SOME FACTORS WHICH INFLUENCE THE REPLICATION OF THE REPLICATIVE FORM OF BACTERIOPHAGE ØX174

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Received November 7, 1966

This report describes experiments to show that in the E. coli cell singly infected with ØX174 in the presence of CAP* at 30 μg/ml, the parental RF molecule, i.e. that which contains the viral DNA strand, undergoes several cycles of semi-conservative replication, the conserved subunits being single polydeoxyribonucleotide chains. In the multiply-infected cell there are several parental RF molecules; evidence is presented that in general only one of these replicates. The formation of parental RF from the infecting virus DNA is not affected by increasing the CAP concentration to 100 μg/ml, but this concentration of the antibiotic severely inhibits RF replication. A more comprehensive account of this investigation will be published elsewhere.

Methods. The technique described in the legend to Fig. 1 was used in all experiments, with the indicated variations in mØ, times of incubation and concentration of CAP.

Results. The large ^{32}P peak in the low multiplicity experiment shown in Fig. 1 (a) represents an average of 0·15 parental RF/cell. Each of these molecules is a 2-stranded hybrid comprising a single light ^{32}P-ØX DNA strand associated with

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* Abbreviations: CAP, chloramphenicol; RF, 2-stranded "replicative form" of ØX DNA; mØ, multiplicity of infection; mRF, mean no. of parental RF molecules/cell, calculated from the ^{32}P data.

one heavy DNA strand; a 4-stranded structure (which would here comprise 1 li and 3 heavy strands) of the type which has been postulated for certain types of replicating DNA (Cavalieri and Rosenberg, 1962) is ruled out by its buoyant

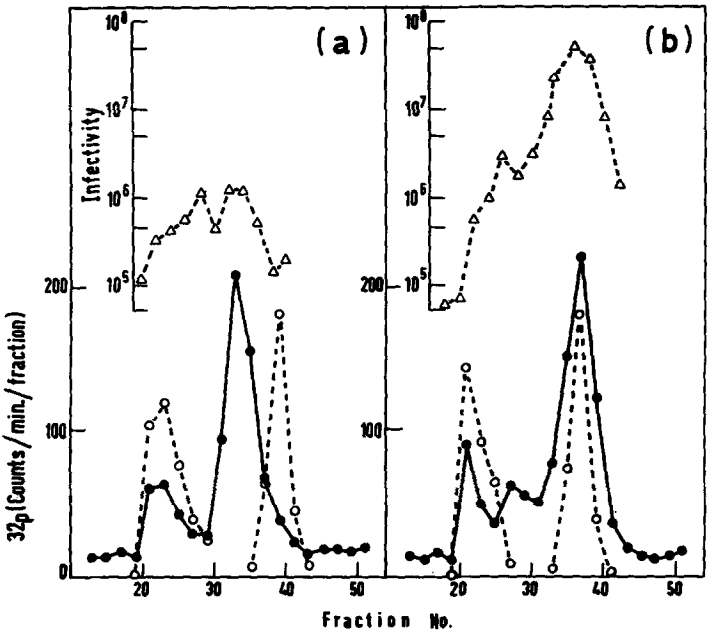

Fig. 1. Density distribution of viral DNA. mØ = 0·2 (mRF = 0·15). CAP = 30 μg/ml. (a) 15 min. in heavy medium; (b) 15 min. heavy then 25 min. light. ●, ^{32}P; Δ , infectivity (single-stranded DNA equiv./fraction); O, ^{3}H (density markers).

GROWTH AND INFECTION OF BACTERIA. E. coli C adapted to "heavy"(^{2}H and ^{15}N) medium (the D-TGL of Sinsheimer et al. (1962) with slight modification was grown to 4 x 10^{8}/ml (Petroff-Hausser counter) and infected in the presence c CAP (30 μg/ml, to block the synthesis of most bacterial proteins and to prevent lysis) with ^{32}P-labelled ØX174 (<1 ^{32}P atom/3 phages; modified from Sinsheime et al., 1962). After 15 min. two 7·5 ml portions of the culture were collected o Millipore filters. The cells from one portion were resuspended in 0·8 ml tris buffer (0·03M, pH 8·0) containing 0·012 M EDTA and lysed by incubation with lysozyme (0·11 mg, 10 min. at room temp.) followed by 5 cycles of freezing and thawing, and their DNA was studied by centrifugation to equilibrium in a CsCl gradient (see below). The cells from the second portion were resuspended in "light" (non-isotopic) medium (the H-TGL of Sinsheimer et al. (1962) with slight modification) containing CAP (30 μg/ml), and after incubation for a further 25 min. they likewise were filtered and lysed and their DNA centrifuged.

CsCl CENTRIFUGATION. Lysates were adjusted to 1·5 ml, CsCl (2·0g) was added to bring the density to 1·72 ± 0·01 g/cm^{3} and the mixtures were centrifuged at 150,000g for 36-40 hr. at 6°. Drops were collected from a pinhole alternately (i) into tubes containing 0·3 ml KH_2PO4 (0·05 M, pH 7·5) supplemented

with EDTA (10 μg/ml), serum albumin (0·1 μg/ml) and yeast RNA (0·1 μg/ml), a portion of which was used for the assay of infective DNA (Guthrie and Sinsheimer, 1963), and (ii) onto filter-paper squares for counting in a Packard Tricarb liquid scintillation spectrometer. Each centrifuge tube yielded 65-75 drops; only the relevant sequences are shown, the density decreasing to the right of the diagrams. DENSITY-MARKER DNA was added to all lysates before centrifugation. The light and heavy markers were extracted from E. coli grown in the presence of ^{3}H-thymidine either on light medium or on heavy medium, and were purified by phenol treatment. Their densities were 1·698 and 1·740 g/cm^3, measured at 6° in the analytical ultracentrifuge. In the experiments shown, the ^{3}H peak fractions contained 80-120 counts/min.; ^{3}H counts have been plotted on scales (omitted from the diagrams) individually chosen for clarity.

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density relative to the fully light and fully heavy markers (1·712 g/cm^3 at 6°). This density and the UV sensitivity of the associated infectivity distinguish it from single-stranded ØX DNA (density = 1·718 g/cm^3). The smaller ^{32}P peak, concentric with the heavy marker, appears to consist of ^{32}P-ØX DNA degradation-products incorporated into the bacterial DNA. The infectivity in the denser fractions indicates that the parental RF has replicated in the heavy medium to produce some fully heavy progeny RF. Fig. 1 (b) shows that when cells which contained hybrid RF were incubated for 25 min. in light medium, the main ^{32}P peak shifted from 1·712 g/cm^3 to 1·698 g/cm^3, the same density as that of RF extracted from cells infected in light medium. (The small ^{32}P peak somewhat less dense than the heavy marker probably represents ^{32}P-ØX DNA degradation-products incorporated into partially replicated bacterial DNA). Thus the parental viral DNA strand has been detached from all heavy DNA and has become associated with light DNA. In heavy samples transferred to light medium for shorter periods, 6 min. and 15 min., respectively 34% and 85% of the hybrid ^{32}P shifted to the lower density. A transfer made after 30 min. in heavy medium instead of 15 min. gave practically the same result, indicating that the parental RF had undergone more than one cycle of replication. These data prove that replication of the 2-stranded form of ØX DNA is semi-conservative, and hence demonstrate the

single-stranded nature of the subunits. A similar conclusion has been reached Denhardt and Sinsheimer (1965). None of these findings excludes the possibility that under certain conditions progeny RF may also arise via a single strand synthesized on a template of parental RF acting as a conserved duplex.

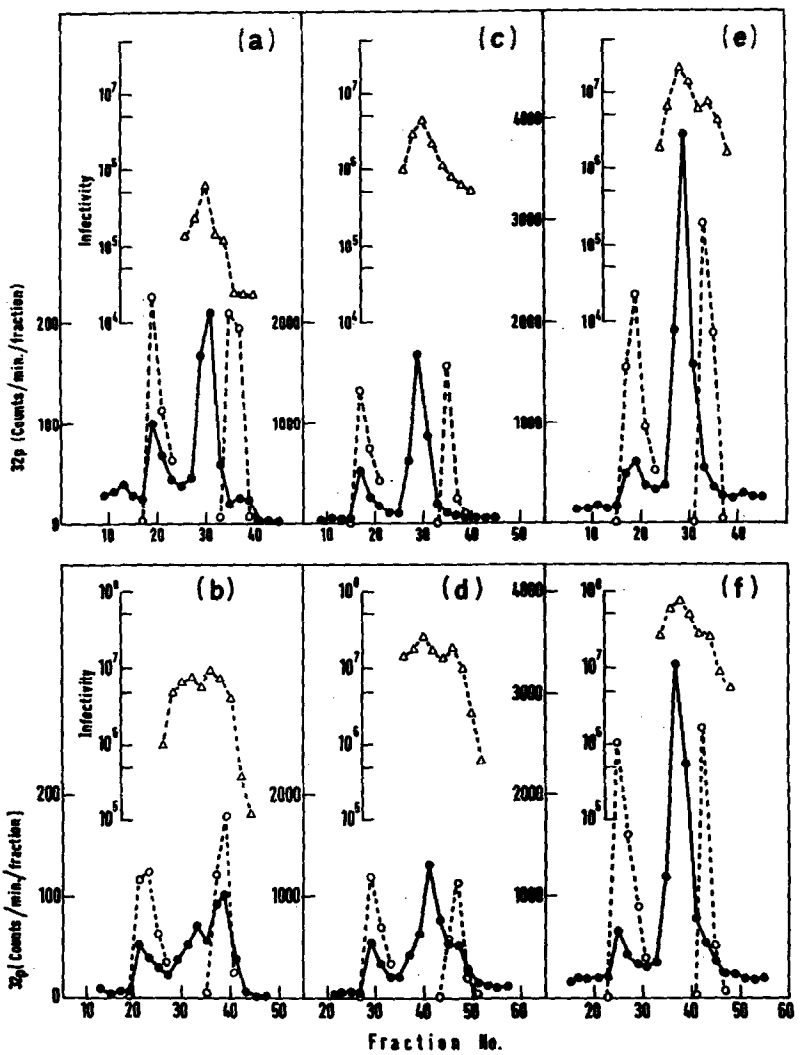

Fig. 2 Density distribution of viral DNA. CAP = 30 μg/ml. (a) (b) mØ = 0·5 (mRF = 0·49); (c) (d) mØ = 4·0 (mRF = 3·7); (e) (f) mØ = 20·0 (mRF = 9·0). (a) (c) (e) 15 min. in heavy medium; (b) (d) (f) 15 min. heavy then 25 min. light. ●, ^{32}P; ▲, infectivity (single-stranded DNA equiv./fraction); O, ^{3}H (density markers).

The denser infectivity peak of Fig. 1 (a) remains in Fig. 1 (b), which suggests that the fully heavy progeny RF has not semi-conservatively replicated. (It is not yet clear whether the large overall increase in infectivity often observed in samples transferred from heavy to light medium is mainly due to the rapid replication of part of the RF population or whether an isotope effect is significantly responsible).

The experiments presented in Fig. 2 were all performed simultaneously with the same preparation of ^{32}P-ØX, using multiplicities of 0·5, 4·0 and 20·0. Although during the 15 min. incubation in heavy medium more parental RF molecules/cell were formed at the higher multiplicities ($\underline{m}$RF = 0·49, 3·7 and 9·0 respectively), relatively less of the hybrid ^{32}P shifted to the lower density after 25 min. in light medium; the remainder stayed at 1·712 g/cm^3. In a similar experiment ($\underline{m}$Ø = 7·0; $\underline{m}$ RF = 2·7) it was shown that extending the incubation in light medium to 75 min. only slightly increased the proportion of ^{32}P which shifted, although there was a several-fold rise in infectivity at the light density. Several experiments of this type have indicated that the cell is incapable of replicating more than a very limited number of parental RF molecules, yet those which do replicate can do so again. The fraction of hybrid ^{32}P which shifts to the lower density approximately fits the expression $\frac{1 - e^{-\underline{m}RF}}{\underline{m}RF}$, which is the fraction expected to shift if 1 parental RF/cell can be replicated, assuming a Poisson distribution of parental RF molecules between the cells.

Fig. 3 illustrates an experiment performed at the same time and with the same ^{32}P-ØX preparation as that shown in Fig. 1; the important difference is the increased CAP concentration (100 μg/ml). The amount of infective parental RF formed has not been affected, nor has its density (nor its UV sensitivity), yet the production of fully heavy infectivity (Fig. 3 (a)) and the ^{32}P shift after transfer to light medium (Fig. 3 (b)) have been markedly reduced. The gross (over 90%)

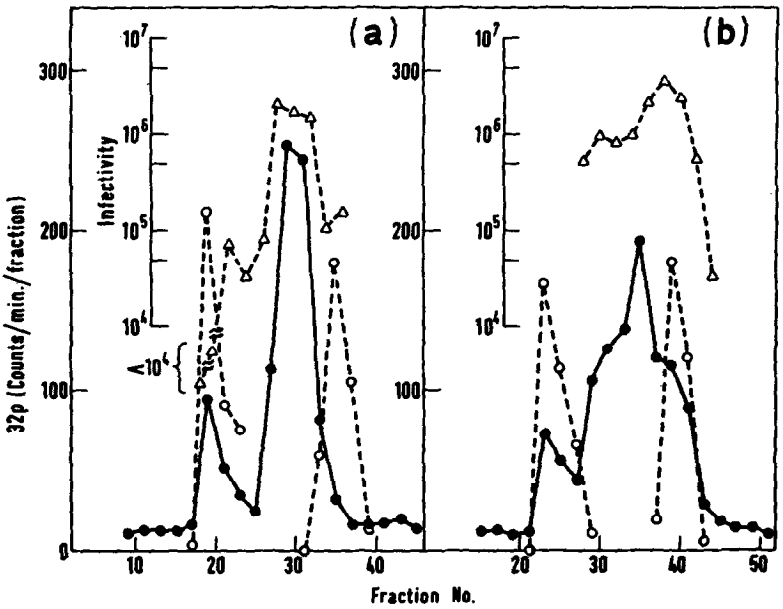

Fig. 3. Density distribution of viral DNA. $m\emptyset$ = 0·2 (mRF = 0·20). CAP = 100 μg/ml. (a) 15 min. in heavy medium; (b) 15 min. heavy then 25 min. light. ●, ^{32}P; Δ , infectivity (single-stranded DNA equiv. /fraction); O, ^{3}H (density markers).

inhibition of RF replication resulting from raising the CAP concentration is also clearly demonstrated by the infectivity patterns of Fig. 4, which shows the results of infecting at a multiplicity of 7·0 and incubating for 45 min. in heavy medium. Again, the conversion of viral single-stranded DNA into infective RF was not affected.

Discussion. The present observations support the following concept: The E. coli cell contains an enzymic mechanism able to synthesize a complementary DNA strand on each of the several single-stranded DNA molecules which may enter it. Only one of the several duplex molecules so produced can be accommodated by a unique cellular component capable of performing some function necessary for the semi-conservative replication of RF. The component may comprise a special

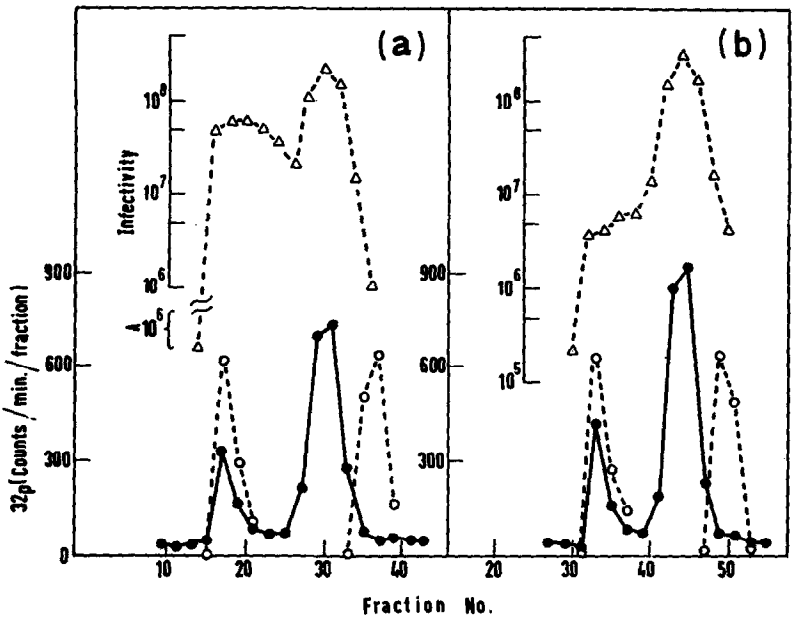

Fig. 4. Density distribution of viral DNA. mØ = 7·0. (a) CAP = 30 μg/ml, 45 min. in heavy medium, mRF = 6·5; (b) CAP = 100 μg/ml, 45 min. in heavy medium, mRF = 7·5. ●, ^{32}P; ▲, infectivity (single-stranded DNA equiv./fraction); O, ^{3}H (density markers).

DNA-polymerase and/or a special attachment site on the bacterial membrane. The functioning of either this component or some other structure involved in RF replication requires the formation of peptide-containing material. If this be the correct interpretation, it would also explain why progeny RF does not replicate.

What yet remains obscure is the relation between the special cellular component capable of replicating one RF molecule and the apparatus which replicates the bacterial chromosome; ØX-infection does not grossly interfere with cellular DNA synthesis (Rueckert and Zillig, 1962; Stone, unpublished data). Also unclear is the relation between the above findings and the data which implicate several polypeptides in the regulation of bacterial chromosome duplication (Lark, 1966). A further question is the possible connection between the enzyme

which converts ØX DNA into RF, the enzyme responsible for the replacement c defective single-strand regions in the DNA of UV- or mustard-treated E. coli (Hanawalt and Haynes, 1965) and the E. coli DNA-polymerase studied by Kornl group (Richardson, Inman and Kornberg, 1964), of which there are perhaps 200-300 molecules/cell (Kornberg, personal communication).

Work in progress is aimed at elucidating the nature of the special cellul component and of the peptide material involved in RF replication.

I wish to thank Prof. J. A. V. Butler, F. R. S., for his interest and hospitality, and Dr. K. S. Kirby for kindly accommodating me in his laboratory.

This investigation was supported by a grant from the Medical Research Council.

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