinsheimer (12), who found a limitation of the number of parental genotypes that could participate in replication. The number of host-cell replication "sites" depended on the physiology of the cell, i.e., starved cells contained generally only one such site. The value of about 4 estimated by these authors for unstarved cells is in good agreement with our observations of the number of initiation events per cell. We conclude, therefore, that the initiation of DNA replication in infected cells requires a complex of the parental RFI, the gene A product, and an essential host-cell component, which is likely to be the same as the essential bacterial site postulated by Yarus and Sinsheimer (12). The availability of sites limits the number of initiation events, i.e., the number of specifically-nicked RFII molecules, but not necessarily the number of molecules with elongated viral strands. The apparent limitation of RI molecules at high multiplicities could be a consequence of the limited number of RFII molecules, rather than being correlated directly to the availability of sites. Whether the site is required at the level of transcription and/or translation, or in connection with the function of the synthesized gene product, is still unknown. It is also not clear which of the components of the complex finally introduces the nick. While it could be the viral gene-product itself, it could also be a host endonuclease (13) contained within the complex, and gene A might only mediate the specific attachment of the DNA to the complex.

The double-infection experiments (Fig. 2 and Table 3) show very clearly that the action of the gene product is *cis*-limited to only its own DNA. While this observation provides some explanation for the known asymmetric complementation properties of gene A (2), the detailed molecular mechanism is still subject to discussion. The most straightforward conclusion is that transcription, translation, and nicking all occur in the same complex. The complex containing am8 RFI with the nonfunctional am8 peptide would then not be accessible to complete gene A products potentially released from an am3 complex after nicking. Alternatively, the gene product could be released from the complex in an inactive form. *In vitro* analysis of such complexes could provide further insight.

Clewell and Helinski (14) have described the isolation of "relaxation complexes" from cells carrying various episomes. For two colicinogenic factors grown in the same cell, they could demonstrate that each complex retained its specific characteristics for *in vitro* relaxation (15), suggesting that a *cis*-acting function may be involved in initiation of DNA replication in this case as well.

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