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*EARLY INTRACELLULAR EVENTS IN THE REPLICATION OF T4 PHAGE DNA, II. PARTIALLY REPLICATED DNA**

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In our previous paper,[1] we showed that early replicated DNA becomes preferentially associated with a protein inside the infected cell. Digestion with pronase allows a quantitative recovery of the early replicated DNA. The analysis of the novel properties of this early replicated and recombining DNA will be the topic of this paper.

Materials and Methods.—All the materials and methods are identical to those previously described.[1]

The method of extraction of intracellular DNA in this case is exclusively restricted to the duponol-pronase-phenol method.

Results.—Isolation and characterization of early, partially replicated DNA: Heavy bacteria were infected with light, radioactive bacteriophage and the injected parental DNA was extracted at early times after infection. Analysis of the replicative pattern of the radioactive DNA revealed that at 5 or 6 min after infection the first replicative form of DNA was of a density intermediate between parental and hybrid. This indicates that newly synthesized material is added to the parental molecule in a proportion approximating only 25 per cent of the mass of the replicating DNA parental molecule. This moiety of DNA is not a hybrid in which the proportion of new and parental is 50:50, but is only partially replicated. We call this partially replicated molecule PRM.

At later times after infection the replicative DNA assumes a hybrid density. At still later times, due to recombinational events, the parental material becomes dispersed and bands closer to the final location of the progeny DNA.

The different stages of replicative DNA—PRM, hybrid, and recombined—were isolated from CsCl fractions (indicated by thin arrows in Fig. 1), dialyzed against CS, and subjected to further analysis, then rerun in CsCl without further treatment, followed by analysis in CsCl after shearing or denaturation and analysis in a sucrose density gradient. The results of the rerun without further treatment (Fig. 1*b*, *f*, and *j*) confirm that the average proportional composition of light to heavy is for PRM, 75:25; for hybrid, 50:50; and for the recombined molecules, 25:75.

After denaturation of the PRM, hybrid, and, to a large extent, the recombined moiety, the newly synthesized strand was separated from the light, P^{32} parental material (Fig. 1*d*, *h*, and *i*). Following shearing, the PRM was dissociated into two

classes—one parental-like and one of an intermediate density between parental and hybrid. The hybrid and recombined moieties which were subjected to shearing yielded the parental label in the form of pure hybrid (Fig. 1*c*, *g*, and *k*).

The isolated PRM, hybrid, recombined, and conservative (nonreplicated) moieties were analyzed in a sucrose gradient centrifugation together with non-sheared H^3 reference DNA. Figure 2 represents the analysis of only the PRM; the hybrid and recombined moieties produced virtually identical distributions.

The immediate conclusion is that a large fraction of partially replicated, hybrid, and recombined DNA assumes a position corresponding to that of molecules of double, quadruple, and larger sizes (Fig. 2*A*). Knowing from CsCl density gradient analysis that, in the case of PRM at least, only partial replication had occurred (Fig. 1*b*), the interpretation of this pattern of distribution in a sucrose gradient demands changes in the tertiary structure of the DNA, mainly circularization or folding. The conservative, nonreplicated moiety isolated from CsCl closely overlaps in a sucrose gradient centrifugation with the H^3 reference.

Denaturation of replicative DNA leads to a change in the sedimentation pattern depicted in Figure 2*B*. This is in agreement with the effect of denaturation on the distribution pattern in a CsCl gradient (Fig. 1*d*, *h*, and *i*).

In order to check the possibility of the formation of a closed circle, replicated DNA was mixed with H^3 reference DNA and part of the mixture was heated to 100°C for 5 min. Both heated and nonheated mixtures were subjected to digestion with exonuclease I. The results are shown in Table 1. It is clearly visible that the nonheated DNA's are not decomposed while both denatured DNA's are decomposed to the same extent, contradicting the possibility of the closed circle.

In another variation of the above experiment, light, cold bacteria were infected with 5-BU cold parental phage. At the time of infection, H^3-thymidine and FUdR were added. Samples were taken at various time intervals. Analyses of the pronase extracted intracellular DNA are shown in Figure 3. Here again the incorporation of H^3 TD to the moiety (PRM) banding halfway between parental (heavy) and hybrid is clearly visible.

Discussion.—The dissociation of the complex with proteolytic enzymes and the efficient extraction of intracellular DNA allowed us to isolate, at early times after infection (5–6 min), the parental DNA, which is only partially replicated. These partially replicated molecules (PRM), when separated in CsCl, showed several new properties. Despite the fact that PRM did not yet complete one cycle of replication (roughly only half of its mass had been replicated), the sedimentation pattern in a sucrose gradient indicated a broad spectrum of apparent sizes, with a large (up to 50%) fraction sedimenting at rates exceeding those for a double-size molecule. This suggests that the sucrose gradient centrifugations discriminate in the case of PRM's on a basis other than actual molecular size. Obviously, a tertiary structure, like circularization and folding, is responsible for these characteristics. How the "replicative large molecules" of T4 compare with "double CM molecules"[3] requires further investigation. The possibility of aggregation as a source of increased sedimentation velocity was excluded by the results of the experiment in which a single multiplicity of infection and smaller quantities of H^3 reference DNA were used. In addition, persistence of the sedimentation pattern

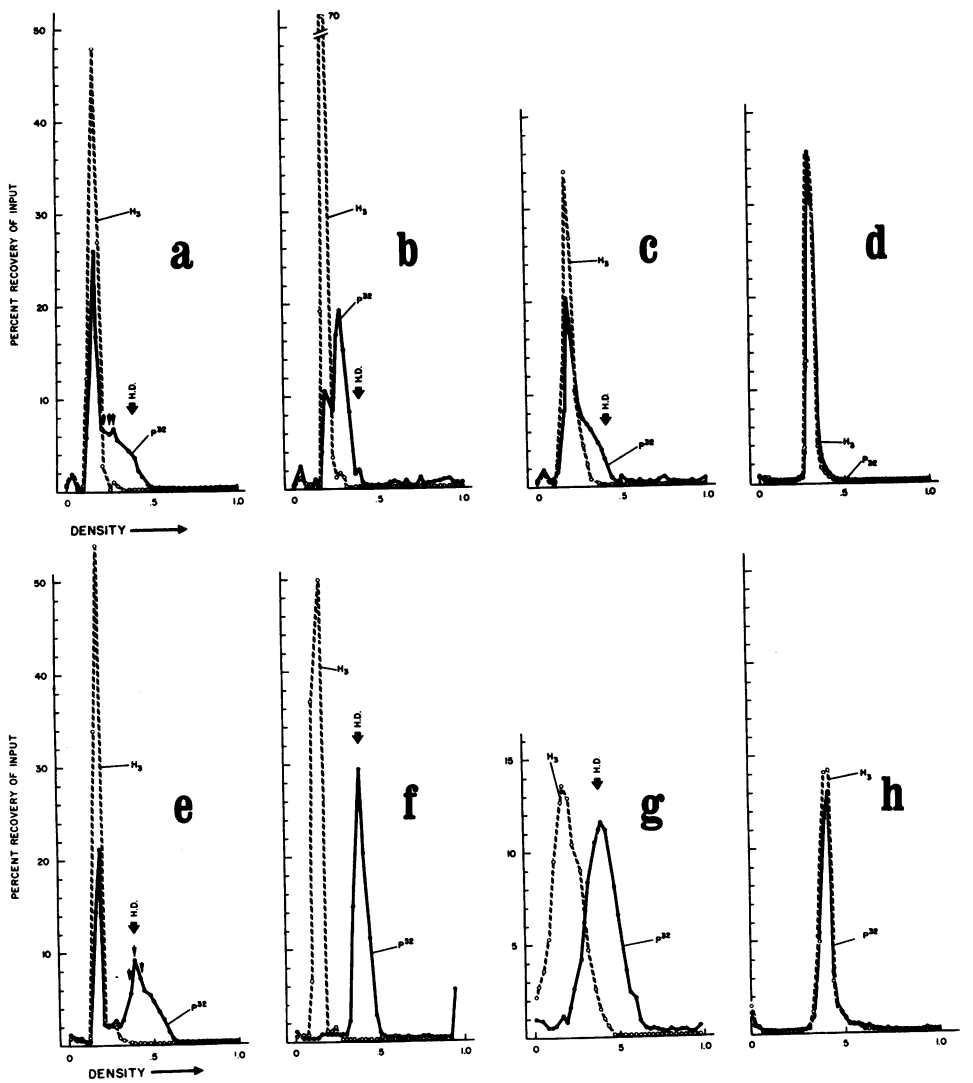

FIG. 1.—CsCl density gradient centrifugation of intracellular DNA extracted by the duponol-pronase-phenol method. In all figures the thick arrows represent the *hybrid* location: broken lines represent H^3 reference light DNA; thin arrows represent fractions used for further analysis. Heavy, cold host; light P^{32} parental; m.o.i., 3.0. (*a*) Fractionation of pronase extract (5 min after infection). (*b*) Rerun of partially replicated fraction [PRM from (*a*)]. (*c*) PRM disrupted by sonication; approximate molecular size, 5×10^6 (Raytheon sonicator; maximum output: 5 min). (*d*) PRM denatured by alkali; 0.2 *M* KOH, followed by dialysis against CSF and centrifuged in CsCl containing 1% formaldehyde. (*e*) Fractionation of pronase extract (7 min after infection). (*f*) Fractions derived from (*e*) rerun. (*g*) Fractions derived from (*e*) sonicated—conditions the same as (*c*). (*h*) Fractions derived from (*e*) denatured by alkali.

over a relatively broad range of concentrations of DNA used in the sucrose gradient centrifugations speaks against the above possibility. It is interesting to note that intracellular DNA extracted with phenol without treatment with pronase is composed mainly of molecules which overlap with the H^3 reference DNA. The ap-

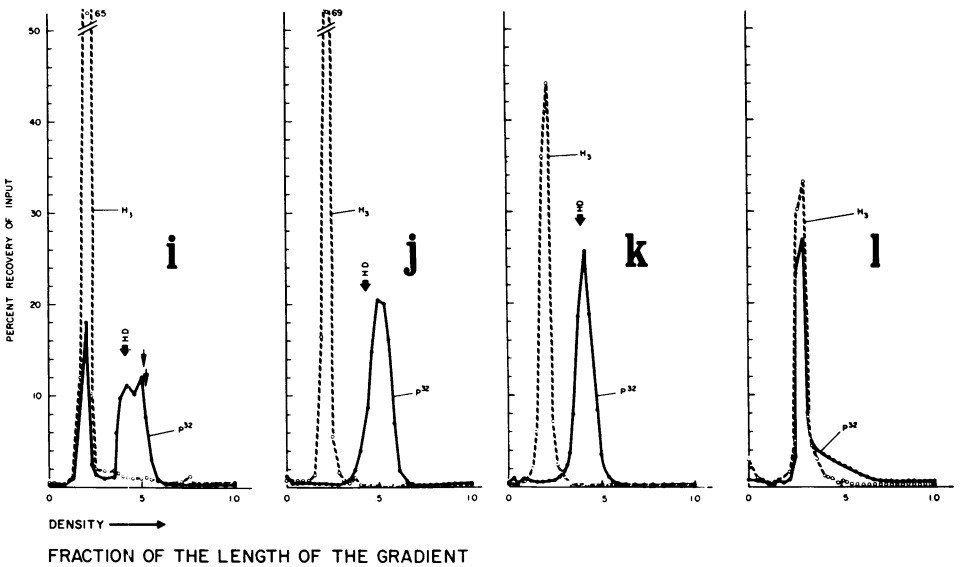

FIG. 1 (cont'd).—(*i*) Fractionation of pronase extract (9 min after infection). (*j*) Fractions derived from (*i*) rerun. (*k*) Fractions derived from (*i*) sonicated. (*l*) Fractions derived from (*i*) denatured by alkali.

propriate controls (not shown in the results), in which light bacteria were infected with light bacteriophage and the intracellular DNA was extracted and analyzed under the same conditions as those previously described, revealed a density overlap of parental label with that of reference. This and the fact that a similar pattern of replication and PRM formation occurred in a reciprocal experiment (not shown here) where light bacteria were infected with heavy radioactive bacteriophage rule out the possibility of a physiological change in the density of DNA due to a mechanism other than insertion of a new strand of different density. (Also, the experiment in which C^{14} 5-BU was added after infection with light, cold bacteriophage indicated unambiguously that the first DNA to acquire label bands at the PRM location.)

A second new fact learned about PRM and the hybrid is that the new strand added to the parental molecule is associated with its matrix only by hydrogen bonding, since it is separated by denaturation from the rest of the molecule. No covalent bonding between the old and new DNA was observed during replication *in vivo*. This is in contrast to the reported behavior of replicative DNA *in vitro*.[4, 5]

The susceptibility of denatured replicated DNA to exonuclease I indicates that circularization does not involve formation of covalent bonding and the resulting

TABLE 1

EFFECT OF EXONUCLEASE I ON REPLICATIVE MOIETIES OF DNA

Type of preparation	Conservative		PRM		Hybrid		Recombined	
	Non-denatured (%)	De-natured (%)	Non-denatured (%)	De-natured (%)	Non-denatured (%)	De-natured (%)	Non-denatured (%)	De-natured (%)
H^{3}*	5	90	6	95	4	94	5	93
P^{32}	6	92	6	93	5	94	7	94

* Figures refer to % of DNA rendered TCA nonprecipitable after digestion. Condition of digestion described by Lehman[7] [*J. Biol. Chem.*, 235, 1479 (1960)], total amount of DNA less than 2 μg/sample.

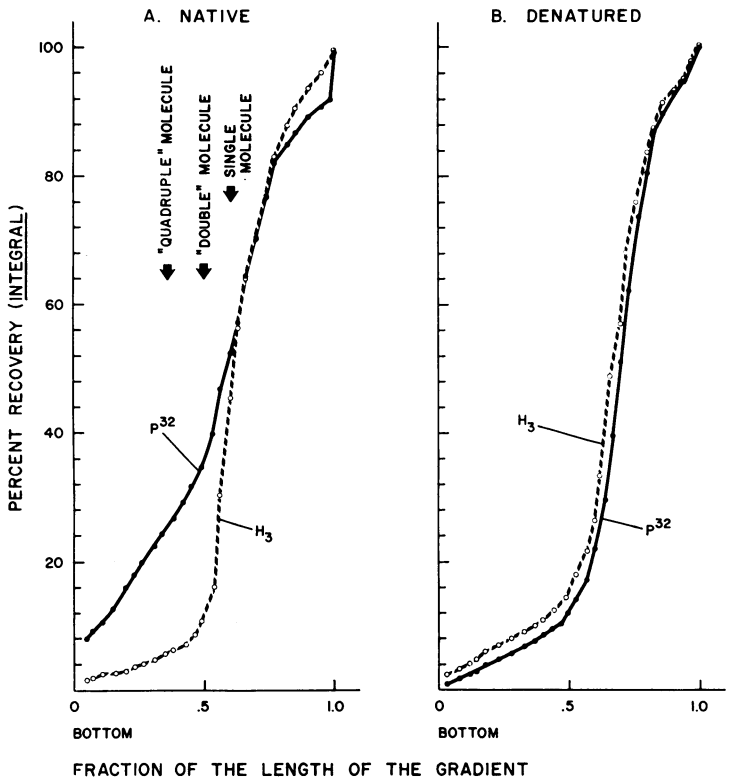

FIG. 2.—Sucrose gradient analysis of PRM. Sucrose gradient analysis of fractions isolated from Fig. 1*a*. Low specific activity H^3-thymidine labeled reference DNA was added. Conditions of centrifugation—25,000 rpm, 150 min, 22°C. The thick arrows represent the areas expected for single, double, and quadruple-size molecules (calculated according to Hershey and Burgi[6]).

closed circle. Alternatively, some limited amounts of breaks could appear within the circular molecule. The last interpretation seems unlikely since denaturation separates all the parental strands from the newly synthesized DNA (Fig. 1*d* and *h*), and this phenomenon would require either a great number of random breaks [contradicted by the sedimentation velocity of denatured PRM indicating no more than one break per strand (Fig. 2*B*)], or again a preferential break at the meeting point between the old and new DNA.

Although in the late stages of intracellular events and in the progeny molecule the continuity of the PNC becomes repaired,[2] we have shown in the present paper that after the first recombinational event there is a discontinuity of the PNC at the intersection of the parental and progeny strands. Therefore, recombination and repair are more than likely not catalyzed by polymerase or recombinase, but rather a separate enzyme, "repairase," seems to be required. An indication of the existence of such an enzyme is provided by our unpublished experiments in which the addition of CM was carefully timed and the following series of events were observed:

When CM was added prior to 5 min after infection, no replication of DNA occurred; CM added 5–6 min after infection—only semiconservative replication,

no recombination; CM added 6–8 min after infection—semiconservative replication and recombination, but the recombined moiety, after denaturation, indicated a lack of repair in the PNC; CM added 9–11 min after infection—recombination occurred, and the recombined moiety, upon denaturation, was repaired as the parental strands did not separate from the progeny molecule.

After sonication, the PRM was dissociated into two classes—one composed of double parental strands which overlapped with H^3 reference DNA, and a second, which, contrary to expectation, was not pure hybrid, but banded at a position intermediate between the parental and hybrid location. This unusual characteristic can be explained if one visualizes the beginning of replication as the addition of new material to parental molecule in discrete "patches." This might be a general preparatory stage for DNA replication; however, further investigation is needed.

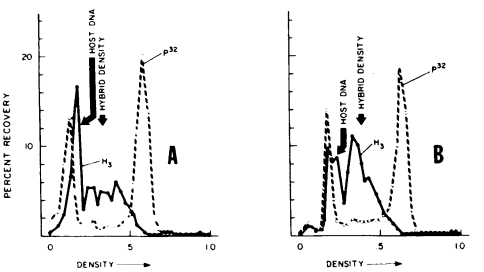

FIG. 3.—H^3-thymidine incorporation into light bacteria infected with cold, 5-BU labeled bacteriophage. Light bacteria were grown in the presence of 10 μg TD/ml and 5 μg FUdR/ml. Infection was with cold, 5-BU phage; m.o.i., 3.0. At the moment of infection, H^3-TD was added to a specific activity of 0.5 mc/mg TD. At 5 (*A*) and 6 (*B*) min, samples were chilled and extracted by the duponol-pronase-phenol method. Extraction was followed by dialysis against CS. An adequate amount of P^{32}-labeled light and heavy DNA was added as reference and is represented by the broken line. *Note:* H^3-TD is also incorporated into bacterial DNA in the fraction of bacteria which remained uninfected. At later times this moiety disappears, being replaced by phage DNA.

An examination of the changes in densities and locations of radioactivity occurring during the first phase of either the replication of a light radioactive parental in a heavy host, or the incorporation of H^3-thymidine into heavy, cold parental DNA in a light host, reveals the same two-step pattern: first, partial replication of DNA, and second, hybrid formation. This can be observed at early stages as independent peaks. Thus, PRM is not a complex with protein, an RNA, an area rich only in one base pair, nor a single-stranded DNA (as in this case a drastic difference would be noticed in replication of parental DNA's of two different densities).

It is quite probable that this partial replication might play an important role in the regulation of protein synthesis, resembling to a certain extent puffing in chromosomes. Although a detailed analysis of the PRM will be the subject of a further paper, the authors cannot resist commenting on other properties of these partially replicated molecules. When analyzed in a protein monolayer in the EM, a significant fraction of the molecules appeared as circle or hairpin-like structures. Such forms have never been observed in a nonreplicating moiety of DNA isolated from CsCl gradient nor from DNA extracted from intact bacteriophage. There are also significant indications that PRM contains a single-stranded region as it is retained on the methylated albumin column and precipitated preferentially by heavy metal ions. The single-stranded areas, however, are not susceptible to the action of exonuclease I.

Our conclusion, therefore, is that at early stages of replication parental DNA becomes circular, the circle being closed by weak forces of the noncovalent type.

Summary.—Soon after infection, partially replicated parental (PRM) DNA can be isolated in CsCl. The newly synthesized strands are associated with the parental molecule only by hydrogen bonding as they can be separated by denaturation. After sonication, this partially replicated DNA is dissociated into two classes—one parental-like, and the other having a density intermediate between parental and hybrid.

A large fraction of partially replicated DNA, when characterized in a sucrose gradient, sedimented at least twice as rapidly as unbroken DNA from T4 particles; therefore, a circular and folded tertiary structure of replicative T4 DNA is postulated.

Abbreviations: DNA, deoxyribonucleic acid; 5-BU, 5-bromodeoxyuridine; FUdR, 5-fluorodeoxyuridine; "hot," labeled with radioactive isotope; "cold," not labeled with radioactive isotope; "heavy," substituted with heavy density marker 5-BU; "light," not substituted with heavy density marker 5-BU; CM, chloramphenicol; m.o.i., multiplicity of infection; CS, citrate salt buffer; EM, electron microscope; TD, thymidine; PRM, partially replicated molecule—replicative DNA, moiety of parental DNA which, during semiconservative replication, acquires a new strand (of a new density in a particular experimental system); PNC, polynucleotide chain; CSF, citrate salt buffer with 1% formaldehyde.

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*PROTEIN-HEME INTERACTIONS IN HEME-PROTEINS: CYTOCHROME C**

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Protoporphyrin IX, when free in solution as hemin, does not rotate the plane of polarized light. Yet, when bound to its apoprotein, as in myoglobin[1] or hemoglobin,[2] its electronic transitions become optically active. The present communication considers the source of this optical activity by enumerating dominant terms in the rotational strength of the Soret transition, and by invoking the sum rule for coupled transitions which requires that other regions of the optical rotatory dispersion (ORD) curves of heme-proteins be altered as a consequence of a simple Soret Cotton effect. These considerations will be applied to ORD curves of cytochrome *c* which on superficial examination appear in contradiction with established views on the optical rotation of proteins and polypeptides. However, the incongruity is only apparent in that it assumes that cytochrome *c* follows a simple polypeptide model. Detailed analysis of the data discloses a protein-heme interaction, and