THE MECHANISM OF REPLICATION OF φX174 DNA:

DISCONTINUOUS SYNTHESIS OF THE φX174 VIRAL STRAND

by

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to my mother

ABSTRACT

The nascent intermediates present during the final stage of ϕX174 DNA replication were studied using a new method of stopping DNA synthesis. About 40 to 50% of the [^{3}H]thymidine incorporated in a brief pulse was found in viral strand molecules shorter than unit length. The proportion of pulse label found in the short intermediates varied with pulse length, stopping procedure, aeration level of the infected culture, and host strain. The short molecules were not generated by the excision of misincorporated uracil since they were equally abundant in *ung* and *ung*$^+$ strains. Approximately 20% of the short molecules had at least one ribonucleotide at the 5' terminus as determined by the spleen exonuclease assay. The short nascent molecules hybridized to all regions of the ϕX genome, but preferentially to the regions around the origin and terminus of replication. After phosphatase treatment, the short molecules were resistant in variable proportions to degradation from the 5' end by spleen exonuclease. Some proteins remained very tightly associated with the nascent molecules when the DNA was isolated without the use of proteolytic enzymes. On the basis of these studies of the short nascent intermediates, we conclude that ϕX viral strand synthesis is a discontinuous process, not continuous as originally proposed by the rolling circle model.

RESUME

Les produits intermédiaires naissants identifiés durant le dernier stade de la réplication de l'ADN du bactériophage ϕX174 furent étudiés en utilisant une nouvelle méthode permettant l'arrêt de la synthèse de l'ADN. Environ 40 à 50% de la thymidine[^{3}H] incorporée par le marquage par impulsion a été trouvé dans les brins viraux plus courts que l'unité. La proportion de marquage retrouvé dans les produits intermédiares courts a varié avec la durée du marquage par impulsion, le procédé d'arrêt, le niveau d'aération de la culture de cellules infectées, et de la souche hôte. Les courtes molécules de l'ADN ne proviennent pas de l'élimination de l'uracile incorporé par erreur puisqu'elles se retrouvaient en quantités égales parmis les souches *ung* et *ung*$^+$. Environ 20% des molécules courtes avaient au moins une molécule de ribonucléotide à la terminaison 5' tel que déterminé par titrage avec l'exonucléase de la rate. Les courtes molécules naissantes furent hybridées à toutes les régions du génôme de ϕX, mais préférentiellement aux régions situées près de l'origine et de la terminaison de la réplication. Suite au traitement par la phosphatase, les molécules courtes de l'ADN furent résistantes en proportion variable à la dégradation de la terminaison 5' par l'exonucléase de la rate. Une certaine quantité de protéines était fermement associée aux courtes molécules naissantes quand l'ADN était isolé sans l'utilisation d'enzymes protéolytiques. A partir de ces études sur les courts intermédiaires naissants, nous pouvons conclure que la synthèse de la chaîne virale du ϕX est un processus discontinu, contrairement au modèle continu du "rolling circle" originellement proposé.

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TABLE OF CONTENTS

This thesis consists of an introductory chapter, two chapters in the form of manuscripts, and a concluding chapter. Because Chapters II and III deal with related material, there is some overlap in the Introduction, Materials and Methods, and Discussion. Most of Chapter II has already been published; parts of Chapter II and III are being prepared for publication.

CHAPTER I

Introduction

PREFACE

Elucidation of the anti-parallel base-paired double helix structure of DNA by Watson & Crick (1953) suggested a mechanism for replication of DNA whereby each strand served as a template for the synthesis of a complementary strand. This hypothesis was supported by the classic density-labelling experiments of Meselson & Stahl (1958), which showed that each progeny duplex consisted of one parental strand and one newly synthesized strand. Radioautographic studies revealed that DNA synthesis proceeds sequentially from an origin (Cairns, 1963). Since the two strands in the duplex are oriented opposite to each other, one of the two strands in every replication fork must be synthesized in the 5' to 3' direction, while the other is synthesized in the 3' to 5' direction. This poses a problem because all of the known DNA polymerases are able to synthesize DNA only in the 5' to 3' direction. Okazaki (1968) suggested that the joining of short chains synthesized in the 5' to 3' direction could result in an overall 3' to 5' direction of strand growth. Thus, the strand that grows in the 3' to 5' direction, often called the lagging strand, could be synthesized as short pieces by a discontinuous mechanism. The strand that grows in the 5' to 3' direction, called the leading strand, could be synthesized continuously as one long molecule. However, in my opinion, most of the evidence appears to favor the hypothesis that the leading strand is discontinuously synthesized, but in longer molecules that are more rapidly joined together. Since it is particularly relevant to my work, I will review this evidence in the prokaryotic systems in which it has been studied.

In addition to polymerizing only in one direction, DNA polymerases are also restricted in being unable to initiate *de novo* synthesis of DNA chains. All known DNA polymerases can only add nucleotides to the 3' OH ends of primers base paired to the template strand. Because synthesis of a DNA chain is necessarily coupled to a priming event, elucidation of the nature of the primer that exists at the 3' end is the ultimate proof that the DNA chain was initiated *de novo*. The demonstration of primers on the 3' ends of short molecules arising from the leading strand is the strongest evidence that the strand was synthesized discontinuously. So I will review also what is known of the mechanisms by which DNA synthesis initiates in those prokaryotic systems where the process has been analyzed.

1. DISCONTINUOUS DNA SYNTHESIS IN PROKARYOTES

(a) Okazaki pieces: intermediates in discontinuous synthesis

The earliest evidence that DNA synthesis of both strands was discontinuous was presented by Okazaki (1968). All of the label incorporated in a brief pulse of [^{3}H]thymidine was found as short molecules with a sedimentation coefficient of about 10S on alkaline sucrose gradients. These 10S molecules, or Okazaki fragments, were observed in several strains of *E. coli*, including B, 15T$^-$, and W3110, in *Bacillus subtilis*, and in bacteriophage T4-infected *E. coli*. Other investigators had difficulty reproducing these results. Iyer & Lark (1970) reported that even with the briefest pulse of [^{3}H]thymidine, more than 50% of the label incorporated was found in high molecular weight DNA. The ^{3}H-labelled DNA in these long molecules was present at the 3' end, since it was degraded by the 3' to 5' exonuclease activity of *E. coli* exonuclease I at a more rapid rate than the ^{14}C-pre-labelled DNA. Since the newly synthesized DNA was covalently attached to pre-existing DNA, it appeared that strands in the replicating fork could be continuously elongated. These *in vivo* results were also found *in vitro* by Olivera & Bonhoeffer (1972). The [^{3}H] TMP incorporated by a crude lysate of *polA*$^-$ cells (deficient in DNA polymerase I activity) on a cellophane disc, to which NMN had been added to inhibit *E. coli* DNA ligase, was found to be equally distributed between the 10S Okazaki fragments and longer DNA molecules with an average sedimentation coefficient of 38S. The 10S and 38S intermediates did not self-anneal to an appreciable extent, but cross-annealed to about 80% (Herrmann *et al.*, 1972) and so appeared to be derived from different strands at the replicating fork. Presumably the longer intermediates are derived from the leading

strand. In an attempt to reconcile their results with Okazaki's, Olivera & Bonhoeffer hypothesized that the length of the intermediates formed reflected a competition between initiation and elongation. If elongation on the leading strand were very rapid, fewer initiations would be able to occur on this strand. Various conditions might favor elongation or initiation.

(b) Nascent intermediates reflect the stopping procedure

One of factors which determines the nascent intermediates observed after a brief pulse of [^{3}H]thymidine is the method used to stop the pulse. Both Okazaki (1968) and Iyer and Lark (1970) stopped the pulses by pouring the cultures onto frozen solutions containing KCN. But ϕX DNA synthesis occurs in the presence of KCN (Cairns & Denhardt, 1968) and *E. coli* DNA ligase is active at 0°C in the presence of azide or KCN (Schekman *et al.*, 1971). Jacobson & Lark (1973) found that adding 10% pyridine to the frozen KCN solution reduced the incorporation of [^{3}H]thymidine; the label found in the 10S molecules remained relatively constant, but much less label was found in high molecular weight DNA. Sternglanz *et al.* (1976) compared the intermediates which were observed when pulses were stopped with pyridine and KCN or with cold acetone (Cairns & Denhardt, 1968). Again the amount of label in the 10S molecules remained relatively constant but substantially less label was found in longer molecules with the cold acetone stop. More than 90% of the [^{3}H]thymidine incorporated by *Bacillus subtilis* was found in Okazaki fragments. Approximately 70 to 80% of the [^{3}H]thymidine incorporated by various strains of *E. coli*, and most of the label incorporated by T7-infected *E. coli* B was found in 10S Okazaki fragments. Sternglanz *et al.* (1976) concluded that DNA

synthesis on both strands was discontinuous, but that some methods of stopping the ~~pulse allowed~~ preferential joining of leading strand Okazaki pieces. Fragments on the strand growing in the 5' to 3' direction have a greater probability of being immediately adjacent to each other with no nucleotides missing between them. On this strand, the 5' end of the newly initiated molecule can be ligated to the 3' end of the preceding molecule before the newly initiated one is completely synthesized. In contrast, on the strand growing in the 3' to 5' direction, the newly initiated molecule must be completely synthesized before its 3' end can be joined to the 5' end of the preceding molecule.

Kurosawa & Okazaki (1975) found that when a brief pulse of [^{3}H] thymidine administered to P2-infected *E. coli* was stopped with an ethanol phenol mixture at room temperature instead of with a frozen solution containing KCN, the total incorporation of [^{3}]thymidine was not changed, but the size distribution was altered, with more label in 10S than in 32S molecules. A larger proportion of the 10S molecules (from 20 to 33% as compared to 8 to 10%) were derived from the strand growing in the 5' to 3' direction. Kurosawa & Okazaki (1975) concluded that both strands of P2 were discontinuously synthesized, but that the rate of joining of the leading strand fragments was much more rapid than the joining of lagging strand fragments. Joining of the leading strand molecules could occur at 0°C in the presence of KCN.

If the leading strand fragments are prematurely joined, that is, joined before they are completely synthesized, one would expect the average size of the leading strand fragments to be shorter than that of the lagging strand fragments. However both for bacteriophage P2 (Kurosawa & Okazaki, 1975) and ϕX (Machida *et al.*, 1977), the leading strand

fragments were found to be longer on average than the lagging strand fragments. Thus a combination of factors - fewer initiations and longer molecules, plus more rapid joining of the molecules - may prevent the short intermediates on the leading strand from being readily observed.

(c) Mutant studies support discontinuous synthesis

When *E. coli* infected with T4 containing a temperature sensitive (*ts*) ligase were pulse-labelled with [^{3}H]thymidine at the non-permissive temperature, there was an accumulation of 10S molecules (Sugimoto *et al.*, 1968). In an *E. coli* mutant with a *ts* ligase, *lig ts* 7, at the non-permissive temperature, all the [^{3}H]thymidine appeared in 10S intermediates under conditions where most of the label in the wild type strain was present in longer molecules. Even at the permissive temperature, there was an enrichment for 10S molecules in *lig ts* 7 (Konrad *et al.*, 1973). Ligase is responsible for joining the 5' P end of one DNA chain to the 3' OH end of another. That all the [^{3}H]thymidine was found in 10S molecules in the absence of ligase activity suggested that ligase was required to join the short molecules on both strands.

In a *polA1* mutant, more than 90% of the newly synthesized DNA was found as 10S molecules (Kuempell & Veomett, 1970). This implicated DNA polymerase I in the joining of the 10S molecules, and led to the suggestion that it was required to fill in gaps between the 3' and 5' ends of adjacent molecules. Schekman *et al.* (1971) also concluded that DNA polymerase I was required to fill in gaps during ϕX RF replication because they observed an accumulation of RF molecules with gaps in a *polA* strain at the non-permissive temperature. The double *polA1 lig4* mutant was not viable at the non-permissive temperature because of a greater than 50 fold

reduction in the joining of Okazaki pieces (Gottesman *et al.*, 1973). Although these experiments demonstrated that both DNA polymerase I and ligase were required to join short molecules on the two strands at the replicating fork, there was the possibility that the enzymes were functioning in post-replicational repair rather than in replication *per se*. So the results are suggestive, not conclusive.

In a *dna*G mutant at the non-permissive temperature, incorporation of label into DNA stopped soon after initiation of Okazaki pieces was inhibited (Lark, 1972). That polymerization cannot continue for very long without initiation implied either that all DNA synthesis, including synthesis of the 5' to 3' strand, occurred via short intermediates, or that synthesis of the two strands at the replication fork was closely coupled, so that inhibition of discontinuous synthesis on the lagging strand prevented continuous synthesis on the leading strand.

(d) Excision of misincorporated uracil as a potential source of Okazaki pieces

The presence of most of the label in short molecules after a pulse of radioactive precursor is generally taken as evidence for the discontinuous synthesis of both strands. However, short fragments can also be produced by post-replicative degradation of DNA. In 1977, Tye *et al.* reported the accumulation of 4S molecules when dUTPase deficient (*dut*$^-$) strains of *E. coli* were briefly pulse-labelled with [^{3}H]thymidine. dUTP can arise intracellularly by the phosphorylation of dUDP, or by the deamination of dCTP. Since DNA polymerase III holoenzyme has the same Km for dUTP and dTTP (Schlomai & Kornberg, 1978), dUTP can be incorporated into DNA as efficiently as dTTP. The misincorporated uracil residues can be recognized and

removed by uracil-DNA glycosylase, and the apyrimidinic sites generated will be cleaved by the appropriate endonuclease (Tye *et al.*, 1977).

Support for this hypothesis has been provided by the finding that uracil can be stably incorporated into DNA in uracil-DNA glycosylase deficient (*ung*) strains (Tye *et al.*, 1978; Warner & Duncan, 1978; Warner *et al.*, 1979). The uracil-containing DNA can be fragmented *in vitro* by successive treatment with uracil-DNA glycosylase and alkali (Tye *et al.*, 1978; Warner & Duncan, 1978).

That excision repair of misincorporated uracil residues does not contribute significantly to the population of Okazaki fragments except in dUTPase-deficient hosts is suggested by several observations. First, the amount of Okazaki pieces is not significantly diminished in *ung* strains, where the excision of uracil is blocked (Tye *et al.*, 1978; Tamanoi & Okazaki, 1978). When the uracil-containing DNA is fragmented *in vitro*, differences in the sedimentation coefficient have led to estimates of the frequency of uracil misincorporation which ranged from 1 per 2000 to 3000 nucleotides (Lehman *et al.*, 1979) to 1 per 5000 nucleotides (Tamanoi *et al.*, 1979). Also *in vitro*, very high levels of dUTP are required to reduce the size of the Okazaki fragments to the 4S range (200 μM dUTP for *dut*$^+$ extracts as compared to 18 μM dUTP for *dut* extracts) (Olivera, 1978). This implies that dUTPase is a very efficient enzyme and rapidly removes dUTP from the intracellular pool, so that very little is available to be incorporated into DNA.

(e) Distribution of label in Okazaki pieces

To determine if the 10S pieces were generated by post-replicational nicking of DNA, Okazaki *et al.* (1968) isolated doubly labelled molecules from *E. coli* 15T$^-$ cells pre-labelled with [^{14}C]thymidine, then pulse-labelled

with [^{3}H]thymidine. When these molecules were degraded with *E. coli* exonuclease I, which hydrolyzes single-stranded DNA from the 3' end, the [^{3}H]pulse-label was released before the [^{14}C]pre-label. Conversely, with the *B. subtilis* nuclease that degrades single-stranded DNA from the 5' end, the [^{3}H] pulse-label was released after the [^{14}C] pre-label. The location of the pulse label at the 3' ends of the 10S molecules indicated that they were synthesized in the 5' to 3' direction. An uneven distribution of label would not be expected if the 10S pieces were caused by post-replicational nicking. Similar results were reported for the 10S molecules isolated from T4-infected *E. coli* (Sugino & Okazaki, 1972). Because the rate of polymerization is so rapid in *E. coli* , in order to obtain doubly labelled 10S molecules, the cells were pulse-labelled at very low temperatures (0°C to 8°C), and so these experiments can be criticized on the grounds they they were performed with DNA synthesized under non-physiological conditions. Also the presence of uniformly ^{14}C-labelled non-nascent DNA molecules of this size (see Section g) was not taken into account by these investigators.

(f) Labelling conditions determine nascent intermediates

Werner's (1971) inability to label Okazaki fragments with pulses of [^{3}H]thymine led him to suggest that the [^{3}H]thymidine-labelled Okazaki pieces were intermediates in repair synthesis. However, the inability to label with [^{3}H]thymine was probably due to the slowness with which thymine equilibrates with the intracellular pool of dTTP relative to thymidine (Cairns & Denhardt, 1968). In order to maximize the rate of uptake of [^{3}H]thymine and to have it incorporated into DNA at full specific activity, Diaz *et al.* (1975) depleted the intracellular nucleotide pools by incubating

thymine-requiring strains in thymineless medium before labelling briefly with [^{3}H]thymine. The [^{3}H]thymine was incorporated into very large numbers of very small DNA chains. If thymine-requiring strains are deprived of thymine, the intracellular dUTP/dTTP ratio would rise, and one might expect to observe an excess of short molecules which arose from an increased frequency of misincorporation of dUTP followed by excision repair.

Brewin (1977) hypothesized that if the short molecules truly represent the beginnings of a large number of molecules at the replication fork, then the label incorporated during a brief pulse should be dispersed over a large stretch of the genome, and interspersed with DNA that was synthesized after the pulse. When thymine-starved cells were pulse-labelled with trace quantities of [^{3}H]thymidine and then chased with 5-bromouracil, and the extracted DNA sonicated to an average size of 2000 nucleotides, most of the [^{3}H]thymidine was found associated with the density-labelled DNA. So Brewin (1977) concluded that the short molecules are not the result of post-replicational repair, but rather, represent the beginnings of large numbers of nascent molecules. Furthermore, these molecules appeared to be synthesized in two steps: an initial slow step which was stimulated by the addition of thymidine, followed by a rapid stage which was not affected by the addition of thymidine (Brewin & Cairns, 1977). The pattern of DNA synthesis in cells deprived of thymine is complex, not easily understood, and perhaps not representative of DNA synthesis in wild type cells.

(g) Nascent and non-nascent short molecules

Pulse-labelling with [^{3}H]thymidine can give a rough indication of the size of the short chain intermediates, but it is not possible to be certain of the size distribution because not all the short molecules may be uniformly labelled throughout their length. Furthermore, since the specific activity of the DNA precursor pool is steadily changing during a pulse, the quantity of DNA and the number of pieces that are made cannot be estimated from the incorporated radioactivity. In order to overcome these difficulties, Anderson (1978) isolated short molecules from cells that had been uniformly labelled with [^{3}H]thymine. She found that a large number of the short molecules did not disappear in a chase period in unlabelled medium. In a *polA1* strain, the number of [^{3}H]thymine-labelled molecules in the 700 to 9000 nucleotide range dropped from 80 to about 50 per cell after the chase. In a *polA*$^+$ strain, there was a drop from 20 to 17 molecules per cell. Molecules shorter than 700 nucleotides were not analyzed because not enough radioactive label was incorporated. Thus only a small proportion of molecules the size of Okazaki fragments in the cell appear to be nascent intermediates.

Jacobson & Lark (1973) had also reported that a large fraction of the short molecules, which they isolated from *E. coli* 15T$^-$ cells and labelled with [γ-^{32}P]ATP and kinase, did not behave as nascent intermediates, since they did not accumulate in ligase-defective strains at the non-permissive temperature, nor disappear in a *dnaG* mutant under restrictive conditions.

Denhardt *et al.* (1979) quantitated the numbers of short molecules in various strains of *E. coli* which did not behave as nascent intermediates. Depending on the strain and growth conditions, there

could be as many as 400 molecules the size of Okazaki fragments per cell, and as many as 2000 very short molecules approximately 30 nucleotides long per cell.

Anderson (1978) suggested that the short non-nascent molecules represent the extreme tail end of a random distribution of breaks scattered throughout the entire bacterial chromosome. Denhardt *et al.* (1979) hypothesized that the short non-nascent molecules inserted between the two strands of the genome play an active role in transposition, recombination or repair. Whatever the cause or purpose of these molecules in the cell, their existence complicates the study of nascent intermediates, since the shortest DNA molecules in the cell are not necessarily the most recently synthesized ones.

(h) Hybridization of Okazaki fragments to separated strands

The 10S molecules isolated from T4- or λ-infected *E. coli* were shown to anneal equally to the separated complementary strands of these phages (Sugimoto *et al.*, 1969; Ginsberg & Hurwitz, 1970). These results were first taken as evidence of the discontinuous synthesis of both strands. However, when it was discovered that the replication of these two bacteriophage was bidirectional, the interpretation became more complicated. In bidirectional replication, short molecules can be synthesized on only one of the strands at each replication fork, but may still hybridize to both strands, if the short molecules come from opposite strands in the two replication forks.

In order to avoid these complications, Kurosawa & Okazaki (1975) studied the replication of bacteriophage P2 because the replication of

this phage proceeds unidirectionally from a fixed point on the genome. A brief pulse of [^{3}H]thymidine was incorporated into two sizes of intermediates: 10S and 32S. Both the amount of label in the intermediates and the strand distribution were determined by the method used to stop the pulse (see section b). Approximately one third of the 10S molecules were derived from the leading strand when the pulse was stopped with an ethanol phenol mixture. In a P2-infected *polA* strain, all the [^{3}H] thymidine was incorporated into 10S molecules which hybridized equally well to both strands. Again DNA polymerase I appeared to be involved in joining the Okazaki pieces.

φX RF is replicated unidirectionally from a fixed origin like P_2. The orientation of the strands in the RF molecule is such that the viral strand is synthesized in the 5' to 3' direction (see Denhardt, 1977 for review). The nascent molecules isolated during φX RF replication contained a single gap in the viral strand, and multiple gaps non-randomly located around the genome in the complementary strand (Eisenberg *et al.*, 1975). This asymmetry in the distribution of gaps was interpreted to indicate that the viral strand was continuously synthesized while the complementary strand was discontinuously synthesized. Furthermore, in a φX-infected *ts* ligase host at the non-permissive temperature, a brief pulse of [^{3}H] thymidine labelled both short and unit length molecules during RF replication. Hybridization studies revealed that 90% of the short molecules were of complementary strand origin, and only 10% of viral strand origin, while almost all of the unit length molecules were viral strands (McFadden & Denhardt, 1975). Again this suggested that only the complementary strand was discontinuously synthesized. However, Machida

et al. (1977) found that during RF replication in φX-infected *E. coli* HF4704, the [^{3}H]thymidine incorporated during a brief pulse into the complementary strand was present mostly as short molecules, but about one third of the label incorporated into the viral strand was also present as short molecules. This indicated that both strands were discontinuously synthesized. Since both Machida *et al.* (1977) and McFadden & Denhardt (1975) used an ethanol phenol mixture to stop the pulse, the differences reported by these investigators probably reflect differences in the host strains. The *ts lig* strain used by McFadden & Denhardt (1975) was not thymine requiring, whereas the strain used by Machida *et al.* (1977) was. Machida *et al.* (1977) mentioned that discontinuities during RF replication were more pronounced in the HF4704 strain than in the wild type strain. This is consistent with our observations during φX viral strand synthesis (see Chapter II).

The closed circular duplex genome of the *E. coli* plasmid ColE1 also replicates unidirectionally from a fixed origin. Sakakibara (1978) found that in a cell extract prepared from a temperature-senstitive ligase-defective strain of *E. coli* , at both the permissive and non-permissive temperature, dTMP is incorporated into ColE1 DNA which ranges from 7S to 17S. The 17S DNA hybridized equally to both strands of ColE1 DNA, the intermediate-sized DNA hybridized preferentially to the lagging strand, while the 7S DNA hybridized preferentially to the leading strand. The accumulation of short molecules was believed to be due to the defective *E. coli* ligase activity. However, the joining of the short chains was not prevented by adding NMN to the system, although NMN will inhibit ligase in the final sealing of two strands and in closing nicks

generated by repair. Sakakibara (1978) suggested that ligase may be NMN resistant because of an association with other replication factors in a complex.

McFadden and Denhardt (1975) also postulated the existence of a free NMN-accessible ligase and an NMN-inaccessible ligase in an attempt to reconcile their results with those of Hess *et al.* (1973). Hess *et al.* had found that the joining of very short ϕX complementary strand molecules was not inhibited by NMN in an *in vitro* system, and concluded that there might be a second ligase activity in *E. coli*. McFadden & Denhardt (1975) found that the complementary strand fragments were not joined *in vivo* in a ligase defective host, which suggested that the known *E. coli* ligase was responsible. NMN may be capable of selectively inhibiting certain ligase activities. Also, the mechanism used to join molecules on the two strands at the replicating fork may differ, with the lagging strand fragments joined by the NMN-sensitive activity while the leading strand fragments are joined by the NMN-resistant activity. If this is the case, Olivera and Bonheffer (1972) may have been selectively inhibiting the joining of lagging strand fragment by adding NMN to their *in vitro* system.

Sternglanz *et al.* (1976) showed that the pulse labelled 10S molecules found in *B. subtilis* hybridize equally well with the separated strands of *B. subtilis* DNA. They also showed that the [^{3}H]thymidine-labelled 10S molecules from T7-infected *E. coli* B hybridize equally well to the separated strands of T7 DNA. Since T7 replication is bidirectional from an origin 17% from the left end of the molecule, and knowing the polarity of the strands relative to the left end, one would predict that if only one strand was synthesized discontinuously,

there would be an asymmetry with the short molecules hybridizing preferentially to one of the strands. That no such asymmetry was observed suggested that both strands were discontinuously copied.

Although the hybridization experiments of Herrmann *et al.* (1972) indicated that 10S and 38S molecules in *E. coli* were derived from separate strands (see Section a), they did not demonstrate that the 10S pieces were derived from the lagging strand. That was done by Louarn & Bird (1974) who pulse-labelled a λ lysogen of *E. coli* and hybridized the labelled DNA to the separated λ strands. λ is integrated into the *E. coli* chromosome at a specific site and with a known polarity. Integrated λ is passively replicated in a specific direction by the *E. coli* replication fork at the same time as the *E. coli* genome replicates. Therefore, it is an excellent probe to study the replication of *E. coli*. When Louarn & Bird (1974) pulse-labelled a λ*polA*$^+$ lysogen, they found the label equally distributed between 10S and 50S DNA molecules. The short and long molecules hybridized preferentially to the λ l and r strands respectively; this indicated that the long molecules were from the leading strand while the short molecules were from the lagging strand. Louarn & Bird (1974) concluded that only the lagging strand was synthesized discontinuously. However, these results may be biased, since the method they used to stop the pulse, a frozen solution containing KCN and pyridine, may allow preferential joining of leading strand molecules (see Section b). Also, in a λ*polA*$^-$ lysogen, Louarn & Bird (1974) found the pulse label predominantly in 10S and intermediate sized molecules which hybridized equally well to both strands of λ DNA. The reason given for this was that initiations occurred more frequently

on the leading strand in the *polA*⁻ strain, so both strands appeared to be discontinuously synthesized. However, it is more likely, in my opinion, that initiations occur equally frequently on the leading strands in both *polA*⁺ and *polA*⁻ strains, but that in the *polA*⁻ strain, the short leading strand molecules are more readily observed, because their joining is retarded by a delay in gap-filling mediated by DNA polymerase I, as originally suggested by Kuempell & Veomett (1970) and Schekman *et al.* (1971).

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2. INITIATION OF DNA SYNTHESIS IN PROKARYOTES

There are two levels of initiation of DNA synthesis: the initiation of a round of replication at the origin, and the initiation of synthesis of individual DNA molecules. The same mechanism may be responsible for both initiation events, although there is evidence in some systems that this is not necessarily so.

Initiation at the origin involves recognition of a specific site by the replication complex. Priming of synthesis of the DNA chains can occur in several ways. The 3' OH end of a nicked parental strand could function as a primer. A short oligonucleotide synthesized *de novo* on the exposed template strand could prime DNA synthesis. Finally, a protein could position a deoxynucleoside triphosphate residue on the template strand in such a way that the 3' OH end would be available to DNA polymerase as a primer. What follows is a detailed description of the events involved in the initiation of DNA synthesis in a number of prokaryotic systems, which include the single-stranded DNA phages, fd, G4, and φX, *E. coli*, the ColE1 plasmid, and bacteriophages T4, T7, P2, and φ29.

(a) Initiation of complementary strand synthesis on the single-stranded DNA phages

The genomes of the single-stranded DNA phages consist of circular molecules approximately 5000 nucleotides long of similar genetic complexity. The replicative cycle of these phages can be divided into 3 stages. The first stage, the synthesis of a complementary strand on the infecting viral strand, depends entirely on host proteins. Yet these

phages can be divided into 3 groups, each of which uses a different set of *E. coli* enzymes to prime synthesis of the complementary strand.

Earliest evidence of a difference came from studies which showed that synthesis of the complementary strand was rifampicin-sensitive for fd (Brutlag *et al.*, 1971; Wickner *et al.*, 1972) but not for ϕX (Silverstein & Billen, 1971). Rifampicin sensitivity suggested the involvement of the *E. coli* RNA polymerase. The reaction was divided into two stages: a rifampicin-sensitive stage which required all 4 rNTPs and a rifampicin-resistant stage where dNTPs were required (Wickner *et al.*, 1972). *In vitro*, a complementary strand could be synthesized on the fd template using only four purified proteins: *E. coli* RNA polymerase, DNA polymerase III holoenzyme, *E. coli* DNA binding protein (DBP), and all 4 rNTPs and 4 dNTPs (Geider & Kornberg, 1974; Hurwitz & Wickner, 1974). Geider & Kornberg suggested that DBP covered the single-stranded DNA in a pre-initiation step which left a specific duplex region exposed. RNA polymerase synthesized a transcript which could be extended by DNA polymerase III holoenzyme. This hypothesis was supported by the localization of a gap in a specific region of the genome on the fd (fd is closely related to f1 or M13) complementary strand (Tabak *et al.*, 1974). Furthermore, in a fd DNA-RNA polymerase-DBP complex, a fragment about 120 nucleotides long was protected from nuclease digestion. This fragment mapped at the origin and possessed double strand characteristics which included resistance to the S1 single-strand specific nuclease (Schaller *et al.*, 1976). It was later found to contain 2 hairpin structures (Gray *et al.*, 1978). Isolation of the transcript revealed that it was 30 nucleotides long and precise localization on the genome showed that it

initiated with ATP at a dTMP residue that was not base paired near the stem of the hairpin structure closest to the origin. One side of the hairpin structure was transcribed, thus disrupting the base-paired duplex and allowing DBP to bind to the exposed strand. Presumably transcription stopped when the RNA polymerase encountered the DBP-template complex, and DNA synthesis initiated at any one of several nucleotides (Geider *et al.*, 1978).

Since the combination of DBP, RNA polymerase and DNA polymerase III holoenzyme can also synthesize a complementary strand on the ϕX viral strand *in vitro*, there must be factors *in vivo* which prevent this from happening (Geider & Kornberg, 1974). Vicuna *et al.* (1977 a, b) found that RNase H, which hydrolyzes the RNA of an RNA-DNA duplex, prevented DNA synthesis by the purified components on both ϕX and fd templates. Two discriminatory factors, α and β, formed a complex with DBP and with fd DNA, but not with ϕX DNA. A complementary strand could be synthesized on the complex composed of the two discriminatory factors, DBP, and fd DNA, by RNA polymerase and DNA polymerase III holoenzyme in the presence of RNase H. So there exist factors *in vivo* which allow certain reactions, but prevent others from happening. Loss of these factors allows reactions to occur *in vitro* which would not normally occur *in vivo*.

Initiation of complementary strand synthesis on the G4 viral strand differed from that on fd in one important aspect. The primer was synthesized, not by RNA polymerase, but by the *E. coli dnaG* protein in a reaction which required only ATP and GTP of the 4 rNTPs, although the possibility that trace amounts of UTP and CTP were present as contaminants was not ruled out (Zechel *et al.*, 1975). Synthesis of the complementary strand on the G4 template required only the *dnaG* protein, DBP, and DNA polymerase III holoenzyme. The transcript was synthesized

at a unique site, suggested by the association of [α-^{32}P]ATP and [α-^{32}P]GTP label with only one small *Eco*RI fragment (Bouche *et al.*, 1975). Sequence complexity of the RNase T_1 fingerprint digest of the primer suggested a length of 20 to 25 nucleotides (Bouche *et al.*, 1975). This was confirmed by sequencing the isolated primer. It was found to start with an ATP residue, to contain a hairpin region of 1 A-U and 7 G-C base pairs (the fd primer also initiated with ATP, but only one side of the hairpin was copied), and to terminate at any one of four nucleotides, which resulted in primers from 26 to 29 residues long (Bouche *et al.*, 1978).

A novel property of the *dnaG* protein was the discovery that it could use dNTPs in place of rNTPs to synthesize primers (Wickner, 1977). The *dnaG* protein is the first known polymerase capable of the *de novo* synthesis of a nucleotide chain starting with a dNTP residue, although it can only synthesize very short DNA chains. The reported precursor requirements of the *dnaG* protein vary; efficient primer synthesis has been said to occur with only dTTP (or UTP) and dGTP (or GTP) in a reaction stimulated by ADP (Wickner, 1977); or only ATP and GTP (Zechel *et al.*, 1975; Rowen & Kornberg, 1978); or ATP (or ADP) and GTP (or dGTP) (Sumida-Yasumoto *et al.*, 1979). The general concensus is that only two or three of the rNTPs and dNTPs were required. Since a minimum of two nucleoside triphosphates appeared to be necessary, it has been suggested that a dinucleotide may function as an efficient primer (Rowen & Kornberg, 1978). Primers composed of dNTPs tend to be much shorter than primers composed of rNTPs (Rowen & Kornberg, 1978; McMacken & Kornberg, 1978; Sumida-Yasumoto *et al.*, 1979). There is some controversy over what occurs in the presence of both rNTPs and dNTPs. Sumida-Yasumoto *et al.* (1979) reported that when both rNTPs and dNTPs were present, even if there was a ten-fold excess of

rNTPs, the primer was composed preferentially of dNTPs. This was contradicted by Kornberg's group (Rowen & Kornberg, 1978; McMacken & Kornberg, 1978) who found that with relatively high rNTP concentrations, rNTPs were much more efficiently incorporated than dNTPs, and since the primers formed with rNTPs were longer, they primed DNA synthesis more efficiently.

The difference between fd and G4 in the initiation of complementary strand synthesis lies entirely in the enzyme which synthesizes the RNA primer: RNA polymerase with an fd template and the *dnaG* protein with the G4 viral strand. It was postulated that a "pilot protein", a minor phage capsid protein that remained associated with the DNA, played a role in recognizing and appropriating a particular replicative system (Jazwinski *et al.*, 1974). However, it later appeared that the nucleotide sequence at the origin, which differs in these two phages, was sufficient to account for the difference. When the G4 origin was cloned into M13, DNA synthesis initiated at the cloned G4 origin, in the presence and absence of rifampicin, *in vivo* and *in vitro* (Kaguni & Ray, 1979).

Initiation of complementary strand synthesis on the ϕX template was found to be a much more complicated process than those described for fd and G4. It was shown *in vitro* (Schekman *et al.*, 1972) that rifampicin-resistant RNA synthesis required all four rNTPs, and that ^{32}P was transferred from α-labelled dNTPs to ribonucleotides after alkaline hydrolysis. This ^{32}P transfer demonstrated a covalent attachment between RNA and DNA chains. These results were contradicted by a report that synthesis of a complementary strand required only ATP of the four rNTPs (Wickner *et al.*, 1972). This discrepancy can be accounted for by

the discovery that short oligoribonucleotides which can prime DNA synthesis on single-stranded ϕX and fd templates were present as contaminants in commercially availably NTP preparations (Masamune & Richardson, 1977). Also, priming of DNA synthesis by the *dna*G protein can occur with only dNTPs (Wickner, 1977; Rowen & Kornberg, 1978; McMacken & Kornberg, 1978).

Using reconstitution and complementation assays, the proteins that were required for ϕX complementary strand synthesis were isolated. Some of them remain genetically and functionally uncharacterized. The essential proteins included the *E. coli* DBP, *dna*B, *dna*C, and *dna*G proteins, and factors x, y, and z, DNA polymerase III plus elongation factors 1 and 2 (Wickner & Hurwitz, 1974) or factors i and n and the DNA polymerase III holoenzyme (Schekman *et al.*, 1975). Factor n was later resolved into n and n' (Meyer *et al.*, 1979), and it is very likely that factors x, y, and z described by the Hurwitz group are identical to factors i, n, and n' described by Kornberg's co-workers.

The reaction was divided into two steps: a pre-initiation step which required only ATP, the *dna*B and *dna*C proteins, DBP, and the three replication factors x, y, z, followed by a synthesis step which required the *dna*G protein, DNA polymerase III plus the necessary factors, and dNTPs plus ATP (Wickner & Hurwitz, 1975; Wainer *et al.*, 1976). It was suggested that the *dna*B and *dna*C proteins, which formed a complex isolatable by gel filtration *in vitro*, participated in a reaction which transferred the *dna*B protein to ϕX DNA, simultaneously releasing the *dna*C protein (Wickner & Hurwitz, 1975). Although the role of factor x was uncertain, it was found that the binding of factors y and z to the DBP-coated

ϕX template preceded binding of the *dnaB* protein (Wickner, 1978a). Once the *dnaB* protein was bound, the *dnaG* protein was able to synthesize primers on the ϕX template (McMacken *et al.*, 1977). Primer synthesis could occur in the absence of DNA synthesis at multiple, probably random, sites (McMacken *et al.*, 1977). The *dnaG* protein synthesized RNA primers which ranged from 14 to 50 nucleotides in length, and DNA primers from 4 to 20 nucleotides long (McMacken & Kornberg, 1978). From 5 to 8 primers were synthesized on each circle at regular intervals along the template strand (McMacken *et al.*, 1978). It was suggested that the *dnaB* protein moved processively along the template, functioning as a "mobile promoter" for primer synthesis. The *dnaG* protein recognized a secondary structure generated by the *dnaB* protein-template complex, and synthesized a primer at this site (McMacken *et al.*, 1978).

To summarize, the synthesis of a complementary strand on the ϕX viral strand is believed to be initiated by the following series of events (Wickner, 1979: Meyer *et al.*, 1979): (1) replication factors y and z bind to the DBP-covered ϕX DNA; (2) the *dnaB* and *dnaC* proteins form a complex in an ATP-dependent reaction; (3) the *dnaB* protein is transferred to the factor y-z-DBP-ϕX DNA complex in a reaction which requires factor x and releases the *dnaC* protein; (4) *dnaB* protein migrates processively along the template, hydrolyzing ATP; (5) *dnaG* protein, or primase, recognizes a structure generated by the *dnaB* protein-template complex, and synthesizes primers at regularly spaced intervals along the genome.

In spite of the capacity of this multienzyme system to transcribe multiple primers on ϕX DNA, it cannot initiate primer synthesis on any other template, presumably because one or more of the proteins which

transfer the *dnaB* protein to the ϕX template recognize a specific site in the ϕX genome (McMacken & Kornberg, 1978). It has recently been found that in the absence of DBP, the *dnaB* and *dnaG* proteins alone are sufficient to synthesize multiple primers on ϕX, G4, and M13 single-stranded DNAs (Arai & Kornberg, 1979). The unique primer is synthesized on the M13 and G4 templates only in the presence of DBP. And several additional proteins are required to bind the *dnaB* protein to the ϕX template in the presence of DBP. The non-random size distribution and unique electrophoretic pattern of the primers made on the ϕX, G4, and M13 templates by the *dnaB* - *dnaG* proteins suggests that certain regions may be preferentially transcribed (Arai & Kornberg, 1979).

(b) Initiation of ϕX viral strand DNA synthesis

Events involved in the synthesis of a complementary strand on the ϕX template *in vitro* are fairly well characterized, and since the same proteins are required to synthesize complementary strands during RF replication (Dumas, 1978), it is possible that the same series of events may occur on the displaced viral strand. Synthesis of the viral strand, however, was believed to occur by a fundamentally different mechanism. The discovery that brief pulses of [^{3}H]thymidine labelled viral strands longer than genome length (Dressler & Denhardt, 1968; Dressler, 1970; Schröder *et al.*, 1973) supported the hypothesis that ϕX viral strands were synthesized by a rolling circle model (Gilbert & Dressler, 1968), where the 3' OH end of a nicked parental strand functioned as a primer for DNA polymerase III. *In vitro* studies which showed that ϕX viral strands could be synthesized with only four proteins (the ϕX gene A protein, *rep*, DBP, and DNA polymerase III holoenzyme) provided additional

support for this model (Eisenberg *et al.*, 1976, 1977; see Chapter IV for details).

However, other investigators found that ϕX viral strand synthesis *in vitro* required in addition to these four proteins, the products of genes *dna*B, *dna*C, *dna*G, and other unidentified proteins (Sumida-Yasumoto & Hurwitz, 1977; Sumida-Yasumoto *et al.*, 1978). It has been reported that the *dna*G protein is required continuously for ϕX viral strand synthesis *in vivo* (McFadden & Denhardt, 1975). Also, there have been reports of short viral strand intermediates both *in vivo* (Machida *et al.*, 1977; Hours *et al.*, 1978) and *in vitro* (Sumida-Yasumoto *et al.*, 1978; 1979). Finally, Machida *et al.* (1977) have demonstrated the presence of ribonucleotides on about half of the short molecules of both viral and complementary strand origin which they isolated during ϕX RF replication, using the spleen exonuclease assay (see next section for detailed description of this assay). These experiments are extremely important because the demonstration of ribonucleotide primers at the 5' ends of short molecules derived from the potentially continuous strand is convincing evidence that the molecules were initiated *de novo*.

Although gene A protein-catalyzed nicking at the origin signals the initiation of a round of ϕX replication (see Denhardt, 1977 for review), synthesis of the short viral strand molecules on the exposed template may be initiated in a manner similar to the complementary strand molecules. The ϕX gene A protein has been described as a multifunctional enzyme in DNA replication (Eisenberg *et al.*, 1977) which (1) nicks the viral strand at the origin to initiate a round of replication; (2) participates in a complex with the *rep* protein which unwinds the two strands at the replicating fork; (3) nicks again at the regenerated origin to release

a full-length viral strand; (4) ligates the excised DNA to form a circular viral strand.

(c) Evidence for RNA primers in *E. coli*

The earliest observation that DNA synthesis in *E. coli* was independent of rifampicin (Silverstein & Billen, 1971) was contradicted by the finding that in cells synchronized by amino acid depletion, a round of replication was completed, but no new rounds were initiated in the presence of rifampicin (Lark, 1972a). Initiation of DNA synthesis at the origin in *E. coli* required the rifampicin-sensitive synthesis of a transcript by RNA polymerase at a time when chloramphenicol-sensitive protein synthesis was no longer required. Further initiation of short DNA chains was independent of rifampicin.

DNA replication in *E. coli* is incredibly complicated; studies with mutants have revealed that a large number of proteins are required (see Wickner, 1978b, for review). The proteins coded by genes *dnaA*, *dnaB*, *dnaC*, *dnaI*, *dnaP*, and RNA polymerase are involved in initiation of a round of replication. DNA elongation requires the products of genes *dnaB*, *dnaC*, *dnaG*, *dnaZ*, *dnaE* (DNA polymerase III), *lig*, *polA*ex (the 5' to 3' exonuclease activity), *cou* and *nalA* (two components of gyrase), and DBP. The products of genes *dnaJ*, *dnaK*, *dnaL*, and *dnaM* remain to be characterized. Since DNA replication occurs on the separated strands at the replication fork, and since *E. coli* requires all of the proteins required to synthesize a complementary strand on the ϕX template, as well as a few additional ones, it is quite possible that the mechanisms involved in the *in vivo* replication of the *E. coli* chromosome will share some similarities with the *in vitro* reactions characterized for ϕX.

The first physical evidence of an RNA primer associated with *E. coli* DNA (Hirose *et al.*, 1973) was a buoyant density shift of the nascent fragments in Cs_2SO_4 gradients. The size of the shift led to estimates of 50 to 100 nucleotide-long RNA molecules attached to the Okazaki fragments. Labelling the nascent molecules with [^{32}P]ATP and kinase, before and after alkali treatment, indicated that one RNA molecule was associated with every DNA molecule (Hirose *et al.*, 1973). Later it was shown that even after denaturation, non-covalent interactions between RNA and DNA molecules could produce shifts in buoyant density gradients (Probst *et al.*, 1974). And the end-labelling results were suspect when it was found that polynucleotide kinase catalyzed an exchange reaction between the γ-phosphate of ATP and the 5' phosphate of a polynucleotide chain (van de Sande *et al.*, 1973).

Also other investigators failed to reproduce Okazaki's results. Jacobson and Lark (1973) could find no evidence that the ^{32}P-label on the 5' ends of short molecules from *E. coli* 15T$^-$ was either alkali or RNase labile. This is probably due to the fact that no attempts were made to minimize or correct for the exchange reaction catalyzed by kinase. Also only a small fraction of the ^{32}P-labelled molecules isolated by Jacobson and Lark (1973) behaved as nascent intermediates. Since end-labelling is not specific for nascent molecules, the presence of large numbers of non-nascent molecules could easily obscure the existence of a few nascent molecules with alkali-labile 5' ends.

When Okazaki *et al.* (1975) found conditions which minimized the exchange reaction catalyzed by kinase and allowed selective labelling of 5' hydroxyl termini, they were able to demonstrate that a *polA*ex1

strain(deficient in the 5' to 3' exonuclease of DNA polymerase I) contained about 5 times as many RNA-linked DNA molecules which could be labelled after alkali treatment as the wild type strain. Furthermore, the number of molecules with alkali labile ^{32}P-labelled ends corresponded well to the number of molecules with hydroxyl termini which could be end-labelled after alkali treatment (Ogawa *et al.*, 1977).

These experiments have been criticized on the grounds that the production of a 5' hydroxyl terminus after alkali treatment does not prove the existence of an RNA chain at the 5' end of an Okazaki piece, since the alkali-labile apyrimidinic sites after uracil excision might also give rise to a 5' hydroxyl terminated chains (Alberts & Sternglanz, 1977). One argument against this was that both alkali and RNase produced similar numbers of 5' hydroxyl termini in nascent molecules (Kurosawa *et al.*, 1975). Also it has recently been demonstrated with chemically synthesized substrates that when molecules containing apyrimidinic sites were hydrolyzed in alkali, 5' phosphate, not 5' hydroxyl, terminated chains were produced (Okazaki *et al.*, 1979).

One limitation of the polynucleotide kinase assay is that it is not specific for nascent DNA. An alternate method was developed which used spleen exonuclease to degrade molecules with 5' hydroxyl termini generated by alkali or RNase-catalyzed removal of ribonucleotides (Kurosawa *et al.*, 1975). This method was specific for [^{3}H]thymidine pulse-labelled nascent molecules and allowed estimates to be made of the numbers of both RNA-linked and RNA-free DNA pieces. Using this assay, Kurosawa *et al.* (1975) showed that RNA-linked DNA molecules were more frequent among shorter molecules, and less frequent among molecules

recovered after longer pulses. Again the *polA*ex1 and *polA*12 (deficient in the polymerase activity of DNA polymerase I) strains were found to contain about 5 times as many RNA-linked pieces as the wild-type strains.

Ogawa *et al.* (1977) isolated the ribonucleotide primers by degrading the 5' terminally labelled nascent molecules with a combination of pancreatic DNase and the 3' to 5' exonuclease of T4 DNA polymerase. Most of the deoxyribonucleotide moiety of the nascent molecules was degraded, and the mixture of di - to tetranucleotides which remained were analyzed for the presence of ribonucleotides. Ribonucleotides at the 5' end were measured by the release of ^{32}P-labelled pNps after alkaline hydrolysis; 88% of the DNase-resistant molecules contained a ribonucleotide at the 5' terminus by this criterion. The 3' ends were analyzed by periodate and aniline treatment. These chemicals will hydrolyze molecules with a 3' ribonucleotide. Only 40% of the di, and 10% of the trinucleotides were sensitive to periodate. Therefore, the majority of the di- to tetranucleotides are co-oligomers of ribo- and deoxyribonucleotides, containing from 1 to 3 ribonucleotides at the 5' end. So the RNA primers in *E. coli* are very short. Labelling the 5' OH groups after alkali with [^{32}P]ATP and kinase, and analyzing the ^{32}P-labelled dNMPs produced after nuclease digestion, revealed that all four dNMPs were present with about equal frequency at the RNA-DNA junctions.

Using the spleen exonuclease assay, Miyamoto & Denhardt (1977) also found evidence for RNA-linked DNA molecules which seemed to be more frequent among the shorter molecules in a *polA*ex2 strain. End-labelling with [^{32}P]ATP and kinase, after correcting for exchange, revealed that the number of ^{32}P-labelled pNps correlated well with the number of

hydroxyl groups which could be labelled after alkaline hydrolysis. Although Ogawa *et al.* (1977) were unable to detect any unique ribonucleotides at the 5' terminus, Miyamoto & Denhardt (1977) found that mostly pAp and pGp were released. This is not too surprising, since the *dnaG* protein which is required *in vivo* to initiate synthesis of Okazaki pieces (Lark, 1972b) has been shown to be able to synthesize primers *in vitro* using only ATP and GTP (Zechel *et al.*, 1975; Rowen & Kornberg, 1978).

Miyamoto & Denhardt (1977) did not find a significant number of ribonucleotide linkages in wild type strains, using either the spleen exonuclease or polynucleotide kinase assays. Also, they found that fewer pNps were released from the shorter than from the longer molecules. But since there are very many (as many as 2000) of these short molecules in the cell, only a small proportion of which may be nascent, it is not surprising that ribonucleotide termini could be detected on very few of them. The evidence is reasonably convincing that at least in the *polA*ex strain of *E. coli*, RNA primers are covalently attached to DNA molecules.

It is generally assumed that the ribonucleotide-terminated molecules are nascent or newly synthesized. This may not be the case, because some of the short non-nascent molecules, recovered under conditions where no DNA synthesis was occurring, were found to have ribonucleotides at the 5' terminus, as measured by the release of ^{32}P-labelled pNps after alkaline hydrolysis (Denhardt *et al.*, 1979). The ribonucleotide primers may not always be very rapidly removed.

(d) Initiation of Col El DNA synthesis

Replication of *E. coli* plasmid ColE1 does not require any plasmid-coded proteins, but uses only host cell proteins (Tomizawa *et al.*, 1975). As with *E. coli*, the initiation of a round of ColE1 replication is rifampicin-sensitive and requires all four rNTPs; continued replication is rifampicin-resistant (Sakakibara & Tomizawa, 1974). This suggested the involvement of *E. coli* RNA polymerase in the synthesis of the early replicative intermediate, the 6S L fragment. Bird & Tomizawa (1978) demonstrated the presence of RNA at the 5' ends of the 6S L fragments by *in vitro* labelling with [α-^{32}P]dNTPs and ^{32}P-transfer after alkaline hydrolysis. It was estimated that 20% of the 6S L fragments contained RNA-DNA junctions. The spleen exonuclease assay revealed that as many as 40% of the molecules could have ribonucleotides at the 5' end.

A 580 nucleotide-long stretch encompassing the origin was found to be essential for ColE1 replication (Backman *et al.*, 1979). It has been suggested that a 100 nucleotide-long transcript synthesized in this region was processed and then annealed to the origin where it served as primer, but supporting data are not yet available (Backman *et al.*, 1979).

Itoh & Tomizawa (1979) have studied the first stage of the replication of the ColE1 plasmid *in vitro*. *In vitro*, ColE1 replication initiated at the origin in the presence of RNA polymerase, RNase H, DNA polymerase I and gyrase. Gyrase was believed to facilitate unwinding of the parental strands. RNA polymerase synthesized the primer which was processed by RNase H. RNase H did not just remove RNA annealed to regions of the genome other than the origin, and so increase the proportion of fragments

which initiated at the origin, it also increased the overall efficiency of DNA synthesis several fold, and so was believed to process the primer RNA, perhaps by nicking at a specific site or creating a specific 3' OH end. Since both the 5' to 3' exonuclease and polymerase activities of DNA polymerase I were required to extend the primer, it could occur by nick translation from a specific site in the primer. The aberrant initiations which occured in the presence of these proteins could be inhibited by another factor which has been partially purified.

The initiation of a round of ColE1 replication, which occurs in this first stage with the synthesis of the 6S L fragment, differs from the stage which follows it. Synthesis of the rest of the molecule requires other proteins used by *E. coli* in its replication, including the *dnaB*, *dnaC*, *dnaG*, and *dnaZ* proteins, and DNA polymerase III (Itoh & Tomizawa, 1979).

(e) Initiation of bacteriophage T4 and T7 DNA synthesis

The bacteriophage T7 genome consists of a 40,000 nucleotide linear duplex. Replication is bidirectional from an origin 17% from the left end, and requires 6 phage-coded gene products *in vivo* (Richardson *et al.*, 1979). Using the spleen exonuclease assay, Okazaki *et al.* (1979) found that approximately half of the 10S fragments labelled *in vivo* with a brief pulse of [^{3}H]thymidine with a *ts* gene 6 T7 mutant at the restrictive temperature (gene 6 codes for a 5' to 3' exonuclease similar to the 5' to 3' exonuclease of DNA polymerase I) were terminated with ribonucleotides. With wild type T7, much lower levels of RNA-linked DNA molecules were found, even in *polA*ex1 strains. Alkaline hydrolysis following end-labelling with kinase and [γ-^{32}P]ATP released predominantly

pAp and pCp (44% each). Since the T7 primers synthesized *in vitro* are terminated exclusively with ATP (Richardson *et al.*, 1979; Scherzinger *et al.*, 1977), the primers on the molecules isolated *in vivo* are probably partially degraded. The ribonucleotide portion of these molecules was recovered after degrading the deoxyribonucleotides with the 3' to 5' exonuclease of T4 DNA polymerase. The tri- to hexanucleotides recovered were analyzed separately by periodate and aniline treatment. The intact primers were found to be predominantly four nucleotides long, terminated at the 5' end with pppApC. This is consistent with *in vitro* results (Richardson *et al.*, 1979; Scherzinger *et al.*, 1977). Okazaki *et al.* (1979) state that if primers are synthesized at every complementary sequence in the T7 template, primers could be synthesized at 20 to 60 nucleotide intervals. The size of Okazaki fragments indicates that primers are elongated only at 1000 nucleotide intervals. Therefore, there must exist other factors which allow the selection of one site from many potential intiation sites *in vivo*.

In vitro, in a reaction not coupled to DNA synthesis, the T7 gene 4 protein synthesized the tetranucleotide pppApCpCpA (Scherzinger *et al.*, 1977) or pppApCpCpC (Richardson *et al.*, 1979) on single-stranded DNA templates. The precursor requirements of the T7 gene 4 protein, like those of the *E. coli dnaG* protein, were satisfied by only two (ATP and CTP) of the four rNTPs. If DNA synthesis with [α-^{32}P]dNTPs was coupled to T7 gene 4 protein primer synthesis, ^{32}P-transfer after alkaline hydrolysis revealed that all four dNTPs were present at the 3' terminus (Richardson *et al.*, 1979). The T7 gene 4 protein has also been called primase, but it differs from the *E. coli dnaG* protein in several important

ways (Hillenbrand *et al.*, 1979): (1) it cannot use dNTPs; (2) it synthesizes primers equally well on several denatured DNA templates; (3) it doesn't require additional replication factors. It has been suggested that the T7 gene 4 protein stimulates DNA synthesis primed by short synthetic oligoribonucleotides by stabilizing the RNA-DNA hybrid until DNA polymerase can extend the primer (Hillenbrand *et al.*, 1979). The T7 primase in conjunction with the T7 DNA polymerase can catalyze strand separation with the concomittant hydrolysis of dNTPs, similar to the *rep* protein of *E. coli*; the two proteins can also initiate DNA synthesis *in vitro* on nicked duplex templates (Hillenbrand *et al.*, 1979; Richardson *et al.*, 1979). So the T7 gene 4 protein can also be called a multifunctional DNA replication enzyme, although the functions it performs differ from those of the ϕX gene A protein.

The bacteriophage T4 genome is a linear duplex of about 200,000 nucleotides. Replication is bidirectional from several specific origins. There are a number of similarities between the T4 and the T7 systems, which suggest that initiation of DNA synthesis by these two phages may occur in essentially the same way. Okazaki *et al.* (1979) determined that primers on the T4 pulse-labelled molecules were predominantly pentanucleotides, terminated at the 5' end with pApC. Since there was no way to enrich for RNA-linked DNA molecules as in the T7 system (by using an exonuclease-deficient mutant) primers were found on less than 10% of the nascent molecules. *In vitro*, the T4 gene 41 and gene 61 proteins together synthesized short oligoribonucleotides on single-stranded DNA templates (Liu *et al.*, 1979). Only ATP and CTP of the four rNTPs were absolutely required, and the primers were 6 to 8

nucleotides long, and terminated at the 5' end with the sequences pppApCpC and pppApCpA (Liu *et al.*, 1979).

(f) Evidence for RNA primers in bacteriophage P2

Using the spleen exonuclease assay, Okazaki *et al.* (1979) showed that 25 to 50% of the short nascent P2 molecules derived from either the L or H strands have at least one ribonucleotide at the 5' terminus. In prokaryotic systems, only for bacteriophage ϕX (Machida *et al.*, 1977) and P2 (Okazaki *et al.*, 1979) have ribonucleotides been associated with Okazaki fragments derived from both strands. These experiments are important, as already mentioned, because the demonstration of ribonucleotide primers on molecules derived from the potentially continuous strand is the most convincing proof that the strand was discontinuously synthesized.

(g) Novel priming mechanism for bacteriophage ϕ29

ϕ29 genome is a linear non-permuted DNA duplex 18,000 base pairs long. A protein has been found covalently associated with the 5' ends of the duplex (Harding & Ito, 1976; Salas *et al.*, 1978). The terminal protein has been tentatively identified as the phage gene 3 protein; this protein is required continuously for ϕ29 DNA replication (Ito *et al.*, 1979). Replicating ϕ29 DNA molecules visualized in the electron microscope consist of linear duplexes with single and double stranded tails and branches (Ito *et al.*, 1979). Since circular replicating forms have not been observed, the mechanism of initiation cannot be by RNA primers, since the removal of the primer would leave a gap at 5' end which could not be filled in by any known polymerase activity.

Although the association of the protein with nascent molecules has not yet been demonstrated, it is possible that the protein covalently attached to the first dNTP residue is positioned on the template so that the 3' hydroxyl end of the dNTP can function as a primer for DNA polymerase. Denhardt (1972) suggested that a protein which positions a dNTP residue in this fashion might function as a primer and called these hypothetical proteins "π proteins".

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CHAPTER II

Evidence that the ϕX174 Viral Strand is Synthesized Discontinuously

* Most of this chapter has been published
[Matthes, M., and Denhardt, D.T. (1980). J. Mol. Biol. 136, 45-63]

1. INTRODUCTION

The replication of bacteriophage ϕX174 has been extensively characterized both *in vivo* and *in vitro* (see Denhardt, 1977 for review). The process can be divided into three distinct stages: (i) conversion of the single-stranded viral DNA to the duplex replicative form (RF), (ii) RF replication, during which multiple copies of RF are synthesized semiconservatively, (iii) synthesis of viral strand DNA using the complementary strand of RF as the template (Dressler and Denhardt, 1968; Sinsheimer *et al.*, 1969). It is generally accepted that synthesis of the complementary strand is a discontinuous process, initiated at many different locations on the viral strand template (Eisenberg *et al.*, 1975; McFadden and Denhardt, 1975). Synthesis of the viral strand, however, is believed to be a continuous process. According to the rolling circle model (Gilbert and Dressler, 1969), the viral strand is elongated from the 3' OH generated by gene A protein nicking at a defined site on the genome, now mapped on the complete ϕX sequence (Langeveld *et al.*, 1978; Sanger *et al.*, 1977). The rolling circle model is supported by *in vitro* evidence that net synthesis of the viral strand requires only 4 proteins: the ϕX-coded gene A protein and *E. coli* proteins *rep*, DBP, and DNA polymerase III holoenzyme (Eisenberg *et al.*, 1976). These observations are difficult to reconcile with the *in vivo* finding that the *dna*G protein is required for both viral and complementary strand synthesis (McFadden & Denhardt, 1975). The *dna*G protein, or primase, has been shown to incorporate deoxyribo- and ribo-nucleotides into short oligonucleotides which then serve as primers for the synthesis of DNA chains (Rowen & Kornberg, 1978; Wickner, 1977). The rolling circle model is

also inconsistent with reports of viral strand intermediates shorter than unit length (Machida *et al.*, 1977; Hours *et al.*, 1978; Sumida-Yasumoto *et al.*, 1978).

Using a new method of stopping DNA synthesis we have studied the nascent intermediates present during the final stage of ϕX174 DNA replication. We find that 40-50% of the [^{3}H]thymidine incorporated in a brief pulse stopped by bringing the infected culture rapidly to 100°C is present in DNA molecules shorter than unit length. Approximately 90% of these molecules are incomplete ϕX viral strands. The pulse-labelled molecules, which range from very short to unit length, are not generated by the stopping and isolating procedure since ^{32}P-labelled infecting parental viral strands remain relatively intact. The proportion of pulse label found in short intermediates varies with pulse length, stopping procedure, aeration level of the infected culture, and host strain. There is no significant difference in the abundance of short nascent intermediates in *ung* and *ung*$^+$ strains, suggesting that the short molecules do not result from the excision of uracil by uracil-DNA glycosylase. The ^{3}H-labelled short molecules hybridize to all regions of the ϕX genome, but preferentially to the region around the origin-terminus of replication. The preferential hybridization of pulse-labelled short molecules to the terminus may result from an oversynthesis of the terminal sequences due to the utilization of an initiation site in the central region of the genome. The relative amount of ^{3}H in the unit-length molecules which hybridizes to restriction enzyme fragments increases from the origin to the terminus, indicating that these molecules were completed during the pulse interval. Sensitivity to spleen exonuclease

after alkali or RNase treatment suggests that some of the ^{3}H-labelled short molecules isolated both during viral strand synthesis and during RF replication have at least one ribonucleotide at the 5' end. We conclude that the major mode of ϕX viral strand DNA replication is a discontinuous process, not continuous as proposed by the rolling circle model.

2. MATERIALS AND METHODS

(a) Bacteria and bacteriophage

φX*am*3 is a lysis-defective gene E mutant. *E. coli* C, CR, and HF4704 were described by Denhardt (1969). BW 264 (*E. coli* C, *ts thyA*, *ung*-1, Tn 10 [55 min], *nad*B) and BW 265 (*E. coli* C, *ts thyA*, Tn 10 [55 min], *nad*B) generously supplied by Bernard Weiss, are P1 transductants of HF4704.

(b) Media, chemicals, buffers, and radioactive compounds

TKCaB medium contained 1% Tryptone, 0.5% KCl, and 1 mM $CaCl_2$. TPG and mT3xD media are described by Denhardt *et al.* (1968).

Salmon sperm DNA was obtained from Sigma. Nitrocellulose (a gift of Hercules Chemical Co.) was prepared for column chromatography as described by Boezi & Armstrong (1967).

TE buffer is 50 mM Tris-HCl (pH 8) 10 mM Na_2EDTA, T2E buffer is 50 mM Tris-HCl (pH 8) 20 mM Na_2EDTA. TEK buffer is TE buffer containing 0.5 M KCl. Borate is 0.05 M sodium tetraborate decahydrate. SSC is 0.15 M NaCl, 0.015 M sodium citrate adjusted to pH 7 with a few drops of 6 M HCl. E buffer is 0.05 M Tris, 0.02 M sodium acetate, 1 mM Na_2EDTA adjusted to pH 7.2 with glacial acetic acid.

[methyl-^{3}H]thymidine (50 Ci/mmol), [methyl-^{3}H]thymine (50Ci/mmol), [^{32}P] phosphate, Aquasol, Aquassure, and Omnifluor were purchased from New England Nuclear. [γ-^{32}P]ATP at a specific activity of 10^6 cpm/pmol, prepared as described by Schendl and Wells (1973), was generously donated by C. Miyamoto.

(c) Infection, pulse-labelling, purification of nascent DNA

*E. coli*C was grown in 200 ml TKCaB medium at 33°C with shaking at 170 rpm in a New Brunswick Scientific Gyratory H_2O bath unless otherwise indicated. Cultures at a cell density of 3 - 4 x 10^8/ml were infected with φX*am*3 at a multiplicity of infection 5 - 10. Thirty minutes later, the infected culture was transferred to a 28°C bath and allowed five minutes to equilibrate at the lower temperature. Thirty-five minutes after infection, 2 mCi [^{3}H]thymidine was injected into the culture, which was immediately and rapidly poured into a half volume of a vigourously boiling solution containing 2% sodium dodecyl sulfate (SDS), 3% phenol (distilled and stored under argon) and 10 mM Na_2EDTA pH 7.2 (Miyamoto & Denhardt, 1977). The mixture was swirled and brought to an incipient boil over high heat on a bunsen burner in less than a minute. After cooling on ice for 45 minues, the cell ghosts and SDS precipitate were removed by centrifugation at 5,000 rpm for 30 minutes in the HG4 rotor of the Sorvall RC3. The supernatant was combined with 30 ml of 3M Na acetate pH 5.5 and 600 ml isopropanol and stored at -20°C overnight. The isopropanol precipitate was pelleted by centrifugation at 5,000 rpm for 30 minutes in the HG4 rotor of the Sorvall RC3, air dried for an hour, resuspended in 10 ml T2E buffer, and combined with 100 µl each of 10% SDS and 10 mg/ml Pronase (self-digested at 37°C for 2 hours). The turbid solution cleared after 2 hours at 37°C, and was then extracted at room temperature with 2 volumes of phenol: chloroform (1:1) equilibrated with borate. The nucleic acids were precipitated in 30 ml corex tubes with sodium acetate and isopropanol as described above and collected by centrifugation at 10,000 rpm for one hour in the HB4 rotor of the Sorvall RC2B. The pellet

was resuspended in 2 ml TEK buffer, heated at 100°C for 60 seconds, then applied to a 1.8 cm by 4 cm nitrocellulose column at room temperature which had been equilibrated with TEK buffer. The column was washed with 100 ml TEK buffer and the DNA was eluted with 50 ml TE buffer; appropriate fractions were precipitated with sodium acetate and isopropanol. The precipitate was resuspended in 0.5 to 1 ml T2E buffer and centrifuged in alkaline or neutral sucrose gradients. Neutral sucrose gradients (36 ml, 5-20% sucrose) containing 1 M NaCl, 1 mM Na_2EDTA, 50 mM Tris-HCl (pH 8), were centrifuged at 22,000 rpm for 17.5 hours at 10°C in the SW27 rotor of the Beckman L2-65B ultracentrifuge. Alkaline sucrose gradients (11 ml, 5-20% sucrose), containing 0.2 M NaOH, 0.8 M NaCl, 2 mM Na_2EDTA, 0.05% sarkosyl, on a 1 ml pad of 1.25 g CsCl/ml 0.2 M NaOH, were centrifuged at 36,000 rpm for 16.5 hours in the SW40 rotor.

Fractions were collected from the top using a Buchler auto-densiflow and polystaltic pump. Aliquots of neutral sucrose gradients were spotted on 1 inch squares of Whatman No. 3 filter paper. Alkaline sucrose gradient fractions were precipitated with two volumes of 10% TCA after adding 200 µg salmon sperm DNA as carrier. The precipitate was collected on Whatman GF filters. The filters were dried and the radioactivity determined by scintillation counting with toluene-Omnifluor.

(d) Preparation of φX viral strand DNA

E. coli C was grown in 500 ml of mT3xD at 37°C with aeration to a concentration of 3×10^8 cells/ml, and infected with φX*am*3 at a multiplicity of infection of 10. The phage were pre-incubated in 1 ml of medium at 37°C for 10 minutes before infection. If ^{32}P-labelled φX DNA was desired, *E. coli* C was grown in TPG and 5 mCi [^{32}P]phosphate was added at the time of infection. If ^{3}H-labelled φX DNA was desired, *E. coli* HF4704 was grown

in mT3xD and from 1 to 5 mCi [^{3}H]thymidine was added in equal aliquots at ten-minute intervals from 5 to 65 minutes after infection.

Two hours after infection, the cells were collected by centrifugation at 10,000 rpm for 20 minutes in the GSA rotor of the Sorvall RC2B. The cells were washed with 100 ml TE and resuspended in 40 ml TE. Lysozyme was added to a final concentration of 100 µg/ml, and the mixture was incubated for 30 minutes at 37°C. After the addition of TE buffer to 150 ml, the solution was adjusted to 1 M NaCl, and stirred at 4°C overnight. The precipitate was removed by centrifugation at 8,000 rpm for 10 minutes in the GSA rotor of the Sorvall RC2B. A total of 15 g PEG 6000 was added to the supernatant which was then stirred at 4°C for 10 to 20 hours. The precipitate was collected by centrifugation at 12,000 rpm for 20 minutes in the HB4 rotor of the Sorvall RC2B, and resuspended in 6 ml borate buffer. After an overnight incubation at 4°C with stirring, the undissolved material was removed by centrifugation at 10,000 rpm for 10 minutes in the SS34 rotor of the Sorvall RC2B. CsCl was added to the supernatant to a final concentration of 0.625 g per g sample. The CsCl gradients were centrifuged at 10°C and 40,000 rpm in the Type 40 or Type 50Ti rotors in the Beckman L2-65B ultracentrifuge. Fractions were collected from the bottom, and the lower phage-containing band was pooled and dialyzed for 24 hours at 4°C against 2 ℓ borate buffer. The dialyzed phage suspension was layered on two 36 ml sucrose gradients (5% to 20% sucrose (w/w), 1 M NaCl in borate buffer). The gradients were centrifuged for 3.5 hours at 25,000 rpm and 5°C in the SW27 rotor. Fractions were collected from the top. The more rapidly sedimenting band was pooled and dialyzed against borate buffer as described above. The phage suspension was extracted with an equal volume of borate-

saturated phenol for 10 minutes at 55°C. THe DNA in the aqueous phase was precipitated with sodium acetate and isopropanol. When the phage were radioactively labelled, gradient fractions were assayed by spotting aliquots on 1 inch squares of Whatman No. 3 filter paper. The papers were dried and the radioactivity determined by scintillation counting in toluene/ Omnifluor. When the phage were not radioactively labelled, gradient fractions were assayed by titre, and the sedimentation velocity centrifugation was omitted. Centrifugation in the Sovall RC2B was always performed at 0°C.

(e) Preparation of φX RF DNA

E. coli CR was grown in 500 ml mT3xD at 37°C with aeration to a concentration of 3×10^8 cells/ml, and infected with φX*am*3 at a multiplicity of infection of 10. The phage were pre-incubated in 1 ml of medium at 37°C for 10 minutes before infection. Chloramphenicol was added to a final concentration of 30 µg/ml 10 minutes after infection. For ^{3}H-labelled RF DNA, from 1 to 5 mCi [^{3}H]thymidine was added in equal aliquots at ten-minute intervals from 5 to 65 minutes after infection. For ^{32}P-labelled RF DNA, *E. coli* C was grown in TPG; 5 mCi [^{32}P]phosphate was added at the time of infection.

Two hours after infection, the cells were collected by centrifugation for 20 minutes at 10,000 rpm in the GSA rotor of the Sorvall RC2B. The cells were washed with 100 ml TE buffer, then resuspended in 36 ml TE buffer, and incubated with lysozyme at a final concentration of 200 µg/ml on ice for an hour. After the addition of SDS and Pronase to final concentrations of 1% and 500 µg/ml respectively, the lysate was incubated at 37°C for 4 hours. NaCl was added to 1 M, and the solution was mixed and left at 4°C overnight. The heavy white precipitate was pelleted at 0°C at 35,000

rpm in the Type 42 rotor and the Beckman L2-65B ultracentrifuge. The supernatant was extracted with an equal volume of phenol (equilibrated with borate buffer) for 15 minutes at 55°C. After cooling on ice, the phases were separated by centrifuging at room temperature for 10 minutes at 2000 rpm in a Sorvall GLC table-top centrifuge. The nucleic acids in the aqueous phase were precipitated with 0.1 volume 3 M sodium acetate (pH 5.5) and 2 volumes isopropanol. After 16 hours at -20°C, the precipitate was collected by centrifugation at 0°C for 1 hour at 12,000 rpm in the GSA rotor of the Sorvall RC2B. The pellet was resuspended in 6 ml T2E buffer, two ml aliquots of which were layered on three 36 ml sucrose (5% to 20% (w/v) sucrose) gradients, containing 1 M NaCl, 1 mM Na_2EDTA, 50 mM Tris-HCl (pH 8). The gradients were centrifuged for 16 hours at 25,000 rpm and 10°C in the SW27 rotor. Fractions containing the RF were precipitated with sodium acetate and isopropanol. The precipitate was collected by centrifugation for 1 hour at 10,000 rpm in the HB4 rotor of the Sorvall RC2B, and resuspended in 9 ml TE buffer. Ethidium bromide and CsCl were added to final concentrations of 500 µg/ml and 0.928 g/g solution respectively. The gradients were centrifuged at 40,000 rpm at 10°C in the Type 40 or Type 50Ti rotors of the ultracentrifuge. The RFI and RFII bands were separately pooled and precipitated twice with sodium acetate and isopropanol to remove ethidium bromide. The DNA was stored at -20°C in a small volume of TE buffer.

(f) Preparation of *E. coli* DNA

E. coli CR was grown in 200 ml mT3xD at 37°C with aeration to a concentration of 3×10^8 cells/ml. If ^3H-labelled *E. coli* DNA was desired.

^{3}H-thymine was added to a final concentration of 2 µci/ml. The cells were grown 90 minutes longer, then collected by centrifugation for 10 minutes at 8,000 rpm in the GSA rotor of the Sorvall RC2B. The cells were resuspended in 10 ml TE buffer, and incubated on ice for an hour with 20 µg/ml lysozyme. Pronase and SDS were added to final concentrations of 100 µg/ml and 1% respectively, and the sample was incubated at 37°C for 16 hours, then extracted three times with 2 volumes of borate-saturated phenol:chloroform (1:1, v:v) at 37°C for 30 minutes. The nucleic acids in the aqueous phase were precipitated with sodium acetate and isopropanol, then banded in CsCl gradients (1.25 g CsCl per g solution) to remove RNA. The DNA was stored at -20°C in a small volume of TE buffer.

(g) Enzymes and enzyme reactions

*Hae*III restriction endonuclease was purified essentially as described by Roberts *et al.* (1975). ϕX RFI was incubated at 36°C in 100 µl containing 10 µg DNA, 6 mM $MgCl_2$, 6 mM mercaptoethanol, 6 mM Tris-HCl pH 7.4, and enough enzyme to produce a limit digest in 24 hours. Na_2EDTA at a final concentration of 20 mM was added to stop the reaction.

*Hin*dII restriction endonuclease was purchased from Boehringer-Mannheim. ϕX RFI, at a concentration of 100 µg/ml, was incubated with 1 unit *Hin*dII per µg DNA in 10 mM Tris-HCl pH 7.6, 50 mM NaCl, 10 mM $MgCl_2$, 15 mM dithiothreitol, for 24 hours at 36°C. The reaction was stopped as described for *Hae*III.

Polynucleotide kinase was purified as described by Richardson (1971) from T4-infected *E. coli* B cells generously provided by Dr. P.D. Sadowski of the University of Toronto. DNA at a concentration of 100 µg/ml in 2 mM KPO_4 pH 7.4, 10 mM $MgCl_2$, 20 mM mercaptoethanol, 25 µM ATP and

4 units/ml polynucleotide kinase were incubated for 2 hours at 37°C. The reaction was stopped by adding 20 mM Na_2EDTA. For quantitative end-labelling, 10 µM [γ-^{32}P]ATP at a specific activity of 10^6 cpm/pmol was substituted for the unlabelled ATP. The unincorporated [γ-^{32}P]ATP was removed by three sodium acetate and isopropanol precipitations, and nitrocellulose column chromatography.

RNase A and T_1, purchased from Worthington, were pre-incubated as a 20 mg/ml solution in 0.1 M sodium acetate pH 5.5 at 80°C for 5 minutes. DNA at a concentration of 100 µg/ml in 0.05 M Tris-HCl pH 7, 0.02 M Na_2EDTA was incubated with 60 units/ml RNase A and 120 units/ml RNase T_1 for 3 hours at 37°C. The reaction was stopped by precipitating the DNA with Na acetate and isopropanol.

Bacterial alkaline phosphatase, purified according to the method described by Weiss *et al.* (1968), was generously donated by Neil Miyamoto. DNA at a concentration of 100 µg/ml in 0.05 M Tris-HCl pH 9 was incubated with phosphatase (2 units/ml) at 65°C for 1 hour. The reaction was stopped by adding EGTA to 6.5 mM and incubating at 65°C an additional 30 minutes.

Spleen exonuclease, purified according to the procedure of Bernardi and Bernardi (1968), was provided by Neil Miyamoto. DNA at a concentration of 100 µg/ml was heated briefly at 100°C, then adjusted to 0.05 M Na_2SO_4, 0.01 M Na_2EDTA, 0.15 M Na acetate pH 5.5. After the addition of 66 µg/ml spleen exonuclease, the reaction was allowed to proceed at 45°C for 1 hour. The extent of the reaction was determined by removing 20 to 60 µl aliquots into a 200 µg excess of salmon sperm DNA on ice, and immediately adding an equal volume of either cold 10% TCA or H_2O. After 15 minutes on ice, the precipitate was sedimented in the HB4 rotor of the Sorvall

RC2B at 5,000 rpm for 5 minutes. The acid-soluble and total radioactivity in the two supernatants was determined in 10 ml Aquasol, to which either TCA or H_2O containing salmon DNA had been added, to ensure equivalent counting efficiencies in the two samples.

The *Neurospora crassa* single-strand specific exonuclease, purified according to the method described by Rabin *et al.* (1971), was a gift from Dr. K. Bartok. DNA at a concentration of 40 µg/ml in 0.1 M NaCl, 10 mM $MgCl_2$, 0.1 M Tris-HCl (pH 8) was incubated at 37°C for 3 hours with 2 units/ml nuclease. 1 unit is defined as that amount of enzyme which will release 1 A_{260nm} unit from 600 µg heat denatured calf thymus DNA/ml in 30 minutes at 37°C. Acid-soluble and total radioactivity was determined as described for spleen exonuclease, except that aliquots were added to a 400 µg excess of salmon sperm DNA.

(h) Hybridization in solution to determine strand polarity

^{3}H-labelled nascent DNA (4.5 µg at a specific activity of 1400 cpm/µg) and ^{32}P-labelled ϕX viral strand DNA (0.7 µg at a specific activity of 10,000 cpm/µg) were combined with 25 µg of unlabelled ϕX RF DNA, ϕX viral strand DNA, or *E. coli* DNA, in a final volume of 150 µl of 0.1 M NaCl, 0.1 M Tris-HCl (pH 8), 0.1 mM Na_2EDTA, and sonicated for 60 seconds at output 60 with an Artek sonic dismembrator. The solutions were transferred to 100 µl capillary pipettes which were sealed by carefully heating both ends in a bunsen burner flame. The capillary pipettes containing the hybridization mixture were submerged in a 100°C bath for 5 minutes, then incubated at 62°C for 42 hours (sufficient to give a Cot of 100). The hybridization mixtures were adjusted to final volumes of 750 µl and treated with the *Neurospora crassa* single-strand specific nuclease.

(i) Preparation of filters and hybridization to filters

*Hae*III and *Hin*dII digests of 10 µg of unlabeled ϕX RF I were adjusted to 0.02% bromophenol blue and 7.5% glycerol. The samples were electrophoresed on a 3 mm thick 1.5% agarose slab gel in E buffer containing 0.5 µg/ml ethidium bromide in a Hoefer Scientific Instruments gel apparatus cooled by a continuous flow of cold H_2O. Electrophoresis was at 150 volts for 6 hours or until the dye had migrated 20 cm into the gel. The DNA was denatured, neutralized, and transferred to nitrocellulose filter papers as described by Southern (1975). The papers, 1.5 cm wide and 15 cm long, were placed in Kapak heat-sealable polyester film pouches with 2 ml pre-incubation medium and submerged in a New Brunswick Scientific shaking H_2O bath at 62°C for 4 hours to minimize non-specific DNA binding to nitrocellulose (Denhardt, 1965). Between 100 and 200 µg of ^{3}H-labelled nascent ϕX DNA at a specific activity of 2 - 3 x 10^3 cpm/µg plus 2 - 6 x 10^5 cpm of uniformly ^{32}P-labelled ϕX*am*3 DNA, or from 20 to 100 µg of ^{32}P-end labelled DNA at a specific activity of 1 - 10 x 10^4 cpm/µg plus 1 - 2 x 10^5 cpm of uniformly ^{3}H-labelled ϕX DNA, in 400 µl 3 x SSC, pH 7, was sonicated for 60 seconds at output 60 with an Artek sonic dismembrator, then heated at 100°C for 5 minutes to ensure complete denaturation. Fresh pre-incubation medium was added to a final volume of 2 ml, and the solution substituted for the solution in the pouch with the nitrocellulose paper. Incubation at 62°C with agitation was continued for an additional 36 to 48 hours. The nitrocellulose papers were washed for 5 minutes at room temperature in 3 x SSC pH 7, then cut into 2 mm wide slices. The slices were dried at 110°C for 30 minutes and the radioactivity determined in toluene-Omnifluor.

(j) Analytical sedimentation velocity centrifugation and alkaline gel electrophoresis

Samples of 100 to 200 µl containing the ^{3}H- and ^{32}P-labelled DNA were layered on 5 ml gradients and centrifuged at 45,000 rpm in the SW50.1 rotor of the Beckman L2-65B ultracentrifuge. Neutral sucrose gradients (5% to 20 % (w/v) sucrose, 1 M NaCl, 1 mM Na_2EDTA, 50 mM Tris-HCl (pH 8)), were centrifuged for 2.5 hours at 10°C. Alkaline sucrose gradients (5% to 20% (w/v) sucrose, 0.2 M NaOH, 0.8 M NaCl, 2 mM Na_2EDTA, 0.05% Sarkosyl) were centrifuged for 4.5 hours at 10°C. Formamide sucrose gradients (5% to 20% (w/v) sucrose in 90% formamide) were centrifuged for 24 hours at 20°C. Fractions were collected onto 1 inch-squares of Whatman no. 3 filter paper from the bottom using a B-D Cornwall syringe. The papers were dried and the radioactivity determined in toluene/Omnifluor.

Alkaline gels containing 1% agarose were prepared with sample wells as described by Anderson (1978). The dimensions of the cylindrical gels were 0.6 cm wide and 18 cm long. Samples of 50 to 100 µl containing less than 10 µg DNA in 0.2 M NaOH, 0.1% bromophenol blue, 25% glycerol were applied. The gels were electrophoresed at room temperature at 3 to 5 mamp/gel until the dye had migrated almost to the bottom of the gel. The gels were cut into 5 mm slices which were autoclaved for 10 minutes with 1 ml dH_2O in Linear Polyethylene scintillation vials purchased from NEN. The radioactivity in the dissolved gel slices was determined in 10 ml Aquassure.

3. RESULTS

(a) Short DNA molecules are not produced by degradation during the stopping and isolating procedure

In our procedure, a brief pulse of [^{3}H]thymidine is stopped by pouring the infected pulse-labelled culture into a boiling solution of SDS-phenol. The mixture is then rapidly brought to a boil over high heat on a bunsen burner. Because this is a fairly harsh treatment, it was necessary to ascertain that the molecules shorter than unit length were not being generated by the stopping procedure. A 200 ml culture of *E. coli* C at a cell density of 4 x 10^8 cells/ml was infected with ^{32}P-labelled $\phi X am3$ at a multiplicity of infection of 10 and specific activity of 5 x 10^{-7} cpm/pfu. Immediately before pulse-labelling, a 40 ml aliquot of culture was removed. The infected cells were collected by low speed centrifugation, resuspended, lysed with lysozyme-EDTA and digested with SDS-Pronase as described by Dressler (1970). The crude lysate was sedimented on a alkaline sucrose gradient.

The sedimentation profile of the ^{32}P-labelled infecting viral strand is depicted in Figure 1. Superimposed is the sedimentation profile of the lysate of the remaining 160 ml of [^{32}P] $\phi X am3$-infected culture which was pulse-labelled with 1.6 mCi [^{3}H]thymidine. The pulse was stopped with boiling SDS-phenol and the DNA isolated as described in Materials and Methods. It is obvious that the ^{32}P-labelled infecting viral strand had not been significantly degraded during the stopping and isolating procedure. Approximately the same amount of ^{32}P-labelled parental strand was present as molecules shorter than unit length regardless of whether or not the culture had been subjected to our stopping

and isolating procedure. A considerably greater proportion of the [^{3}H]thymidine incorporated in a very brief pulse was always present in molecules shorter than unit length. In this particular experiment, it was 45% of the total. Hybridization experiments indicated that by 35 minutes after infection, approximately 90% of the radioactivity in the ^{3}H-labelled short molecules was ϕX specific and of viral strand polarity (Section 3g). Thus, the ^{3}H-labelled short nascent molecules cannot be the result of either host cell DNA synthesis or ϕX complementary strand synthesis. Furthermore, molecules longer than unit length were not observed in appreciable amounts; they could account for a maximum of 20% of the total pulse-labelled DNA (Section 3j). Since more than 90% of the radioactivity applied was recovered from the alkaline sucrose gradients, no significant fraction of the nascent DNA was not accounted for.

(b) Proportion of pulse label in short molecules depends on pulse length

As the length of the pulse was increased, there was a net accumulation of unit-length molecules in ϕX-infected *E. coli*C. While the amount of radioactivity present as shorter than unit length molecules increased as well, proportionately it represented a smaller fraction of the total. In Figure 2, three gradient profiles of increasing pulse lengths are superimposed to facilitate comparison. As the pulse length increased from 10 to 20 to 50 seconds, the proportion of pulse label in short molecules decreased from 40% to 30% to 15%. Note that in the 50 second pulse, the DNA shorter than unit length represented only a trailing edge, rather than a definite shoulder, of the peak of unit-length molecules. In longer pulses, the presence of the short molecules may be masked by the accumulation of unit-length molecules.

When a 10 second pulse was chased for 50 seconds by the addition of 10 µg/ml unlabelled thymidine to the medium, the gradient profile of the ^{3}H-labelled DNA resembled that observed for the 50 second pulse in Figure 2 (data not shown). The total incorporation of [^{3}H]thymidine in the one minute interval encompassing the pulse and chase (15×10^5 cpm) was approximately ten times the amount incorporated in the 10 second pulse (1.2×10^5 cpm) and half the amount incorporated in the 50 second pulse (29×10^5 cpm). More label was incorporated during the chase than was incorporated during the pulse itself. The addition of [^{3}H]thymidine to a final concentration of 0.2 nmoles/ml in the pulse was apparently sufficient to saturate the intracellular precursor pools (Cairns & Denhardt, 1968; Neuhard & Thomassen, 1971). The subsequent addition of a vast excess of unlabelled thymidine did not immediately dilute the intracellular pool and reduce the incorporation of [^{3}H]thymidine. This leads to ambiguity in the interpretation of the results of a typical pulse-chase experiment such as that described by Hours *et al.* (1978), since only a small fraction of the labelled intermediates observed after the chase were actually labelled during the pulse.

The data described here do not allow a precise kinetic analysis, first because the experiments are not performed under steady-state conditions (in the first few seconds of pulse-labelling, the intracellular pool of radioactive precursor is expanding), and second, because of the difficulties inherent in quantitation, since the short and unit-length molecules are not completely resolved on sucrose gradients. Nevertheless, the accumulation of radioactivity in unit-length molecules at a rate which exceeds that in short molecules suggests a precursor-product relationship.

(c) Proportion of pulse label in short molecules depends on stopping procedure

Figure 3 shows that when a very brief pulse of [^{3}H]thymidine was stopped by rapid cooling instead of rapid heating, a smaller proportion of the pulse label was present in short molecules. When the pulse was stopped by pouring the infected culture on crushed ice containing KCN and NaN_3 in a dry ice-ethanol bath as described by Baas *et al.* (1978), 15% of the pulse-labelled DNA was shorter than unit length (Figure 3a). When the pulse was stopped by pouring the infected culture into an equal volume of acetone at -70°C as described by Dressler & Denhardt (1968), 30% of the pulse-labelled DNA was shorter than unit length (Figure 3b). The total incorporation of [^{3}H]thymidine into nascent DNA was similar with all three stopping procedures, but the proportion that was present in shorter than unit length molecules was reduced when the pulse was stopped by rapid cooling. The implication of this observation is that ligation of the short molecules occurs extremely rapidly, and may occur even when net polymerization has ceased. Note that with the cold stopping procedures, there is a further delay before the cells are actually lysed with lysozyme and EDTA.

(d) Abundance of short DNA molecules depends on aeration level

Variability in the proportion of [^{3}H]thymidine incorporated into short molecules in early experiments was eventually traced to the level of aeration of the infected culture. This is illustrated in Figure 4. When the φX-infected *E. coli* C culture was aerated slowly by shaking at 70 rpm in a gyratory water bath, 70% of the pulse label was incorporated into short molecules (Figure 4a); when aeration was by bubbling air

through the culture, 50% of the [^{3}H]thymidine label was shorter than unit length (Figure 4b); when the culture was aerated rapidly by agitation at 170 rpm, 40% was present as short molecules (Figure 4c). The last condition was employed in all other experiments described in this thesis.

(e) Host strain affects abundance of short molecules

Figure 5 shows that a higher proportion of the [^{3}H]thymidine pulse label was incorporated into short molecules in thymine-requiring host strains. *E. coli* HF4704 is a *ts* thymine-requiring strain, and when grown at the permissive temperature, 60% of the pulse label was found in short molecules (Figure 5a). *E. coli* CR is a strigent thymine-requiring strain that will not grow in the absence of thymine, and 75% of the pulse label incorporated during ϕX viral strand synthesis was present in short molecules (Figure 5b). These thymine-requiring strains will grow in TKCaB without additional thymine. However, to verify that thymine was not a limiting factor, the medium was supplemented with 10 µg/ml thymine; the same distribution of pulse label was observed (data not shown). The proportion of ^{3}H-labelled short molecules in ϕX-infected *E. coli* CR decreased substantially during a 50 second chase interval (Figure 5c), or during a 50 second pulse (data not shown) with a concommitant accumulation of ^{3}H-labelled unit-length molecules.

(f) Comparison of pulse-labelled molecules in *ung* and *ung*$^+$ strains

If the short molecules were generated by the combined action of uracil-DNA glycosylase and an apyrimidinic endonuclease at sites where dUMP rather than dTMP had been incorporated into nascent DNA, one would expect to see relatively fewer short molecules in a host strain deficient

in uracil-DNA glycosylase. Figure 6 shows that this was not the case. In both the *ung* and *ung*$^+$ strains, approximately 60% of the [^{3}H]thymidine incroporated in a brief pulse was present in molecules shorter than unit length. The sedimentation profile of the nascent pulse-labelled ϕX DNA in these strains was comparable to that observed with *E. coli* HF4704 (Figure 6a, b and Figure 5a). This is not surprising since these strains have similar genetic backgrounds.

We do not attach any significance to the fact that more [^{3}H] thymidine was incorporated into nascent DNA by the ϕX-infected *ung* cells than by the *ung*$^+$ cells. The total incorporation of [^{3}H]thymidine varied from experiment to experiment because it was difficult to be precise with pulses of less than 5 seconds long, and also because during the first few seconds of pulse-labelling, the intracellular pool of [^{3}H]thymidine is expanding. It ranged from 5 x 10^3 to 30 x 10^3 cpm per 200 ml culture of ϕX-infected *ung* or *ung*$^+$ cells.

(g). Strand polarity of the short pulse-labelled molecules

The hybridization experiments which revealed that by 35 minutes after infection 90% of the radioactivity in the ^{3}H-labelled short molecules was ϕX specific and 95% of the ϕX-specific radioactivity was in the viral strand (Hours *et al.*, 1978) were done with DNA isolated from slowly aerated cultures of ϕX-infected *E. coli* C. Since the level of aeration affects the proportion of pulse-label found in short molecules, and could conceivably also affect the proportion of ^{3}H-labelled short molecules which are of ϕX viral strand origin, the hybridization experiments were repeated with nascent DNA isolated from vigorously aerated cultures of ϕX infected *E. coli* C. ^{3}H-labelled nascent short molecules and uniformly

^{32}P-labelled ϕX viral strands were hybridized to an excess of unlabelled ϕX RF DNA, ϕX viral strand DNA, or *E. coli* DNA. The DNA which did not anneal was degraded by the *Neurospora crassa* single-strand specific exonuclease. Table I shows that as much as 90% of the ^{3}H-labelled short nascent molecules was protected from degradation by hybridization to an excess of ϕX RF DNA, implying that the sequences are ϕX specific. Furthermore, 90% of the ^{3}H-labelled short nascent molecules did not anneal to an excess of ϕX viral strand DNA, suggesting that the sequences are of viral strand origin. Finally, as much as 20% of the ^{3}H-labelled nascent DNA and 10% of the ^{32}P-labelled ϕX viral strand DNA hybridized in the presence of an excess of *E. coli* DNA. To rule out the possibility that the enzyme digestion had been incomplete, the experiment was repeated with twice the amount of enzyme. Essentially the same result was observed. The most likely explanation is the the ^{3}H-labelled nascent and ^{32}P-labelled viral strand sequences hybridized to ^{3}H-labelled and unlabelled complementary strand sequences. The method of isolation does not discriminate between pulse-labelled and pre-existing unlabelled short molecules; also the size fractionation by velocity sedimentation is not absolute. A low level of homology between ϕX and *E. coli* DNA could account for less than 1% the hybridization which occurs with an excess of *E. coli* DNA (Denhardt, 1968). Although the ^{3}H-labelled short molecules isolated from rapidly aerated cultures of ϕX infected *E. coli* C represent a smaller proportion of the total pulse label than is found in less well aerated cultures, they still consist predominantly (approximately 90%) of ϕX viral strand sequences.

(h) Localization of the short molecules on the ϕX174 genome

Preliminary experiments revealed that the short nascent molecules hybridized to all regions of the ϕX genome as efficiently as the unit length (but sonicated) nascent molecules (Hours *et al.*, 1978). The hybridization experiments were repeated with uniformly ^{32}P-labelled ϕX DNA as an internal marker to determine whether a gradient of [^{3}H] thymidine pulse label around the genome could be detected. To quantitate the relative amounts of nascent [^{3}H] DNA annealing to different positions of the genome, it was necessary to correct for the thymine content of the various restriction enzyme fragments in the viral strand. The uniformly labelled [^{32}P] DNA served as a internal control for both the efficiency of hybridization and the size of the DNA; the $^{3}H/^{32}P$ ratio corrected for thymine content is a measure of the specific activity of the newly synthesized DNA. The data are presented in Figure 7.

Assuming that the pulse time is less than the time it takes to synthesize one viral strand, there will be a gradient of label from the origin to the terminus in those molecules that have completed a round of replication in the pulse interval. Labelling will be more extensive at the terminus than at the origin. Therefore, the $^{3}H/^{32}P$ ratio, or specific activity, for a restriction enzyme fragment close to the terminus should be greater than the ratio for a fragment close to the origin. This was observed for the unit-length DNA molecules (Fractions 19 to 25 of the neutral sucrose gradient in Figure 8a). When the specific activity was plotted versus position around the genome, it increased with distance from the known origin of DNA replication in an approximately linear fashion (Figure 7a).

The specific activity of the short molecules (Fractions 5 to 18 of the neutral sucrose gradient in Figure 8a) was higher in the origin region than in the central portion of the genome, and increased again as the terminus was approached (Figure 7b). This was not expected. In an unsynchronized culture, the specific activity of the total DNA should be constant across the genome since to our knowledge there is no amplification of any particular ϕX sequence. Thus, any gradient of label in the completed, or unit-length, molecules should be compensated for by a complementary gradient in uncompleted molecules. One would expect the specific activity of the short molecules to decrease from the origin to the terminus. That it increased as the terminus was approached suggests that the terminal sequences of the ϕX genome are synthesized in excess.

In mitochondrial DNA replication, there is an overproduction of 7S origin sequences, 75% of which are displaced from the genome, and only 25% of which are incorporated into newly synthesized molecules (Robberson & Clayton, 1973). If a similar situation occurred in the terminal region of the ϕX genome, one might expect to find an excess of abortive replication intermediates derived from the terminus, if one examined the total population (nascent plus non-nascent) of shorter-than-unit-length ϕX molecules in the cell. Short DNA molecules were isolated from ϕX-infected cultures that had not been pulse-labelled with [^{3}H]thymidine and were end-labelled with [γ-^{32}P]ATP and polynucleotide kinase. The sedimentation profile of the ^{32}P- end-labelled DNA on a neutral sucrose gradient is shown in Figure 9. Because of the existence of a very large number of short molecules, the "C" molecules first described by Miyamoto and Denhardt (1977), most of the ^{32}P-label is found near the top of the gradient.

The top four fractions of the gradient containing the "C" molecules, and the region encompassed by fractions 5 to 18 of the gradient, where the [^{3}H] pulse-labelled molecules shorter than unit length are found (see Figure 8a), were separately combined with uniformly ^{3}H-labelled ϕX viral strands and hybridized to Southern blots containing *Hae*III restriction enzyme fragments of ϕX RF.

The ^{32}P/^{3}H ratios corrected for thymine content of the DNA hybridized to any particular restriction enzyme fragment are an index of the number of ϕX molecules in the cell with 5' termini located in that region of the genome. When the ^{32}P/^{3}H ratios of the short and "C" molecules are plotted versus position on the genome in Figure 10, the ratios are highest in the center of the genome, lower near the origin, and lowest at the terminus. The high ^{32}P/^{3}H ratios in the center of the genome suggest that there are a large number of short ϕX DNA molecules in the cell whose 5' termini are located in this region.

The first attempts to hybridize the ^{32}P-end-labelled short molecules from ϕX-infected cells to the ϕX RF fragments on the Southern blots were unsuccessful because the ^{32}P-end-labelled DNA annealed much less efficiently than the uniformly ^{3}H-labelled ϕX viral strands. This is shown in the data in Table II. Although low, the hybridization efficiency of the ^{32}P-end-labelled molecules from ϕX infected cells is significantly greater than that of ^{32}P-end-labelled molecules from uninfected cells, so the hybridiztion cannot be attributed to a low level of homology between ϕX and *E. coli* sequences. If the ^{3}H- and ^{32}P-labelled DNAs were composed entirely of ϕX sequences, they would hybridize to the ϕX RF DNA on the Southern blots with equal efficiency, although the overall

efficiency would vary from experiment to experiment, depending on the specific activity of the DNA being hybridized and on the amount of DNA on the filter available for hybridization. That the ^{3}H-labelled viral strand DNA hybridizes to the filters more than ten times as efficiently as the ^{32}P-end-labelled short molecules suggests that at most 10% of the ^{32}P-end-labelled short molecules are ϕX specific. Similarly, the fifty-fold greater hybridization efficiency of the ^{3}H-labelled viral strand DNA relative to the ^{32}P-end-labelled "C" molecules indicates that as little as 2% of the ^{32}P-end-labelled "C" molecules may be ϕX specific, assuming that the smaller size of the "C" molecules does not significantly alter their hybridization efficiency. The data in Table II show that only a small portion of the total DNA isolated by our procedure comprises ϕX sequences, but do not allow any estimates to be made of what fraction of the population of ϕX molecules isolated are nascent. The recovery of large numbers of short non-nascent *E. coli* molecules has been reported by Anderson (1978) and Denhardt *et al.* (1979). We find that the same short non-nascent molecules are present in ϕX-infected *E. coli* cells.

(i) Analysis of the 5' ends of the short molecules

The spleen exonuclease method was used to test for the presence of ribonucleotides at the 5' ends of the molecules shorter than unit length during viral strand synthesis. The final step in the purification of the DNA was neutral sucrose gradient sedimentation immediately after heat denaturation; typical profiles are depicted in Figure 8. Uniformly ^{32}P-labelled ϕX DNA of the same size as the nascent DNA was obtained by briefly sonicating viral strand circles. [^{3}H]thymidine pulse-labelled

nascent φX DNA and uniformly labelled non-nascent [^{32}P]DNA were combined and treated with polynucleotide kinase and ATP to ensure phosphorylation of all 5' ends. The sample was divided into four parts. NaOH to a final concentration of 0.4 N was added to one, then all four were incubated at 37°C for 8 hours. A second sample was then treated with RNase A and RNase T_1. Ribonucleotide linkages would be hydrolyzed by the alkali or RNases generating 5' OH ends. One of the remaining non-alkali treated samples was digested with bacterial alkaline phosphatase to remove 5' P termini. All four samples were then subjected to spleen exonuclease treatment.

Spleen exonuclease will degrade molecules with 5' OH termini but not molecules with 5' phosphoryl termini. In the alkali and RNAse-treated samples, only those molecules which had been linked to ribonucleotides would be degraded by spleen exonuclease. Acid solubility was measured as described in Materials and Methods. The data are summaried in Table III. After kinase treatment, both nascent and non-nascent molecules were similarly insensitive to spleen exonuclease digestion. After alkali and RNase treatment, nascent [^{3}H]DNA became significantly more susceptible to spleen exonuclease. Note that both alkali and RNase resulted in similar increases in sensitivity to spleen exonuclease. There was an increase in acid-solubility of almost 5% for the ^{3}H-labelled nascent viral strand molecules, of about 10% for the ^{3}H-labelled nascent RF molecules, and approximately 1% for the ^{32}P-labelled non-nascent DNA in all cases except for the RNase-treated RF sample which had a slightly higher background. After phosphatase treatment, the ^{32}P-labelled non-nascent DNA became 25% acid-soluble on exposure to spleen exonuclease. Longer incubations with spleen exonuclease resulted in higher levels of acid solubility. A second experiment gave essentially the same results as presented in Table III.

Although spleen exonuclease may not degrade 5' OH-terminated chains to completion, degradation from the 5' OH end proceeds at similar rates along DNA molecules of different chain lengths, and therefore, the data provide an estimate of the number of molecules with alkali-labile sites (Kurosawa *et al.*, 1975). Assuming uniform labelling along the DNA, we infer that approximately 20% of the short molecules during viral strand synthesis and approximately 40% of the short molecules during RF replication have at least 1 ribonucleotide at the 5' end.

The short molecules isolated during viral strand synthesis were divided into two pools. When the shortest molecules (fractions 5 to 11 of the neutral sucrose gradient depicted in Figure 8a) and the intermediate-sized molecules (fractions 12 to 18) were treated separately as described above, it became evident that the ribonucleotides were located predominantly on the shortest molecules (Table IV). If the ribonucleotide(s) function as primers, which are very quickly removed once synthesis of a DNA chain is initiated, then one would expect to find them most frequently on the shortest molecules.

One curious observation was that after phosphatase treatment, ^{3}H-labelled nascent DNA was not as susceptible to spleen exonuclease as the ^{32}P-labelled non-nascent DNA. When both ^{3}H and ^{32}P-labelled DNAs were not nascent, they were degraded by spleen exonuclease to the same extent (Table III). Several explanations can account for the lower release of ^{3}H from the nascent molecules by a 5' end-specific nuclease. There could be an increase in secondary structure in these nascent molecules. Their average size may be greater than the average size of the ^{32}P-labelled DNA. There could be an uneven distribution of [^{3}H] thymidine in the nascent molecules, with more label located at the 3' ends. Finally, the 5' ends could be blocked with an unidentified structure which renders them resistant to spleen exonuclease.

(j) Molecules longer than unit length

When the ^{3}H-labelled nascent DNA was sedimented on alkaline sucrose gradients, only a small fraction of the total pulse label appeared to be in molecules longer than unit length (Figure 1). On neutral sucrose gradients, however, up to 20% of the total pulse label could be observed sedimenting more rapidly than unit-length ϕX molecules. To rule out the possibility that the rapidly sedimenting ^{3}H-labelled nascent DNA on neutral sucrose was an aggregate, it was combined with ^{32}P-labelled ϕX viral strands, heated briefly at 100°C, then resedimented on neutral sucrose. Figure 11(a) shows that the ^{3}H-labelled DNA sedimented as a broad peak ahead of the ^{32}P-labelled ϕX viral strand marker. On an alkaline sucrose gradient, however, the same ^{3}H-labelled DNA cosedimented with ^{32}P-labelled ϕX viral strand marker (Figure 11b). This discrepancy could be explained by the existence of an alkali-labile site in the long molecules which was hydrolyzed to yield unit-length and shorter molecules. But when the ^{3}H-labelled DNA longer than unit length was sedimented on denaturing formamide gradients, it again sedimented with the ^{32}P-labelled viral strand marker (Figure 11c), so there was no alkali-induced cleavage of the molecules longer than unit length. Because of the relatively weak dependance of sedimentation coefficient on molecular weight of single-stranded DNA under these conditions, it is possible that the longer molecules were simple more readily observed on neutral than on denaturing gradients. If this were the case, then a more sensitive method would be required to demonstrate a difference in size between the longer and unit-length molecules under denaturing conditions.

This was achieved on an alkaline agarose gel where the ^{3}H-labelled DNA migrated as a broad peak behind the ^{32}P-labelled ϕX viral strand marker. The gel shown in Figure 11(d) shows very convincingly that a substantial portion of the approximately 20% of the initial pulse-labelled DNA that sediments rapidly is in longer-than-genome-length intermediates which are not well resolved from unit length ϕX viral strands on denaturing sucrose gradients.

4. DISCUSSION

There are several explanations for the existence of the short molecules that we observe during φX viral strand synthesis: (i) They could be an artefact of our stopping and/or isolating procedure. (ii) They could be caused by the incorporation of uracil (from dUTP) followed by excision-repair or by other repair events. (iii) They could be due to a second nicking event by the gene A protein at the origin on intermediates longer than unit length. (iv) They could be the result of the "nicking-closing" activities of topoisomerases: (v) They could be true intermediates in the synthesis of the viral strand, indicating that the viral strand is synthesized discontinuously.

Provided that there is nothing special about nascent DNA (e.g. enhanced sensitivity to a heat-activated endonuclease), we have eliminated the possibility that the short viral strand molecules are generated by our stopping procedures. The arguments for this are that the infecting ^{32}P-labelled viral strands were not significantly affected (Fig. 1), and that in longer pulse intervals, or chases, 80-90% of the ^{3}H pulse label was recovered in unit-length viral strands (Figure 2). However, the stopping procedure is critical since it determines the nascent intermediates that are observed. With the two "cold" stopping procedures described in Results (used by Baas *et al.*, 1978; and Dressler & Denhardt, 1968), actual lysis of the cells was not achieved until some time after the pulse was ostensibly stopped. It is possible that the short molecules were ligated during this interval with the consequence that fewer short molecules were observed. Our data (Figures 2 and 3) indicate

that the combination of long pulse lengths and less efficient stopping procedures may have prevented other investigators from observing these short molecules. If synthesis of both strands of RF were stopped with equal efficiency during RF replication, 56% of the [^{3}H]thymidine in a short pulse should be incorporated into the viral strand and 44% into the complementary strand because of the difference in thymine content of the two strands (Sanger *et al.*, 1977). (We assume synthesis of both strands draws on the same precursor pool.) Baas *et al.* (1978) found that with their "cold" stopping procedures, which used frozen KCN-NaN_3, up to 80% of a brief pulse label during RF replication was viral strand specific. With our "hot" stopping procedure, less than 70% was viral strand specific (Hours *et al.*, 1978). Thus while neither method seems to stop synthesis of both strands equally, it is apparent that the "hot" stopping procedure is more effective in shutting off viral strand synthesis than the "cold" stopping procedures. The short molecules are observed in abundance only when the briefest of pulses is stopped with the most efficient procedure.

The host strain also affects the abundance of short molecules. In comparison with strains like *E. coli* C, which does not require thymine, the thymine auxotrophs, *E. coli* CR, *E. coli* HF4704, *ung*$^+$, and *ung*, yield a larger proportion of the pulse label in short ϕX DNA molecules. Machida *et al.* (1977) also found a more pronounced degree of discontinuous ϕX DNA synthesis for both strands during RF replication in a thymine-requiring host. It has been reported that if thymine-requiring *E. coli* strains are briefly deprived of thymine and pulse-labelled with either [^{3}H]thymidine (Brewin, 1977) or [^{3}H]thymine (Diaz *et al.*, 1975), the label

is incorporated almost entirely into small chains. However, we have not been growing or infecting these thymine-requiring strains under conditions of thymine deprivation (see Results). Nevertheless, as reiterated recently by O'Donovan (1978), the dTTP pool in thymine-requiring strains is usually substantially less than in normal strains, and it is possible that in ϕX-infected, thymine-requiring hosts, initiation of the short molecules is not impaired, but elongation is slowed because of a lower precursor (dTTP) concentration. As the consequence of an increased half-life, more short molecules are observed.

A similar explanation may account for the fact that more short molecules were observed in the ϕX-infected wild-type strain when the aeration was reduced. When the cellular oxygen supply is decreased, cellular metabolism is slowed, and energy in the form of ATP may become rate-limiting for many intracellular processes. It is possible that elongation of the short molecules is slowed because of a reduction in the size of the precursor pools. Alternatively, it is possible that the mechanism responsible for joining the short molecules is dependent on high ATP levels.

A low level of uracil incorporation into nascent DNA, followed by post-replication excision repair by uracil-DNA glycosylase and an apyrimidinic endonuclease, could explain the existence of short molecules. It could also account for the accumulation of short molecules in thymine-requiring strains, since more dUMP would be incorporated into nascent DNA in these strains than in the wild-type host (Tamanoi & Okazaki, 1978; Duncan *et al.*, 1978). However, no significant difference in the size distribution of pulse-labelled ϕX viral strand DNA was observed between

the uracil-DNA glycosylase-deficient *ung* strain and its isogenic *ung*$^+$ strain (Figure 6). This suggests that the short molecules were not generated by uracil-induced repair of nascent ϕX DNA. If the short molecules were caused by uracil excision, one would expect to see substantially fewer in the *ung* strain, since uracil incorporated into DNA would persist as a result of the defective uracil-DNA glycosylase (Tye *et al.*, 1978; Warner & Duncan, 1978). Note that the recovery of high molecular weight T_4 and *E. coli* DNA containing uracil from *ung dut* strains implies that neither residual uracil-DNA glycosylase activity nor alternate uracil-removal pathways are sufficient to generate these small nascent molecules *in vivo*. Our results are consistent with findings that short nascent molecules in *E. coli* cannot be attributed to the action of uracil-DNA glycosylase (Tye *et al.*, 1978; Tamanoi & Okazaki, 1978) except in dUTPase-deficient (*dut*) hosts (Tye *et al.*, 1977).

If the short molecules were caused by secondary nicking by the gene A protein one would expect them to hybridize exclusively to the origin region. This was not observed. The short nascent molecules hybridized to all regions of the ϕX genome, but preferentially to both the origin and the terminus region (Figure 7b).

The preferential hybridization of the pulse-labelled short molecules to the terminus region suggests that the terminal sequences of the ϕX genome are synthesized in excess. This interpretation is supported by the observation that there are a large number of short ϕX molecules in the cell whose 5' termini have been located in the center of the genome by end-labelling and hybridization. There is a sequence in the central region of the genome, in the *Hae*III fragment Z8, which resembles the

sequence at the origin in the gene A region where a round of replication is initiated (Weisbeek, P., personal communication). It appears that the similarity is sufficient to allow this site to be recognized by the gene A protein, and other proteins involved in initiating a round of ϕX replication, with the resulting synthesis of half genome length ϕX viral strands. The accidental encapsidation of these incomplete viral strands could result in the production of some of the defective phage particles which are observed in variable amounts in every phage preparation (unpublished observation).

Short molecules could be generated by the action of topoisomerases. However, it seems more likely to us that these enzymes would cleave the parental DNA ahead of the replicating fork to facilitate separation of the pre-existing strands, rather than the newly synthesized DNA behind the replicating fork. Furthermore, molecules which arose from nicks introduced by topoisomerases would not be expected to possess a ribonucleotide at the 5' end.

Since none of the explanations considered seem able to account for the existence of all the short molecules, and since we know of no other explanations, we are left with the conclusion that at least some of the short molecules are nascent intermediates which arise from the discontinuous synthesis of the viral strand from a defined origin (region of the gene A protein nick) unidirectionally around the genome.

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Legend to Table I:

^{3}H-labelled nascent molecules shorter than unit length and uniformly ^{32}P-labelled ϕX viral strand DNA were combined and hybridized to an excess of unlabelled DNA in solution. The DNA which did not anneal was degraded by the *Neurospora crassa* exonuclease. Total and acid-soluble radioactivities were determined as described in Materials and Methods, Section 2(g).

TABLE I

Strand Polarity of ^{3}H-labelled Molecules Shorter than Unit Length

Total DNA (total cpm)	DNA which did not anneal (acid-soluble cpm)	% DNA which did not anneal	when hybridized to an excess of
^{3}H 2270	200	9	φX RF DNA
^{32}P 2260	140	6	
^{3}H 2260	2040	90	φX viral strand DNA
^{32}P 2540	2510	100	
^{3}H 2310	1850	80	*E. coli* DNA
^{32}P 2770	2500	90	
^{3}H 1260	980	78	*E. coli* DNA
^{32}P 1620	1450	90	

Legend to Table II:

The DNA shorter than unit length, recovered from cultures which had not been pulse-labelled with [^{3}H]thymidine, was end-labelled with [^{32}P]ATP and kinase. The ^{32}P-end-labelled DNA was divided into two fractions: the short molecules are fraction 5 to 18 of the sucrose gradient depicted in Figure 9; the "C" molecules are fractions 1 to 4 of the same gradient. The ^{32}P-end-labelled DNA was combined with uniformly ^{3}H-labelled ϕX viral strand DNA, and hybridized to Southern blots containing *Hae*III restriction enzyme fragments of ϕX RF, as described in Materials and Methods, Section 2(i). Two filters were incubated in each bag with the DNA obtained from cultures of ϕX-infected *E. coli* C, and only 1 filter in each bag with the DNA from cultures of uninfected *E. coli* C. Background was subtracted from the sum of the radioactivity hybridized to <u>all</u> of the restriction enzyme fragments on the filters. The hybridization efficiency is the % of the total ^{32}P- or ^{3}H-labelled DNA which hybridized to the ϕX DNA on the filter.

TABLE II

Comparison of the Hybridization Efficiency of ^{32}P-end-labelled DNA Shorter Than Unit Length and Uniformly ^{3}H-labelled φX Viral Strand DNA to φX RF DNA

Short Molecules

	φX-infected *E. coli* C				Uninfected *E. coli* C	
	^{3}H		^{32}P		^{3}H	^{32}P
total cpm	190,000		1,500,000		270,000	1,400,000
cpm hybridized/filter	14,000	13,000	9,100	9,300	18,000	750
% cpm hybridized filter	7.5	7	.62	.64	6.8	.05

"C" Molecules

	φX-infected *E. coli* C				Uninfected *E. coli* C	
	^{3}H		^{32}P		^{3}H	^{32}P
total cpm	105,000		1,800,000		110,000	3,100,000
cpm hybridized/filter	19,000	23,000	4,600	5,900	15,000	2,400
% cpm hybridized/filter	18	22	.26	.33	14	.08

Legend to Table III:

ϕXDNA shorter than unit length and either pulse-labelled with [^{3}H] thymidine or uniformly-labelled with [^{3}H]thymidine was combined with [^{32}P] ϕX viral strand DNA, uniformly labelled and sonicated to the same size. The DNA was treated with polynucleotide kinase and ATP to phosphorylate 5' ends, then divided into 4 parts. One received no further treatment. Of the remaining samples, one was digested with RNase A and RNase T_1 and another was treated with NaOH. These treatments will generate 5' OH termini where ribonucleotides are removed. The final sample was digested with bacterial alkaline phosphatase (BAP) to expose 5' OHs on all termini. All samples were then incubated with spleen exonuclease. Total and acid-soluble radioactivity was determined as described in Materials and Methods, Section 2(g). Total cts/min of either ^{3}H- or ^{32}P-labelled DNA ranged from 5,000 to 10,000 per sample. Acid soluble cts/min ranged from 100 to 2,500.

TABLE III

Spleen Exonuclease Sensitivity of Shorter-Than-Unit-Length DNA

ϕX DNA	Treatment	% Acid-Soluble Radioactivity ^{3}H	^{32}P
pulse-labelled with	none	1.8	1.8
[^{3}H]thymidine during	+RNase	6.5	3
viral strand	+NaOH	6.1	2.8
synthesis	+BAP	11	26
pulse-labelled with	none	2	1.8
[^{3}H]thymidine during	+RNase	15	5.2
RF replication	+NaOh	13	2.8
	+BAP	19	25
uniformly-labelled and	none	2.3	1.9
sonicated viral strands	+BAP	20	20

Legend to Table IV:

ϕX DNA pulse-labelled with [^{3}H]thymidine was divided into two size classes: the very short molecules (fractions 5 to 11 of the neutral sucrose gradient in Figure 8(a)), and the short molecules (fractions 12 to 18 of the same gradient). These were separately combined with uniformly labelled [^{32}P] ϕX viral strand DNA of the same size obtained by sonication. The DNA was treated with polynucleotide kinase and ATP to phosphorylate 5' ends, then divided into 3 parts. One received no further treatment. One was treated with 0.4 M NaOH at 37°C for 8 hours. The final sample was digested bacterial alkaline phosphatase (BAP). All three samples were then incubated with spleen exonuclease. Total and acid-soluble radioactivity was determined as described in Materials and Methods, Section 2(g). Total cts/min of either ^{3}H- or ^{32}P-labelled DNA ranged from 5,000 to 10,000 per sample. Acid-soluble cts/min ranged from 100 to 2,500.

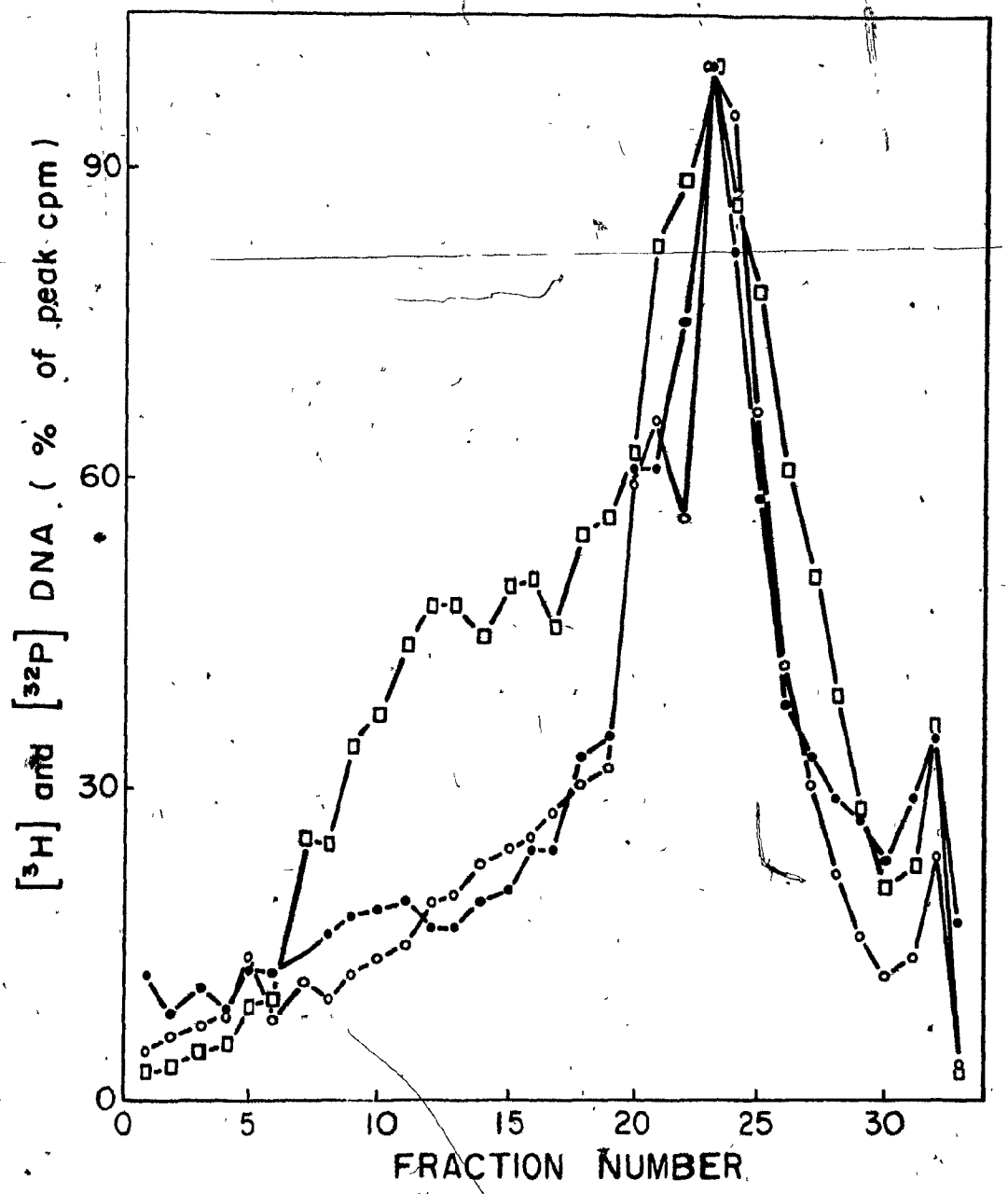
TABLE IV

Spleen Exonuclease Sensitivity of Very Short and Short ϕX DNA Pulse-Labelled with [^{3}H]thymidine During Viral Strand Synthesis

ϕX DNA	Treatment	% Acid-Soluble Radioactivity ^{3}H	^{32}P
Very Short	none	4	3.2
	+NaOH	11	3.7
	+BAP	24	34
Short	none	2.4	2.5
	+NaOH	3.8	2.9
	+BAP	6	15

Legend to Figure 1:

Fate of the ^{32}P-labelled infecting ϕX viral strand in *E. coli* C during the stopping and isolation procedure. Sedimentation was from left to right on alkaline sucrose gradients; composition of the gradients and sedimentation conditions are described in Materials and Methods Section 2(c). Two separate gradient profiles were superimposed to facilitate comparison. One gradient represents the ^{32}P-labelled infecting viral strand in a portion removed before pulse-labelling (● - ● - ● -; input cpm: 3,500) and lysed using conventional procedures (Dressler, 1970). The second gradient contained the ^{32}P-labelled infecting viral stand in the remaining culture which was pulsed, stopped, and purified by our procedure (- o - o - ; total cpm: 12,600); and the [^{3}H]thymidine labelled nascent ϕX DNA in the same experiment (- □ - □ -; input cpm: 23,500).


[3H] and [32P] DNA (% of peak cpm)
90
60
30
0
0
5
10
15
20
25
30
FRACTION NUMBER

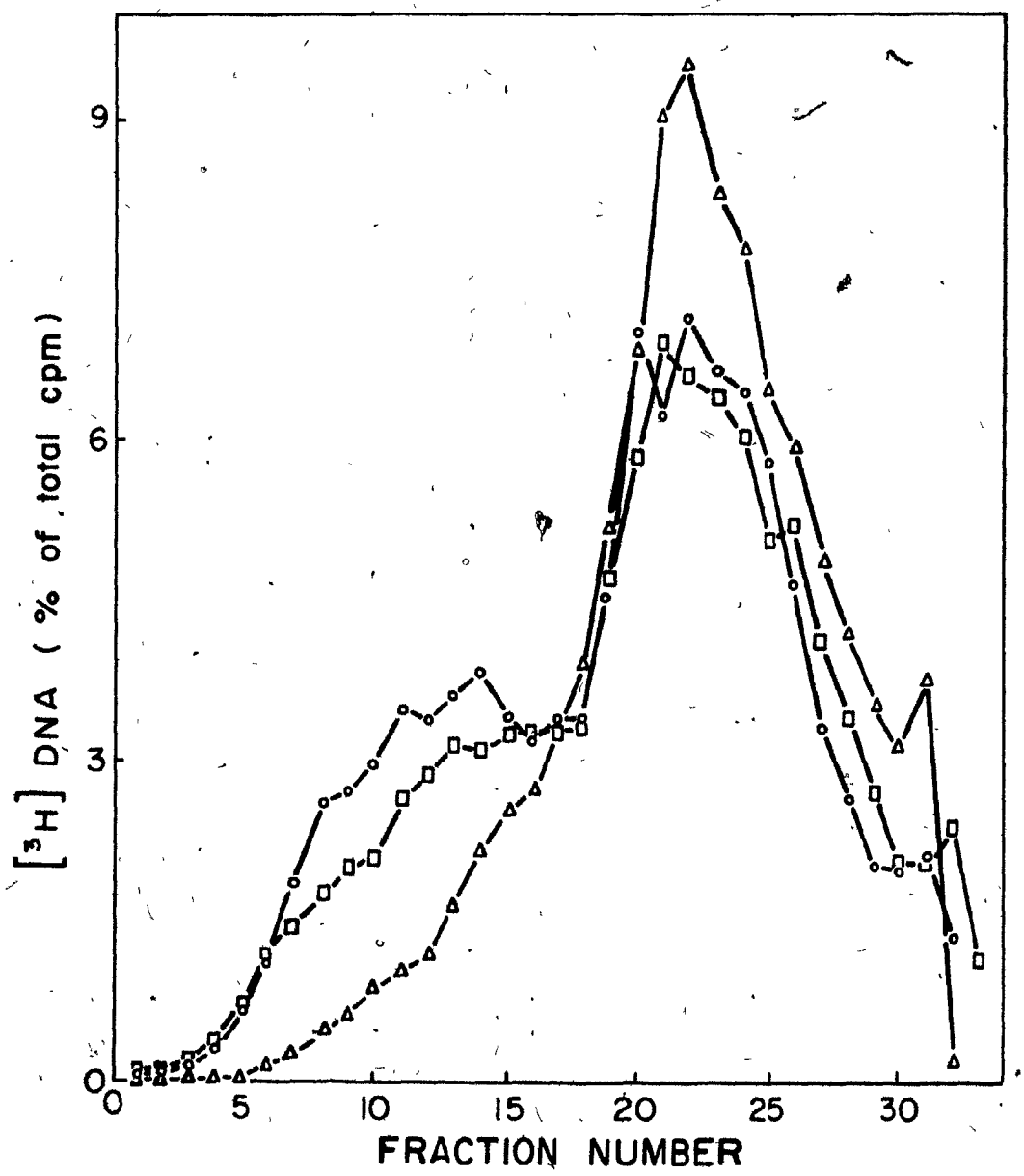
Legend to Figure 2:

Effect of the pulse on [^{3}H]thymidine pulse-labelled nascent ϕX DNA in *E. coli* C. Sedimentation was from left to right on alkaline sucrose gradients. Composition of the gradients and sedimentation conditions are described in Materials and Methods Section 2(c). Three gradient profiles were superimposed to facilitate comparison of the effect of increasing pulse length:

(o - o - o) 10 seconds; input cpm: 1.2×10^5

(□ - □ - □) 20 seconds; input cpm: 3.2×10^5

(Δ - Δ - Δ) 50 seconds; input cpm: 29×10^5

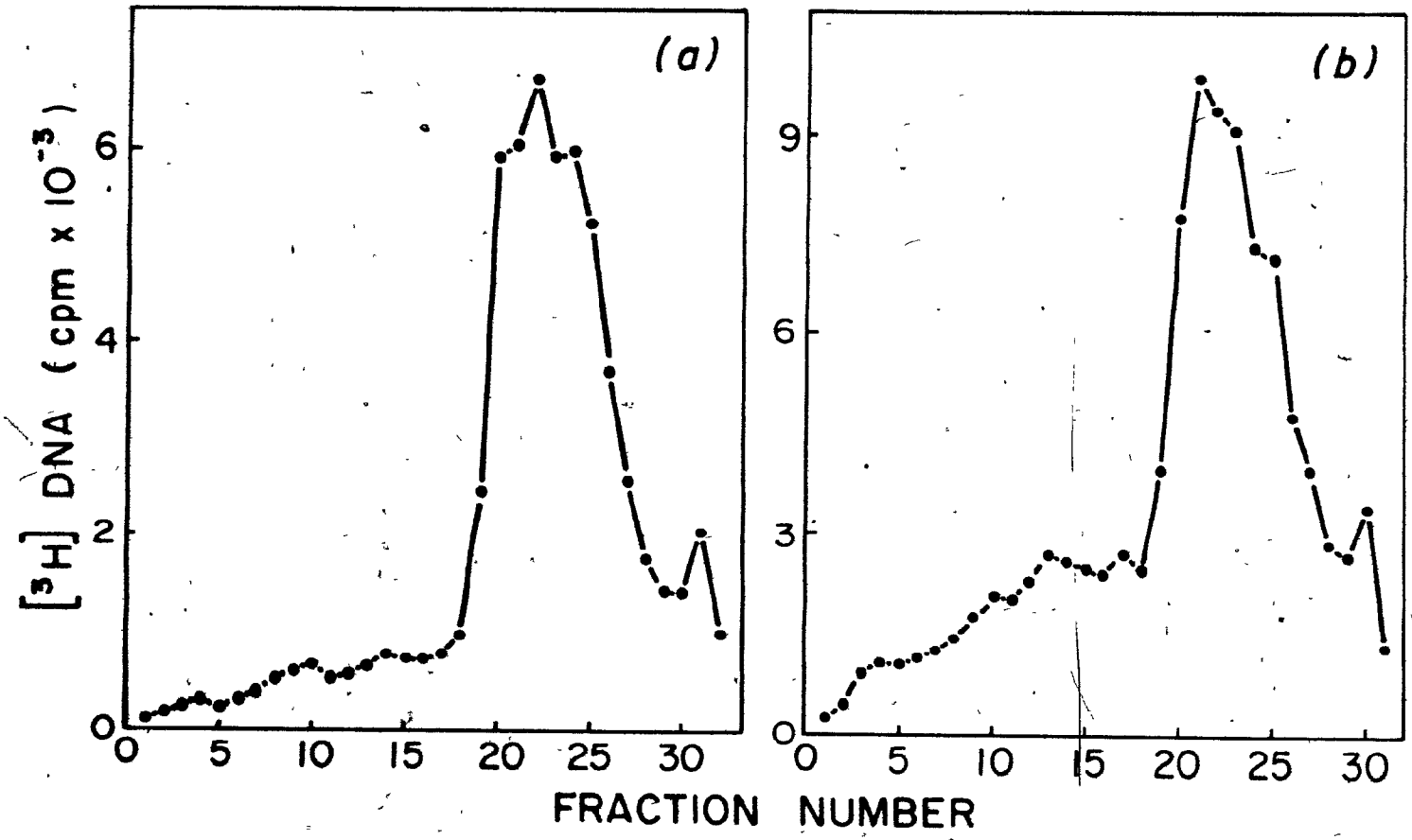

[3H] DNA (% of total cpm)
0
3
6
9
0
5
10
15
20
25
30
FRACTION NUMBER

Legend to Figure 3:

Effect of the stopping procedure on [^{3}H]thymidine pulse-labelled nascent ϕX DNA in *E. coli* C. Sedimentation was from left to right on alkaline sucrose gradients; composition of the gradients and sedimentation conditions are described in Materials and Methods, Section 2(c).

(a) 10-second pulse stopped by pouring infected cultures onto crushed frozen NaN_3-KCN solution as described by Baas *et al.* (1978).

(b) 10-second pulse stopped by pouring infected culture into acetone at -70°C as described by Dressler (1970).


(a)
(b)
[3H] DNA (cpm x 10-3)
FRACTION NUMBER
0
2
4
6
3
9
0 5 10 15 20 25 30

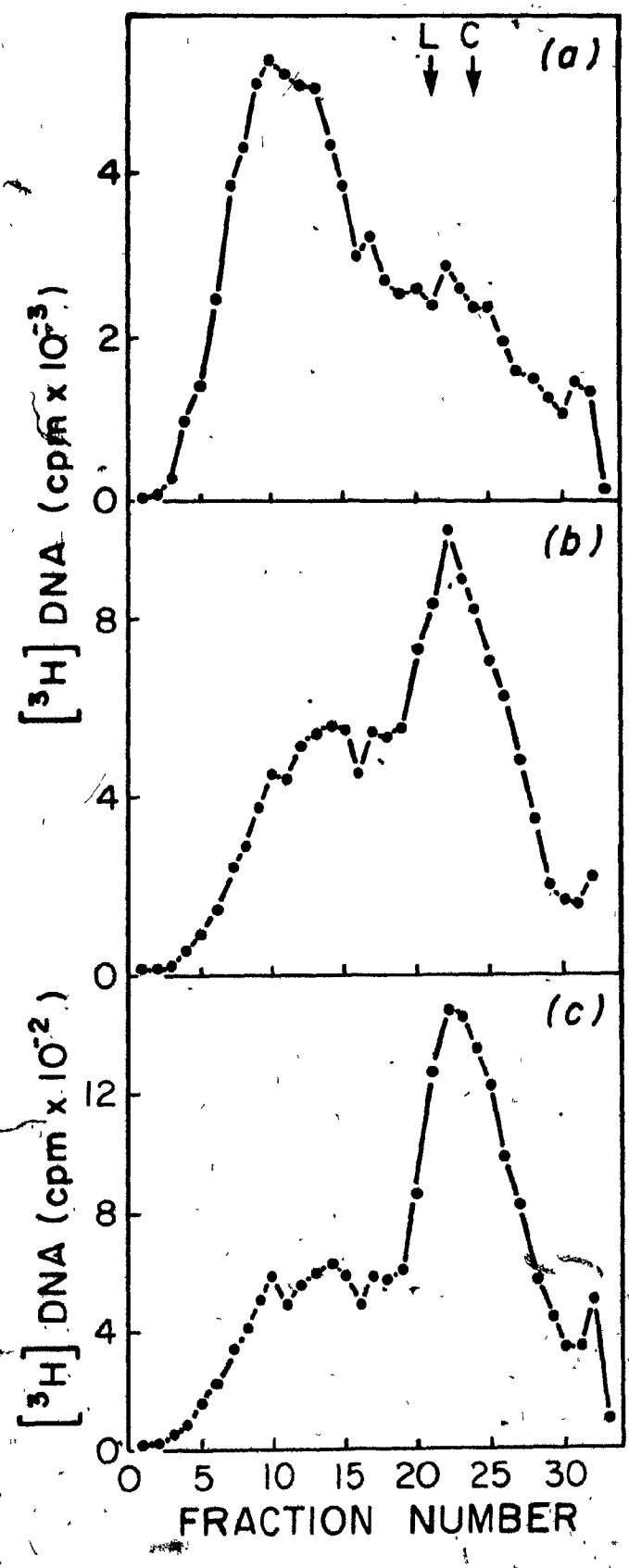
Legend to Figure 4:

Effect of the aeration level on [^{3}H]thymidine pulse-labelled nascent ϕX DNA in *E. coli* C. Sedimentation was from left to right on alkaline sucrose gradients; the composition of the gradients and sedimentation conditions are described in Materials and Methods, Section 2(c). Arrows indicate the positions of linear (L) and circular (C) ^{32}P-labelled ϕX DNA.

(a) Slow aeration at 70 rpm in a New Brunswick Scientific shaking water bath.

(b) Aerated by bubbling air vigorously through infected culture.

(c) Rapid aeration at 170 rpm in shaking water bath.


L
C
(a)
(b)
(c)
[3H] DNA (cpm x 10-3)
[3H] DNA (cpm x 10-2)
0
2
4
8
12
0 5 10 15 20 25 30
FRACTION NUMBER

Facing page 90

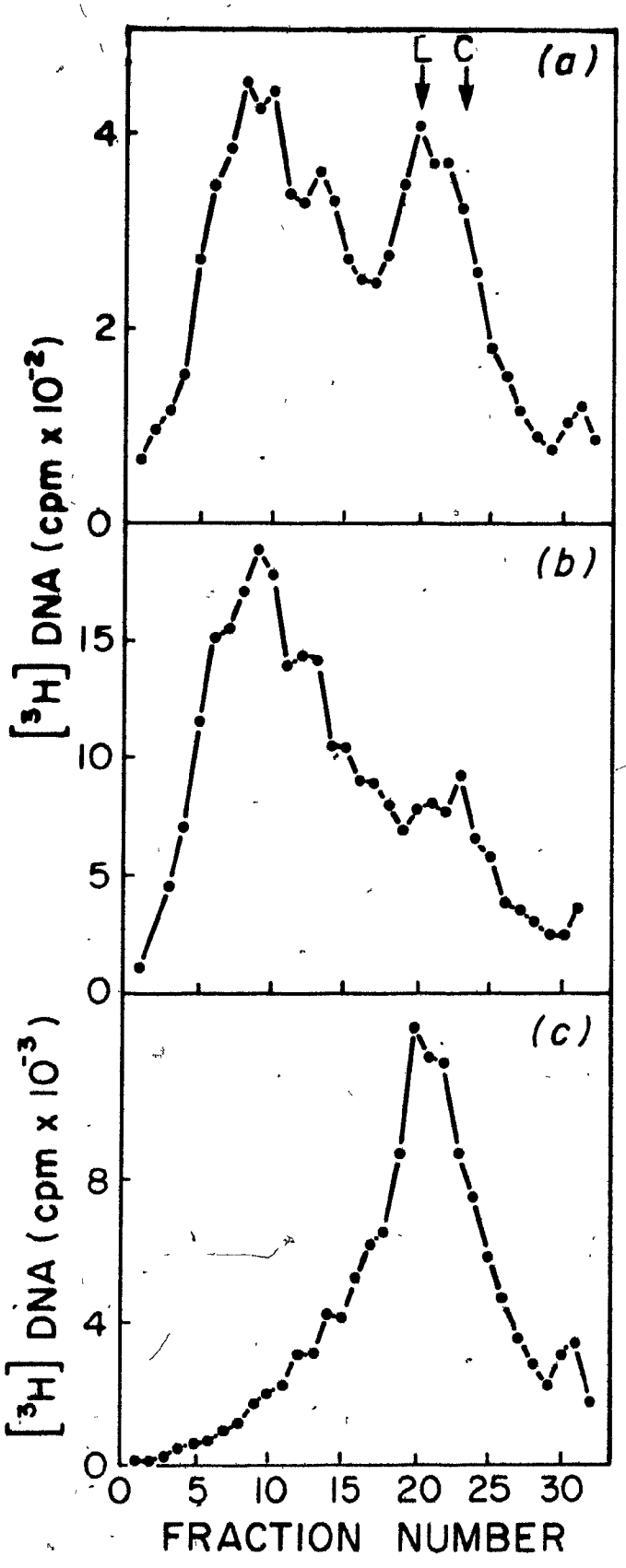
Legend to Figure 5:

Effect of host strain on [^{3}H]thymidine pulse-labelled nascent ϕX. Sedimentation was from left to right on alkaline sucrose gradients; composition of the gradients and sedimentation conditions are described in Materials and Methods, Section 2(c). Arrows indicate the positions of linear (L) and circular (C) ^{32}P-labelled ϕX DNA.

(a) ϕX infected *E. coli* HF4704, grown at 28°C in TKCaB medium, pulsed 50 minutes after infection.

(b) ϕX-infected *E. coli* CR, grown at 33°C in TKCaB medium, pulsed at 28°C 35 minutes after infection.

(c) 5 second pulse described in (b) followed by a 50 second chase with 10 μg/ml thymidine in ϕX-infected *E. coli* CR.

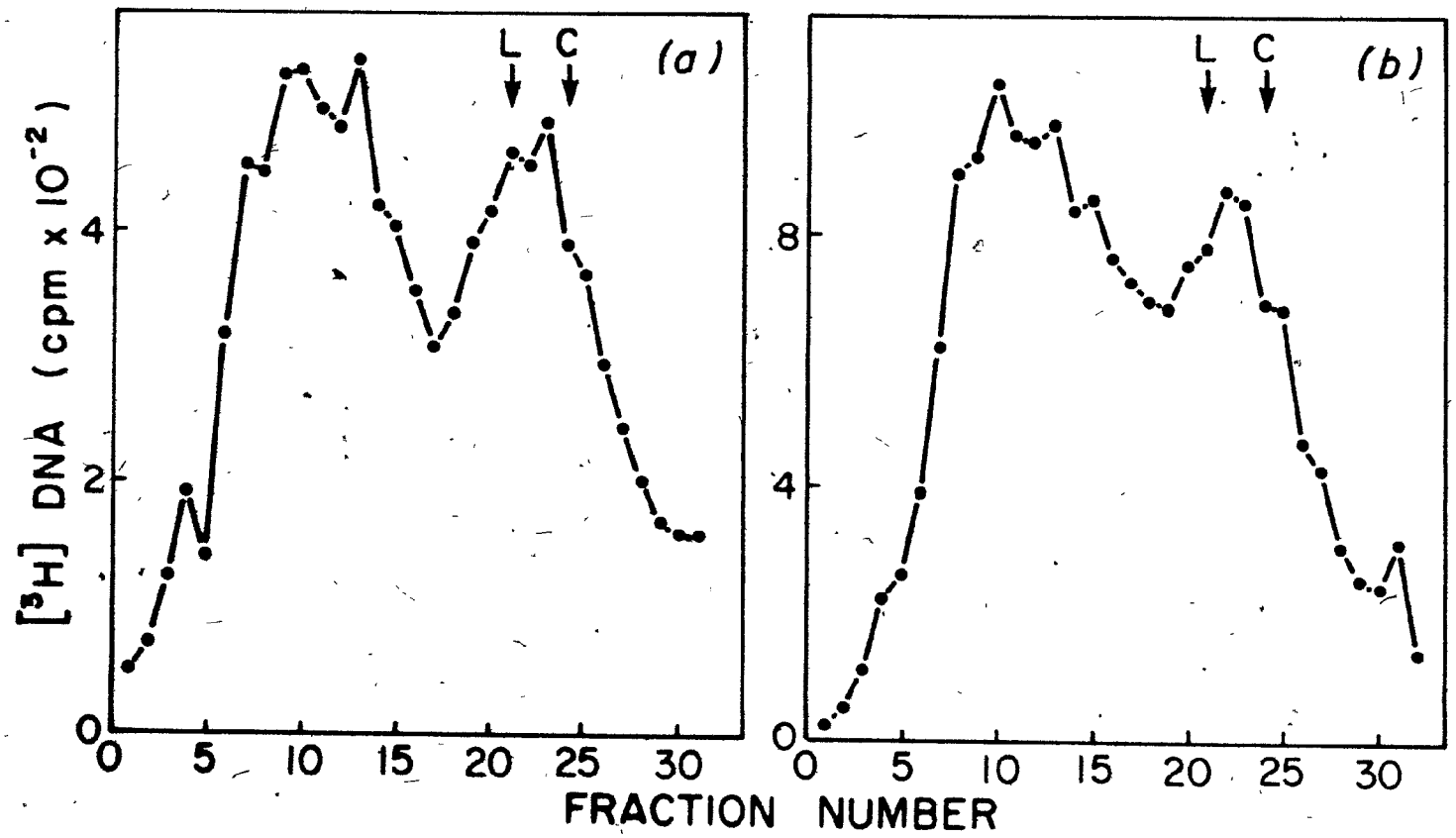

L
C
(a)
(b)
(c)
[3H] DNA (cpm x 10-2)
[3H] DNA (cpm x 10-3)
0
2
4
5
10
15
8
0
5
10
15
20
25
30
FRACTION NUMBER

Legend to Figure 6:

Comparison of [^{3}H]thymidine pulse-labelled nascent ϕX DNA in *ung* and *ung*$^+$ host strains. Sedimentation was from left to right on alkaline sucrose gradients; composition of the gradients and sedimentation conditions are described in Materials and Methods, Section 2(c). Arrows indicate the positions of linear (L) and circular (C) ^{32}P-labelled ϕX DNA. Cultures were grown at 28°C in TKCaB medium and pulse-labelled 50 minutes after infection.

(a) BW 265: *ung*

(b) BW 264: *ung*$^+$

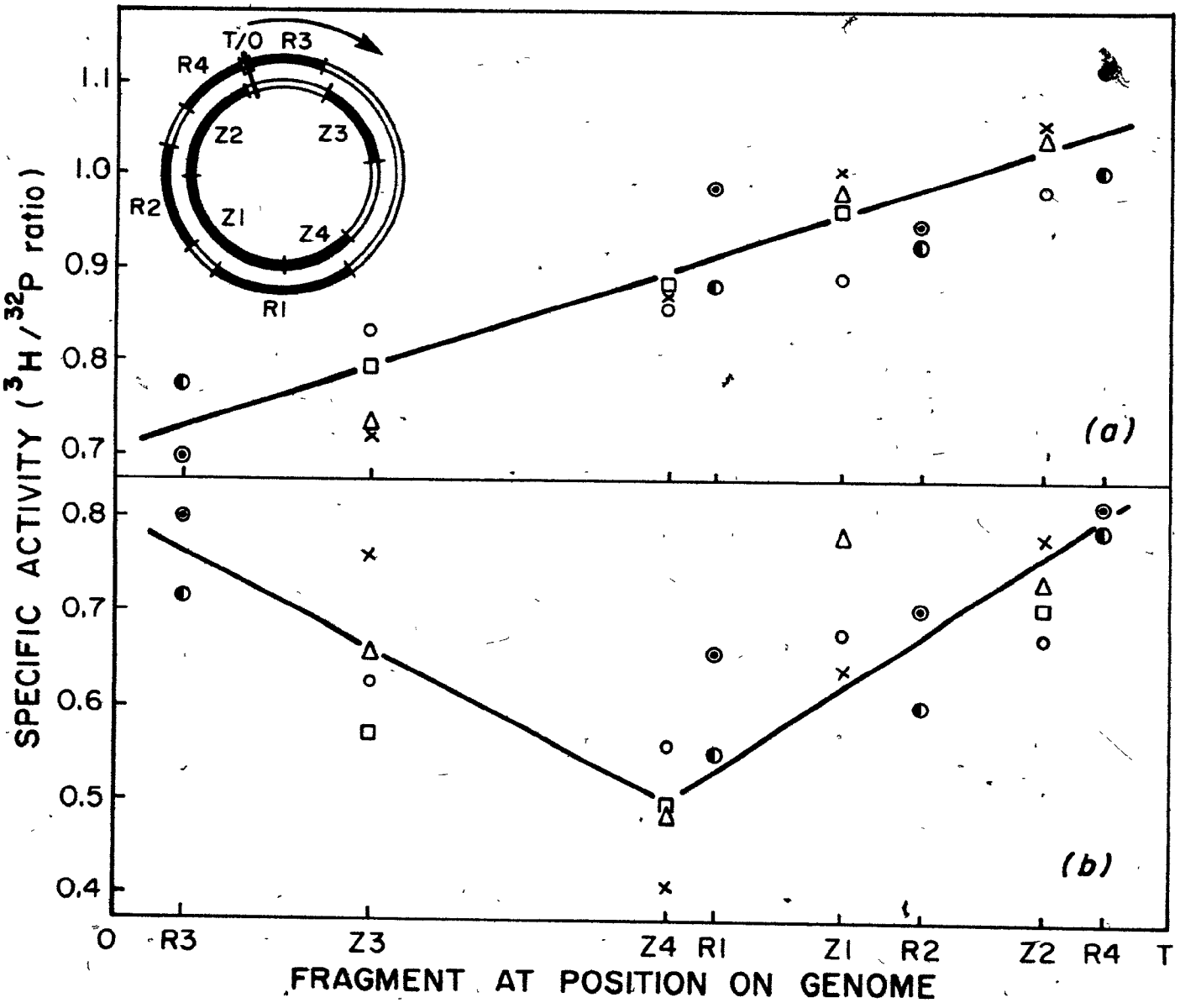

L
C
(a)
L
C
(b)
[³H] DNA (cpm x 10⁻²)
0
2
4
0
4
8
0 5 10 15 20 25 30
0 5 10 15 20 25 30
FRACTION NUMBER

Legend to Figure 7:

Plot of the specific activity of the DNA hybridized to the larger *Hae*III and *Hin*dII fragments of ϕX RF against their positions on the genetic map. Background was subtracted from the total [^{3}H]nascent and [^{32}P]non-nascent DNA hybridized to each restriction enzyme fragment on the nitrocellulose filter and the $^{3}H/^{32}P$ ratio determined. Only the four largest fragments were well enough resolved to permit such quantitation. The specific activity [$^{3}H/^{32}P$ ratio] of each fragment was corrected for thymine content (assuming uniform labeling within the fragment) and plotted against the midpoint of the fragment position around the physical map determined by Lee & Sinsheimer (1974). The origin of a round of replication, indicated by O, is at the far left; the terminus, indicated by T, is at the far right on the linear map. The circular map, reproduced from Denhardt (1977), shows the approximate size and location of the various *Hae*III (Z1, Z2, Z3, Z4) and *Hin*dII (R1, R2, R3, R4) fragments around the ϕX genome. The arrow indicates the direction of replication from the origin to the terminus. Each point (Δ,□ , O, X, ●, ◐) represents a separate experiment.

(a) unit-length nascent ϕX DNA

(b) shorter than unit-length nascent ϕX DNA


T/O R3
R4
Z2
Z3
R2
Z1
Z4
R1
SPECIFIC ACTIVITY ($^3H/^{32}P$ ratio)
1.1
1.0
0.9
0.8
0.7
(a)
0.8
0.7
0.6
0.5
0.4
(b)
O R3 Z3 Z4 R1 Z1 R2 Z2 R4 T
FRAGMENT AT POSITION ON GENOME

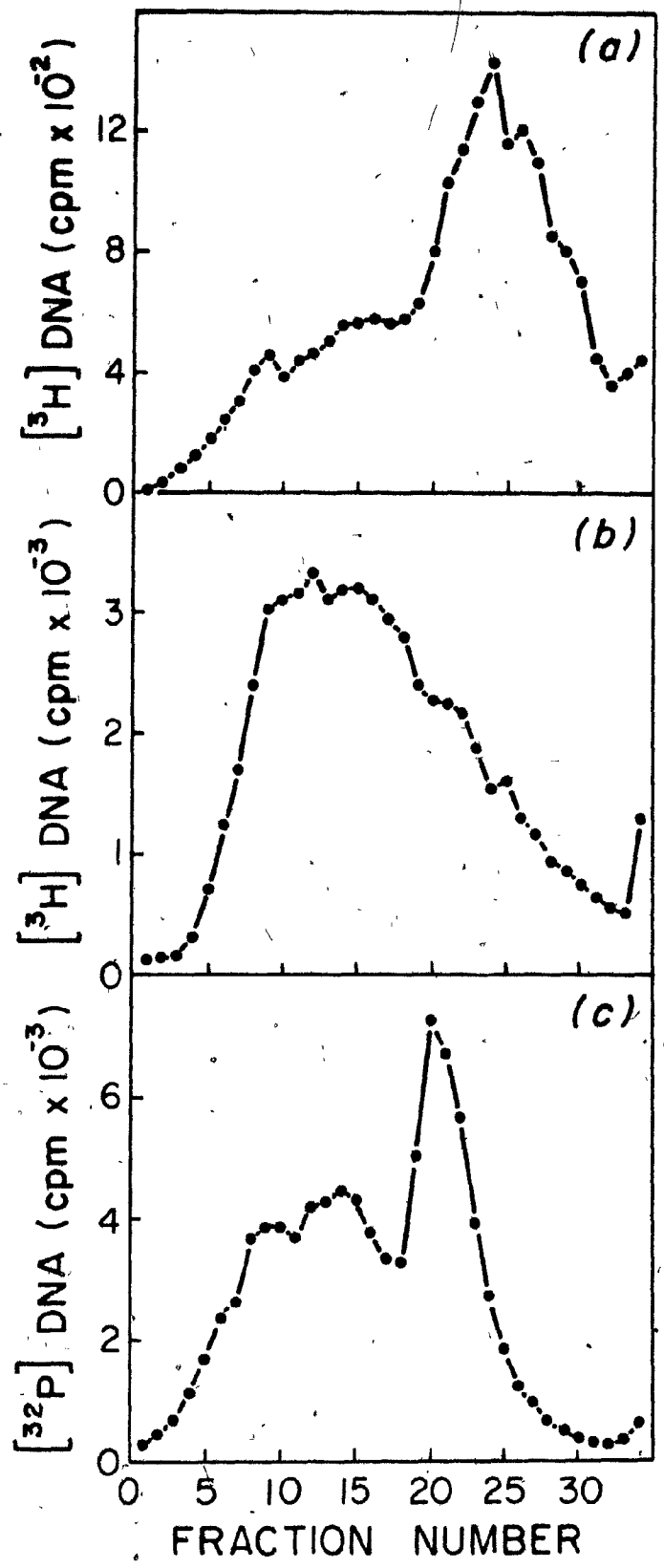
Legend to Figure 8:

Neutral sucrose gradient sedimentation of ϕX DNA for 5' end analysis with spleen exonuclease. DNA was isolated and sedimented on neutral sucrose gradients; composition of the gradients and sedimentation conditions are described in Materials and Methods, Section 2(c). Direction of sedimentation is from left to right:

(a) [^{3}H]thymidine pulse-labelled nascent ϕX DNA in *E. coli* C during viral strand synthesis 35 minutes after infection.

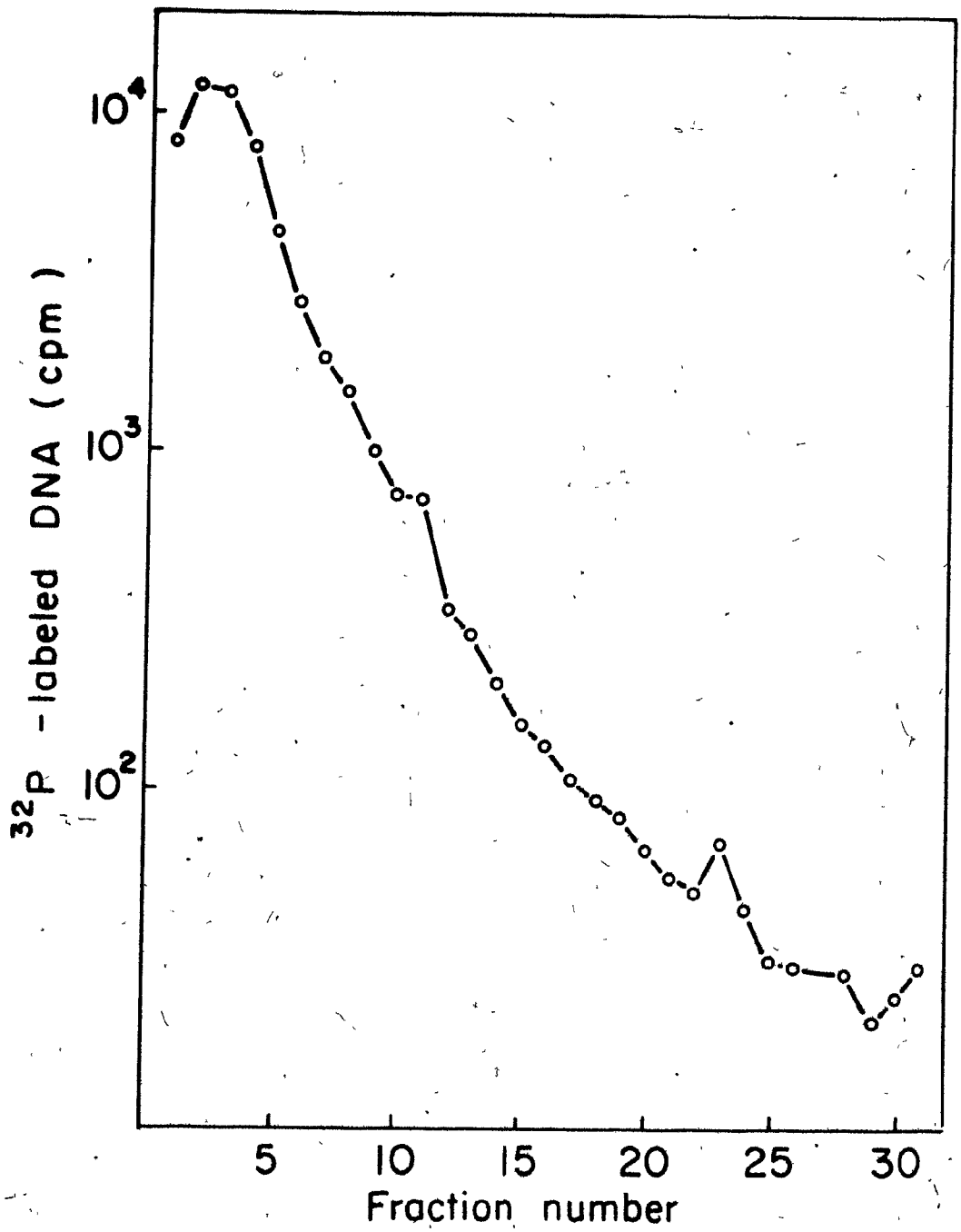
(b) [^{3}H]thymidine pulse-labelled nascent ϕX DNA in *E. coli* C during RF replication. Chloramphenicol at a final concentration of 30 μg/ml was added 5 minutes after infection, and the culture was pulse labelled 15 minutes later.

(c) Uniformly ^{32}P-labelled ϕX viral strand DNA, sonicated for 30 seconds at output 60 with an Artek sonic dismembrator.


(a)
[3H] DNA (cpm x 10-2)
12
8
4
0
(b)
[3H] DNA (cpm x 10-3)
3
2
1
0
(c)
[32P] DNA (cpm x 10-3)
6
4
2
0
0 5 10 15 20 25 30
FRACTION NUMBER

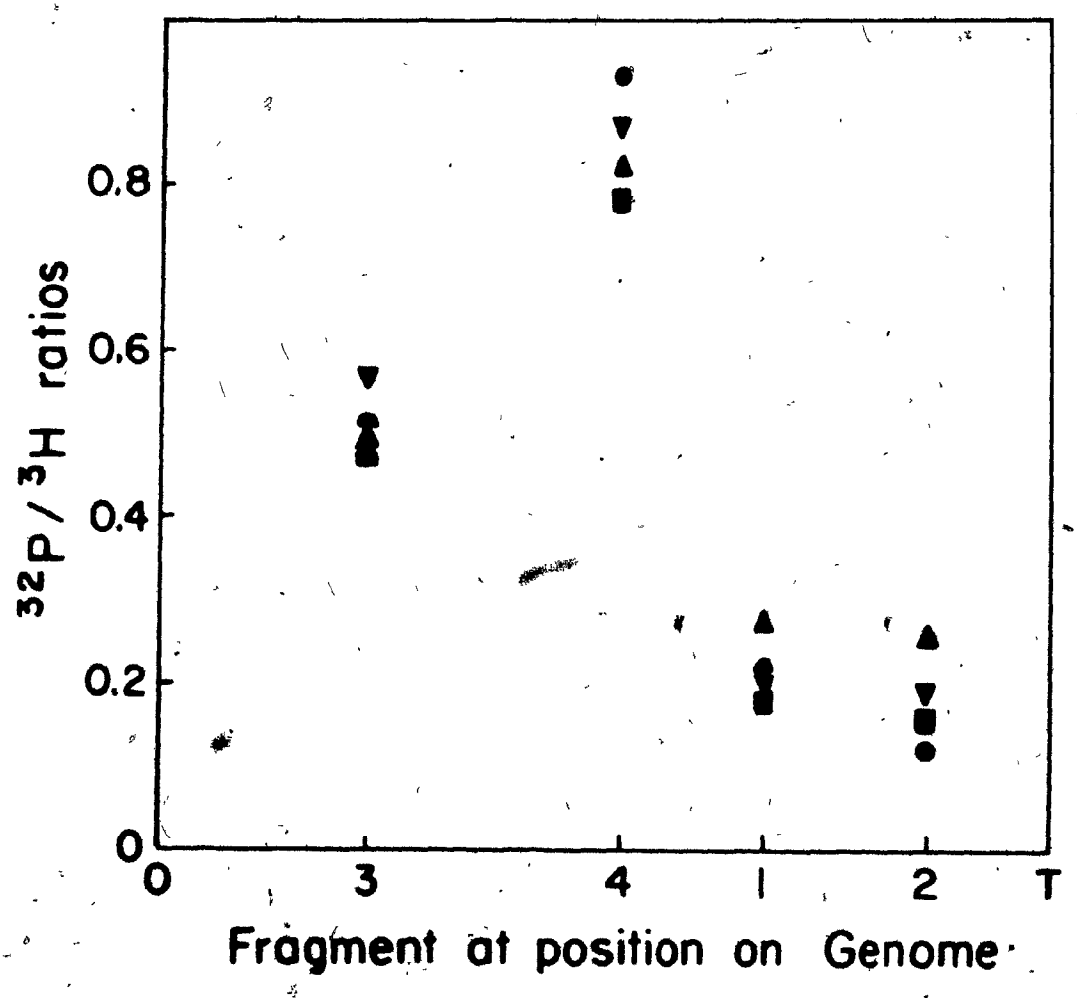
Legend to Figure 9:

Neutral sucrose gradient sedimentation of ^{32}P-end-labelled DNA. The DNA shorter than unit length, recovered from φX-infected cultures of *E. coli* C which had not been pulse-labelled with [^{3}H]thymidine, was end-labelled with [γ-^{32}P]ATP and polynucleotide kinase. After three sodium acetate and isopropanol precipitations and nitrocellulose chromatography to remove the unincorporated [γ-^{32}P]ATP, the ^{32}P-end-labelled DNA was sedimented on a neutral sucrose gradient in the SW 27 rotor. Composition of the gradient and centrifugation conditions are described in Materials and Methods, Section 2(c). Direction of sedimentation was from left to right. Fractions 1 to 4, containing the "C" molecules, and fractions 5 to 18, containing the short molecules, were separately pooled and precipitated with sodium acetate and isopropanol.


10^4
10^3
10^2
^{32}P - labeled DNA (cpm)
5
10
15
20
25
30
Fraction number

Legend to Figure 10:

Plot of the hybridization of ^{32}P-end-labelled shorter than unit length φX DNA against position on the genetic map.
The ^{32}P-end-labelled DNA shorter than unit length recovered from the sucrose gradient depicted in Figure 9, and uniformly ^{3}H-labelled φX viral strand DNA, were hybridized to Southern blots containing *Hae*III restriction enzyme fragments of φX RF, as described in Materials and Methods, Section 2(i). Background was substracted from the total ^{32}P-end-labelled and uniformly ^{3}H-labelled DNA which hybridized to each of the four largest fragments. The ^{32}P/^{3}H ratios were determined, and corrected for the thymine content of the individual fragments. Then the ^{32}P/^{3}H ratios were plotted versus the midpoint of the fragment position on the genetic map determined by Lee and Sinsheimer (1974). O and T denote the origin and terminus of a round of φX replication on the linear map. The approximate size and location on the genome of the four *Hae*III fragments are shown on the inner circle of the insert in Figure 7a.
(●, ■) ^{32}P-end-labelled short molecules
(▲, ▼) ^{32}P-end-labelled "C" molecules


0.8
0.6
0.4
0.2
0
$^{32}P / ^{3}H$ ratios
0
3
4
1
2
T
Fragment at position on Genome

Facing page 96.

Legend to Figure 11:

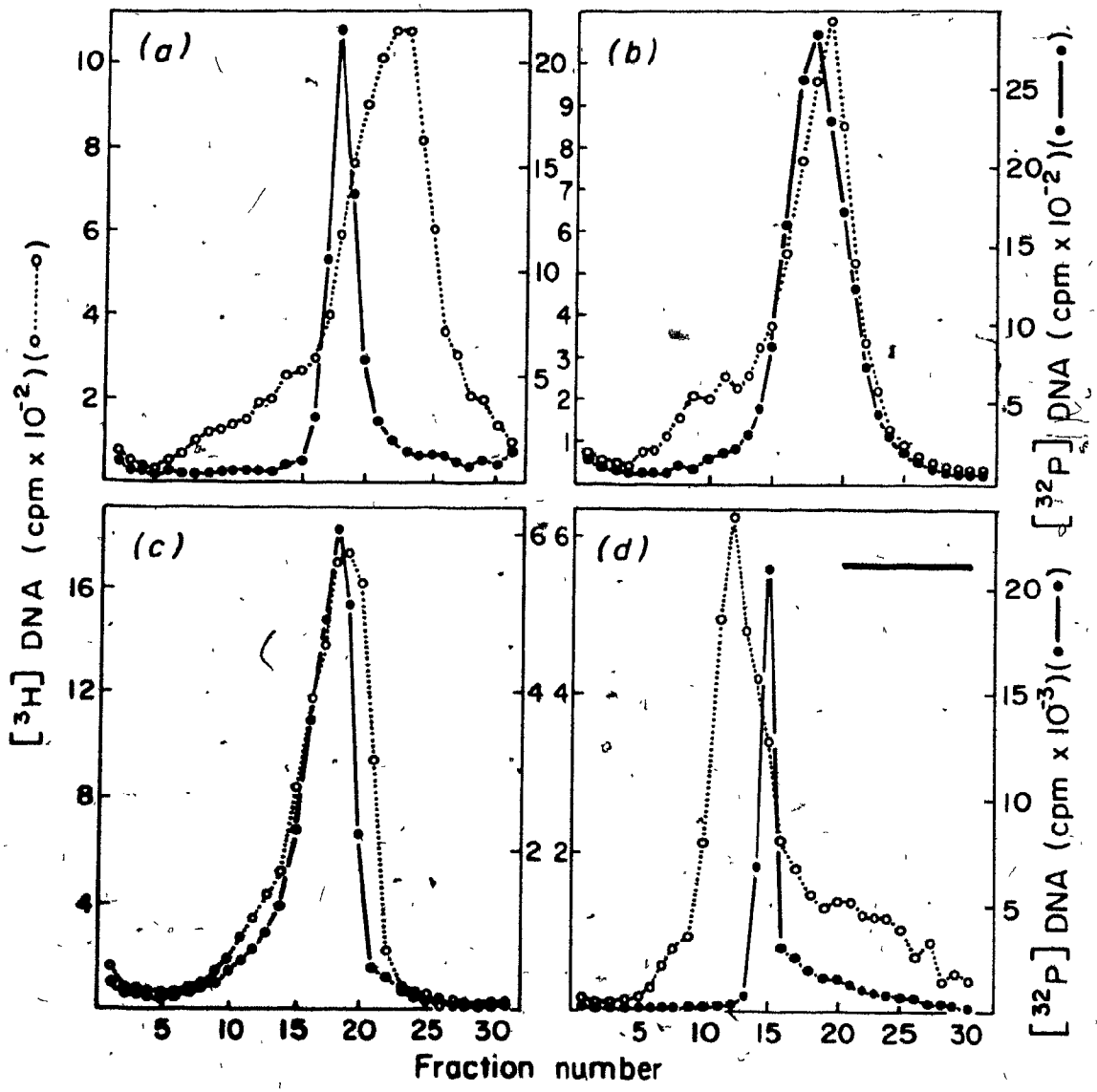
Analysis of molecules longer than unit length. The ^{3}H-labelled nascent ϕX DNA which sedimented ahead of the position expected for unit length ϕX viral strand DNA on the preparative neutral sucrose gradient shown in Figure 8(a) (- o - o - o -) and ^{32}P-labelled unit length ϕX viral strand DNA (● - ● - ●) were analyzed by sedimentation and electrophoresis. The composition of the gradients and gels, plus the sedimentation and electrophoresis conditions, are listed in Materials and Methods, Section 2(j). Direction of sedimentation and electrophoresis was always from left to right.

(a) neutral sucrose gradient

(b) alkaline sucrose gradient

(c) formamide sucrose gradient

(d) alkaline agarose gel; the heavy line indicates the position of the bromophenol blue marker dye.


(a)
(b)
(c)
(d)
[3H] DNA (cpm x 10-2)(o——o)
[32P] DNA (cpm x 10-2)(•——•)
[32P] DNA (cpm x 10-3)(•——•)
Fraction number

CHAPTER III

Characterization of ^{3}H-labelled Short Nascent ϕX Molecules

1. INTRODUCTION

The replication of bacteriophage ϕX174 can be divided into three distinct stages: (1) conversion of the single-stranded viral DNA to the duplex replicative form (RF); (2) RF replication, during which multiple copies of RF are synthesized semi-conservatively; (3) synthesis of viral strand DNA using the complementary strand of RF DNA as the template (Dressler & Denhardt, 1968; Sinsheimer *et al.*, 1969). We have established that synthesis of the viral strand is at least in part a discontinuous process (Matthes & Denhardt, 1980), not continuous as orginally proposed by the rolling circle model (Gilbert & Dressler, 1969). A substantial portion of the [^{3}H]thymidine incorporated during the final stage of ϕX replication when exclusively viral strands are made is found in molecules shorter than unit length. These short molecules are observed in similar amounts in uracil-DNA glycosylase deficient (*ung*) and wild type strains, indicating that they do not result from the excision of misincorporated uracil. Using the spleen exonuclease assay (Kurosawa *et al.*, 1975), we estimated that approximately 20% of the ^{3}H-labelled short molecules have at least 1 ribonucleotide at the 5' terminus.

Spleen exonuclease is a 5' end-specific nuclease. It was found that after phosphatase treatment, the ^{3}H-labelled nascent molecules were not as extensively degraded by spleen exonuclease as ^{32}P-labelled non-nascent molecules of approximately the same size. Data are presented here which indicate that the resistance of the ^{3}H-labelled DNA molecules to degradation by spleen exonuclease is due neither to an enrichment for secondary structure in the nascent molecules, nor to an uneven distribution of [^{3}H]thymidine label in the nascent molecules. We are left with the conclusion that an

unidentified structure is blocking the action of spleen exonuclease on the nascent molecules. Only prolonged incubation in strong alkali removed the block.

There are a number of reports of proteins covalently attached to the 5' ends of DNA molecules. These include the ϕX gene A protein (Eisenberg *et al.*, 1977; Ikeda *et al.*, 1979; Dubeau & Denhardt, submitted for publication), the Col E 1 protein (Guiney & Helinski, 1975), the ϕ29 protein (Ito, 1978; Salas *et al.*, 1978), the *E. coli* DNA gyrase (Morrison & Cozzarelli, 1979), and the adenovirus terminal protein (Carusi, 1977; Rekosh *et al.*, 1977). In some of these cases, the proteins render the DNA molecules resistant to the action of 5' end-specific nucleases. To determine if a protein could be causing the ^{3}H-labelled nascent DNA to be resistant to degradation by spleen exonuclease, the ^{3}H-labelled DNA was isolated without the use of proteolytic enzymes. Protein(s) was found tightly asspciated with the ^{3}H-labelled nascent DNA as evidenced by a Pronase-sensitive shift to a lighter density in neutral CsCl gradients. The protein(s) could be removed by alkali, and varied in amounts proportional to the length of the DNA molecule.

2. MATERIALS AND METHODS

(a) Bacteria and bacteriophage

$\phi X am3$ is a lysis-defective gene E mutant. *E. coli* C was described by Denhardt (1969).

(b) Media, chemicals, buffers, and radioactive compounds

Cultures were grown in TKCaB medium (1% Tryptone, 0.5% KCl, 1 mM $CaCl_2$).

Isoamyl nitrite was from Matheson, Coleman, & Bell Chemical Co., and reagent grade hydroxylamine hydrochloride, purchased from Fisher Scientific, was prepared as described by Gumport & Lehman (1971). Salmon sperm DNA was purchased from Sigma.

TE buffer is 50 mM Tris-HCl (pH 8), 10 mM Na_2EDTA. TEK buffer is TE buffer containing 0.5 M KCl; TEN buffer is TE buffer containing 0.5M NaCl. Borate is 0.05 M sodium tetraborate decahydrate. SSC is 0.15 NaCl, 0.015 sodium citrate, adjusted to pH 7 with a few drops of 6 M HCl.

[methyl-^{3}H]thymidine (50 Ci/mmole), [^{32}P]phosphate, Aquassure and Omnifluor were purchased from New England Nuclear. [γ-^{32}P]ATP, prepared as described by Schendel & Wells (1973), was generously provided by C. Miyamoto. The specific activity of the [γ-^{32}P]ATP was 10^6 cpm/pmol.

(c) Preparation of DNA

The ^{3}H-labelled nascent DNA was isolated as described in Chapter II Section 2(c) unless specified otherwise. The preparation of ^{3}H-labelled and ^{32}P-labelled ϕX viral strand DNA was described in Chapter II Section 2(d). Unlabelled ϕX RF DNA was prepared as described in Chapter II Section 2(e).

The ^{3}H-labelled nascent DNA was purified without the use of proteolytic enzymes by the following procedure: Infection, pulse labelling, and stopping were carried out as described in Chapter II Section 2(c). The first isopropanol precipitate from a 200 ml culture was resuspended in 10 ml TEN buffer. The turbid solution was extracted at room temperature with 2 volumes of phenol/chloroform (1:1, v:v) saturated with borate buffer. After centrifugation for 10 minutes at room temperature on a Sorvall table-top centrifuge at 70 volts, the lower phenol phase plus the material at the interphase were precipitated with 2 volumes of 95% ethanol. After 16 hours at -20°C, the precipitate was collected by centrifugation at 0°C for 1 hour at 10,000 rpm in the HB4 rotor of the Sorval RC2B. The pellet was resuspended in 7 ml 4 M Guanidine·HCl; heating at 100°C for 3 minutes and vortexing vigorously were required to dissolve the pellet. CsCl was added to a final concentration of 0.525 g per g of solution, and the nucleic acids and proteins were centrifuged for 20 to 60 hours at 40,000 rpm and 10°C in the Type 65 or Type 50 Ti rotors in a Beckman L2-65B ultracentrifuge. Fractions of 300 µl were collected from the bottom with a B-D Cornwall syringe, and aliquots were counted on filter paper in toluene/Omnifluor. The peak of ^{3}H-labelled material was pooled, diluted three fold with TE buffer, and precipitated with 0.1 volume of 3M sodium acetate (pH 5.5) and 2 volumes of isopropanol. The pellet was collected in the HB4 rotor as described above, resuspended in 2 ml 4 M Guanidine·HCl, heated for 2 minutes at 100°C, then layered on a 36 ml sucrose (5% to 20%, w/v) gradient containing 4 M Guanidine·HCl, 10 mM Tris-HCl (pH 8), 1 mM Na_2EDTA. The gradient was centrifuged in the L2-65B ultracentrifuge and SW27 rotor at 22,000 rpm and 10°C for 24 hours. Fractions were

collected from the top using a Buchler Auto densiflow and polystaltic pump. Aliquots were spotted on 1 inch squares of Whatman no. 3 filter paper and dried. The radioactivity was determined by scintillation counting in 10 ml toluene/Omnifluor. The appropriate fractions were pooled and diluted about three fold with TE buffer; the DNA was precipitated with sodium acetate and isopropanol, and finally resuspended in TE buffer.

(d) Enzymes and enzyme reactions

Pancreatic DNase was purchased from Worthington. A 1 mg/ml stock solution of the enzyme in 1 mg/ml BSA was stored for several months at 4°C. When required it was diluted 1:1000 in 50 mM Tris-HCl (pH 8), 50 mM $MgCl_2$, and 0.1 volume of this 1 µg/ml DNAse solution was incubated with 100 µg/ml ϕX DNA in 50 mM Tris-HCl (pH 8), 4 mM $MgCl_2$ for 15 minutes at room temperature. The reaction was stopped by adding Na_2EDTA and SDS to final concentrations of 50 mM and 0.1% respectively.

Spleen exonuclease, purified according to the procedure of Bernardi & Bernardi (1968), was provided by Neil Miyamoto. DNA at a concentration of 100 µg/ml was heated briefly at 100°C then adjusted to 0.05 M Na_2SO_4, 0.01 M Na_2EDTA 0.15 M sodium acetate (pH 5.5). After the addition of 66 µg/ml of spleen exonuclease, the reaction was allowed to proceed at 45°C for intervals which ranged from 20 minutes to 72 hours. For long incubations, additional enzyme was added at 12-hour periods after the initial 12 hours.

Snake venom phosphodiesterase was generously donated by Dr. J.H. Spencer. DNA at a concentration of 100 µg/ml in 50 mM Tris-HCl (pH 9), 20 mM $MgCl_2$, was incubated with enough enzyme to completely degrade the DNA in 2 hours at 37°C.

Neurospora crassa nuclease, purified according to the procedure of Rabin *et al.* (1971), was the generous gift of Dr. K. Bartok. DNA at a concentration of 40 µg/ml in 0.1 M Tris-HCl (pH 8), 0.1 M NaCl, 10 mM $MgCl_2$ was incubated at 37°C with 3 units/ml nuclease. This was sufficient to completely degrade the DNA in 2 hours. One unit is defined as that amount which will cause the release of 1.0 A_{260nm} unit of acid-soluble material from 600 µg heat-denatured calf thymus DNA/ml in 30 minutes at 37°C.

The spleen exonuclease, snake venom phosphodiesterase and *Neurospora crassa* nuclease reactions were stopped by removing aliquots at appropriate intervals into a 200 µg excess of salmon sperm DNA on ice, and immediately adding an equal volume of either cold 10% trichloracetic acid or water. After 15 minutes on ice, the samples were centrifuged for 10 minutes in an Eppendorf microfuge at 4°C. The acid-soluble or total radioactivity in the supernatants was determined in 10 ml Aquassure to which either water or trichloroacetic acid containing salmon sperm DNA had been added to ensure equivalent counting efficiencies in the two samples.

The spleen exonuclease and snake venom phosphodiesterase reactions were preceded by bacterial alkaline phosphatase treatment, unless specified otherwise. Phosphatase was purified by Neil Miyamoto according to the method described by Weiss *et al.* (1968). DNA at a concentration of 100 µg/ml in 50 mM Tris-HCl (pH 9) was incubated with phosphatase (2 units/ml) at 65°C for 1 hour. The reaction was terminated by adding EGTA to a final concentration of 50 mM.

Polynucleotide kinase was purchased from PL Biochemicals. DNA at a concentration of 100 µg/ml in 2 mM KPO_4 (pH 7.4), 10 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 25 µM ATP, and 4 units/ml polynucleotide kinase were

incubated for 2 hours at 37°C. The reaction was stopped by adding Na_2 EDTA to a final concentration of 20 mM. For quantitative labelling of 5' termini, [γ-^{32}P]ATP was added to a final concentration of 10 µM. The [γ-^{32}P] ATP labelled DNA was purified by three isopropanol precipitations and nitrocellulose column chromatography.

Pyrophosphatase, purchased from Sigma, was generously donated by Dr. D. Skup and Dr. S. Millward. DNA at a final concentration of 160 µg/ml in 50 mM sodium acetate (pH 5.5), 10 mM 2-mercaptoethanol, 1 mM Na_2EDTA was incubated with 0.68 units/ml pyrophosphatase for 4 hours at 37°C. One unit of pyrophosphatase releases 1 pmol of ^{32}P from [γ^{32}P]ATP in 30 minutes at 37°C.

Pronase was purchased from Calbiochem-Behring Corp. A 10 mg/ml stock solution in 50 mM Tris-HCl (pH 8) was autodigested for 1 hour at 37°C. DNA at a concentration of 10 to 100 µg/ml was incubated with 1 mg/ml pronase and 0.1% SDS for 3 hours at 37°C. The mixture was extracted with an equal volume of borate-saturated phenol at room temperature for 10 minutes. The DNA recovered in the aqueous phase was precipitated with isopropanol.

The DNA was routinely precipitated with 0.1 volume of 3 M sodium acetate (pH 5.5) and 2 volumes of isopropanol, incubated overnight at -20°C, then collected by centrifugation at 0°C in the HB4 rotor and the Sorvall RC2B centrifuge at 10,000 rev/min for 1 hour, air-dried, and resuspended in the appropriate buffer.

(e) Treatment with isoamyl nitrite and hydroxylamine hydrochloride

Two samples, containing from 10 to 20 µg of DNA in 800 µl of a buffer composed of equal volumes of pyridine and acetic acid, were prepared;

100 µl of isoamyl nitrite was added to one. Both were thoroughly mixed and left at room temperature for 4 hours. The volatile solvents were evaporated on a Rotary Evapo-Mix, and the residual nucleic acids were resuspended in 900 µl TE buffer. After two precipitations with sodium acetate and isopropanol, the DNA was purified by nitrocellulose chromatography. It was found that if nitrocellulose chromatography was ommitted, trace amounts of chemicals interfered with subsequent phosphatase and spleen exonuclease reactions. Recovery was greater than 70%.

From 10 to 20 µg DNA in 200 µl 3.86 M hydroxylamine hydrochloride (pH 4.75) or 4 M sodium acetate (pH 4.75) was incubated at 37°C for 30 minutes. Then the samples were diluted to 900 µl with dH_2O, precipitated twice with sodium acetate and isopropanol, and purified by nitrocellulose chromatography before phosphatase and spleen exonuclease reactions. Recovery was greater than 90%.

(f) Nitrocellulose column chromatography

Nitrocellulose, a gift of Hercules Chemical Co., was prepared for column chromatography as described by Boezi & Armstrong (1967). Columns 3 cm high were prepared in sterile pasteur pipettes and pre-equilibrated with 15 ml TEK buffer. It was found that recoveries greater than 90% could be obtained from nitrocellulose columns, if nonreversible DNA binding sites in the column were saturated by running an excess (300 µg) of denatured salmon sperm DNA through the column before use. From 10 to 20 µg of DNA in 200 to 400 µl TEK buffer was applied, the column was washed with 15 ml TEK buffer, and the DNA was eluted with 4 ml TE buffer and precipitated with sodium acetate and isopropanol. If necessary, 10 to 20 µg heat-denatured salmon sperm DNA was added as carrier.

(g) Preparation of filters and hybridization to filters

Schleicher & Schuell BA85 nitrocellulose filters, 25 mm in diameter, containing 6 µg denatured ϕX RF DNA, were prepared as described by Lane & Denhardt (1974). The DNA to be hybridized was resuspended in 400 µl 4 x SSC, sonicated for 1 minute at output 60 on an Artek sonic dismembrator, then heated at 100 °C for 5 minutes to ensure complete denaturation. An equal volume of formamide purified by passage through a column containing Norite and Dowex 1-X8 was added, and the solution was placed on a nitrocellulose filter in a sterile scintillation vial. After incubation at 42°C for 48 hours, the filters were washed in 2 x SSC 50% formamide, then in 3 x SSC, and dried. The radioactivity was determined in toluene/ Omnifluor.

(h) Analytical equilibrium centrifugation and agarose gel electrophoresis

The DNA to be analyzed was added to 3 ml TE buffer containing 100 µg BSA and 60 µg heat-denatured salmon sperm DNA in a 10 ml Oakridge polypropylene centrifuge tube. CsCl (1.25 g per g solution) was added and the solutions centrifuged for 40 to 60 hours (40,000 rpm, 10°C, Type 50 Ti or Type 40 rotor, Beckman L2-65B.) Fractions of 100 µl were collected from the bottom onto 1 inch squares of Whatman no. 3 filter paper. After drying the papers, the radioactivity was determined in toluene/ Omnifluor.

Alkaline gels, containing 1% agarose, were prepared with sample wells as described by Anderson (1978). The gel dimensions were 0.6 cm wide and 18 cm long. Samples of 50 to 100 µl, containing about 10 µg DNA in 0.2 M NaOH, 0.1% bromophenol blue, 25% glycerol, were applied.

The gels were electrophoresed at room temperature at 5 mamp/gel until the dye had migrated close to the bottom of the gel. The gels were cut into 5 mm slices which were autoclaved for 10 minutes in 1 ml dH_2O in scintillation vials. The radioactivity in the dissolved gel slices was determined in 10 ml Aquassure.

3. RESULTS

(a) Properties of the spleen exonuclease reaction

Spleen exonuclease cleaves DNA sequentially starting at the 5' end of the molecule. It is absolutely specific for a hydroxyl group at the 5' end of a DNA chain. Bacterial alkaline phosphatase removes 5' terminal phosphate groups. Figure 1(a) shows that when a mixture of ^{3}H-labelled nascent and ^{32}P-labelled non-nascent DNA molecules of the same size were exposed to spleen exonuclease without prior phosphatase treatment, neither was degraded to a significant extent. After treatment with phosphatase, both the ^{3}H- and ^{32}P-labelled DNAs were rendered partially acid-soluble on exposure to spleen exonuclease. If the phosphatase reaction was followed by phosphorylation of the 5' ends with polynucleotide kinase and ATP, both the ^{3}H- and the ^{32}P-labelled DNAs again became resistant to degradation by spleen exonuclease.

Figure 1 depicts the release of acid-soluble material by spleen exonuclease as a function of time. After the initial three hours, the rate of the reaction decreased considerably. Figure 1(a) shows that between eight and twenty-four hours after the start of the reaction, both 5' OH and 5' P terminated chains were degraded at a similar, slow, relatively constant rate. This suggests that there may be a low level of endonuclease contaminating the spleen exonuclease preparation. In long incubations, the endonuclease would generate molecules with 5' OH ends which are consequently sensitive to the exonuclease.

Spleen exonuclease does not easily degrade DNA chains to completion (Kurosawa *et al.*, 1975). Other things being equal, the extent of degradation is proportional to the length of the DNA chain; the longer the chain,

the smaller the fraction of it which can be degraded. Figure 1(c) shows that after 2 hours, the ^{32}P-labelled very short molecules* became 60% acid soluble, while the ^{32}P-labelled short molecules, which are approximately twice as long, became 30% acid soluble. Since 60% of a molecule 500 nucleotides long is equivalent to 30% of a molecule 1000 nucleotides long in terms of total nucleotides hydrolyzed, it appears that spleen exonuclease cleaves a relatively constant number of nucleotides from the 5' end of a DNA chain and then stops.

Spleen exonuclease is highly specific for single-stranded DNA. Longer molecules may not be completely degraded by spleen exonuclease because of internal regions of secondary structure. If the partially degraded molecules were heat-denatured and exposed to fresh enzyme, there was no increase in the rate of digestion, as illustrated in Figure 1(b). It is possible that spleen exonuclease lost activity after degrading a certain fixed length of a DNA molecule. That the addition of more enzyme did not enhance the rate or extent of the reaction as shown in Figure 1(b) could be explained if the inactive enzyme remained associated with the partially degraded DNA molecule.

It is apparent in Figure 1(c), that the extent of degradation by spleen exonuclease is dependent on the size of the molecule. However, when the same size molecules were compared, the ^{32}P-labelled non-nascent molecules were degraded to a greater extent than the ^{3}H-labelled nascent molecules. There was some variability from experiment to experiment in the degradation of the ^{3}H-labelled nascent DNA relative to the ^{32}P-labelled DNA. The resistance of the ^{3}H-labelled nascent DNA to spleen exonuclease was not always as pronounced as in the experiments presented in Figures 1 and 2.

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* Very short molecules are found in fractions 5 to 11 of the neutral sucrose gradient depicted in Chapter II, Figure 8 a,c; short molecules are found in fractions 12 to 18 of the same gradients.

The uniformly ^{32}P-labelled DNA molecules were obtained by briefly sonicating φX viral strands. Sonication fragments DNA molecules, and may cause other damages which are not readily apparent, but which enhance the activity of spleen exonuclease on a sonicated substrate. To rule out the possibility that this artefactual explanation could account for the difference in sensitivity to spleen exonuclease between the ^{3}H-labelled nascent and ^{32}P-labelled non-nascent DNAs observed in Figures 1 and 2, the susceptibility to spleen exonuclease of short molecules obtained by sonication and by digestion with pancreatic DNase was compared; the results are given in Table I. The ^{3}H-labelled molecules were degraded to the same extent as the ^{32}P-labelled molecules, regardless of whether the ^{3}H-labelled molecules had been obtained by sonication or by DNase digestion. Furthermore, when both ^{3}H- and ^{32}P-labelled DNAs were non-nascent, there was no significant difference in the extent of degradation by spleen exonuclease.

(b) Comparison of degradation of nascent DNA by various nucleases

Spleen exonuclease is a 5' end-specific nuclease. Figure 2 shows a comparison of the degradation of ^{3}H-labelled nascent and ^{32}P-labelled non-nascent DNA by spleen exonuclease. The release of ^{32}P-label exceeds the release of ^{3}H-label for both the very short and the short molecules. The spleen exonuclease reaction does not readily go to completion, but can be coaxed to do so by prolonged incubations (up to 72 hours) with more enzyme added at 12-hour intervals. The curves in Figure 2 are biphasic because most of the release of acid-soluble material in the second half of the reaction seems to be mediated by the low level of contaminating

endonuclease which exposes termini that can be attacked by spleen exonuclease. When the ^{32}P-labelled non-nascent DNA had been completely degraded, more than 10% of the very short and more than 20% of the short ^{3}H-labelled nascent DNA had not been degraded. These values are probably underestimates of the proportion of the ^{3}H-labelled molecules that are resistant to spleen exonuclease, because the endonuclease that allows the reaction to go to completion would not discriminate between molecules with 5' ends which resist the action of spleen exonuclease and those which are sensitive. If the initial parts of the curves are extrapolated to 100%, it becomes evident that as much as 40 to 60% of the ^{3}H-labelled nascent DNA may be resistant to degradation from the 5' end by spleen exonuclease.

The slightly higher initial release of ^{3}H-label relative to ^{32}P-label from the 3' end by snake venom phosphodiesterase, followed by the slightly lower release of ^{3}H-label relative to ^{32}P-label as the reaction approaches completion, illustrated in Figure 3, indicates that there may be a slight bias in the distribution of [^{3}H]thymidine in some of the nascent molecules. This bias is not large enough to account for the much lower release of ^{3}H-label from the 5' end by spleen exonuclease shown in Figure 2.

Extensive regions of secondary structure in the nascent molecules would block the action of spleen exonuclease. However, the nascent molecules do not contain a detectable enrichment for duplex regions since brief heating, followed by rapid cooling, and the addition of fresh enzyme, did not result in an increase in the rate or extent of release of acid-soluble ^{3}H-label relative to ^{32}P-label as shown in Figure 1(b).

Furthermore, the *Neurospora crassa* single-strand specific nuclease, which discriminates strongly against duplex DNA structures*, degrades the ^{3}H-labelled nascent and ^{32}P-labelled non-nascent DNAs at the same rate and to the same extent. The data are plotted in Figure 4.

(c) Attempts to remove the resistance of the nascent DNA to spleen exonuclease

Since neither an uneven distribution of label nor an enrichment for secondary structure seem able to account for the resistance of the nascent molecules to spleen exonuclease, we next consider the alternative that an unidentified structure is blocking the 5' ends of the nascent molecules. A number of methods to remove the hypothetical 5' block were tried in the hope that what removed the block would reveal some insight into its nature.

Figure 5 presents the effects of alkali. When the ^{3}H-labelled nascent and ^{32}P-labelled non-nascent DNAs were exposed to 0.25 M NaOH for 6 hours at 37°C, there was no increase in the extent of degradation of the ^{3}H-label relative to the ^{32}P-label, even after a second phosphatase treatment, compared to control samples which had not been treated with alkali. However, when the DNAs were incubated in 1 M NaOH for 24 hours at 37°C, there was a significant increase in the release of acid-soluble ^{3}H-labelled DNA relative to ^{32}P-labelled DNA, compared to the control samples. When a second phosphatase treatment followed the exposure to alkali, the ^{3}H- and ^{32}P-labelled DNAs were degraded at the same rate and to the same extent by spleen exonuclease. The most obvious interpretation of these results is that 1 M NaOH for 24 hours at 37°C hydrolyzed

* Seven times the amount of enzyme required to completely degrade a certain amount of single-stranded DNA will not result in the release of any detectable acid-soluble material from an equivalent amount of double-stranded DNA in the same length of time at 37°C.

the structure blocking the 5' ends of some of the nascent molecules, leaving molecules with a mixture of 5'-P and 5' OH termini. When the 5' P were removed by a second phosphatase reaction, the ^{3}H-labelled nascent DNA was degraded as readily by spleen exonuclease as the ^{32}P-labelled non-nascent DNA.

If alkali removed the block, it should be possible to quantitate the number of molecules which were blocked, by end-labelling with [γ-^{32}P]ATP and polynucleotide kinase after alkali and a second phosphatase treatment. The [^{32}P]end-labelled [^{3}H]pulse-labelled DNA was hybridized to unlabelled ϕX RF on nitrocellulose filters to avoid the ambiguity due to the large excess of unlabelled *E. coli* molecules which would also be labelled with [γ-^{32}P]ATP and kinase (see Chapter II, Section 3(h)).

The $^{32}P/^{3}H$ ratio is an index of the number of ends which are labellable with [γ-^{32}P]ATP and kinase. The data summarized in Table II reveal that the $^{32}P/^{3}H$ ratios increased after exposure to alkali, and increased even more if the alkali was followed by a second phosphatase treatment, for both the very short and the short nascent molecules. However, the $^{32}P/^{3}H$ ratios increased in a similar way when the [^{3}H]DNA was obtained by sonicating uniformly-labelled ϕX viral strands. That alkali exposes approximately the same number of ends in both nascent and non-nascent ^{3}H-labelled DNA that can be labelled with ^{32}P suggests that alkali is not specifically removing a block, but is nonspecifically hydrolyzing the DNA.

That alkali degrades the DNA is confirmed by the data presented in Figure 6. Here we examined the electrophoretic mobility in agarose gels of ^{3}H-labelled nascent and ^{32}P-labelled non-nascent DNA relative to a

^{32}P-labelled ϕX viral strand marker before and after exposure to alkali. There was a significant increase in the electrophoretic mobility of both the ^{3}H- and ^{32}P-labelled DNAs after exposure to alkali. The cumulative plots in Figure 6(c) reveal that both ^{3}H-labelled nascent and ^{32}P-labelled non-nascent DNAs were fragmented by alkali to the same extent. There was no evidence of an enrichment for internal alkali-labile sites in the nascent DNA. This confirms the results presented in Chapter II, Section 3(f). Apurinic and apyrimidinic sites which occur as the result of the excision of misincorporated bases are alkali-labile (Shapiro & Chargaff, 1964). That internal alkali-labile sites are not more frequent in nascent than in non-nascent DNA implies that potential intermediates in an excision repair process do not accumulate to a significant extent in nascent DNA, and are unlikely to be responsible for generating the molecules shorter than unit length that are observed during ϕX viral strand synthesis.

Siegman and Werner (1976) reported that short [^{3}H]thymine pulse-labelled *E. coli* molecules were resistant to degradation by spleen exonuclease. Later, Werner *et al.* (1979) suggested that a short oligonucleotide joined by a pyrophosphate bond was responsible for blocking the 5' termini of the nascent molecules. More recently they have concluded that contaminating RNA was responsible for the block (Siegman & Werner, 1980). Nevertheless, to determine if a pyrophosphate bond could be linking an unusual structure to the 5' ends of our ^{3}H-labelled nascent ϕX molecules, a combination of ^{3}H-labelled nascent and ^{32}P-labelled non-nascent ϕX DNA was treated with pyrophosphatase. The data in Table III show that there was no difference in the release of

^{3}H-label relative to ^{32}P-label by spleen exonuclease between the sample which was treated with pyrophosphatase and the untreated control.

If the 5' terminal blocking structure were a protein covalently attached to the DNA, the few amino acids left after Pronase treatment should still be sufficient to inhibit the action of spleen exonuclease. Two types of covalent attachments between DNA and protein have been characterized: phosphoamide and phosphoester linkages (Shabarova, 1970). Hydrolysis of phosphoamide linkages can be catalyzed by isoamyl nitrite or hydroxylamine hydrochloride at pH 4.75 (Shabarova, 1970). The data in Table IV indicate that neither of these compounds removed the block, since there was no increase in the release of ^{3}H-label relative to ^{32}P-label in the samples treated with the chemicals compared to the untreated controls.

(d) Evidence that protein is very tightly associated with the nascent DNA

In spite of the fact that a phosphoamide linkage could not be demonstrated, there was still the possibility that a phosphoester or some other bond could be linking a few amino acids to the 5' ends of the nascent molecules. Proteins blocking the 5' ends of the nascent molecules would be easier to identify if they could be isolated intact. To do this, a method was developed to purify the ^{3}H-labelled nascent molecules without the use of proteolytic enzymes. When the nucleic acids were phenol extracted* without Pronase digestion, from 30 to 70% of the ^{3}H-labelled DNA was recovered in the phenol phase*. Difficulty in quantitating the amount of material at this stage is probably due to variable contamination by acid-soluble [^{3}H]thymidine.

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* For simplicity, the phenol/chloroform extraction, and the phenol/chloroform phase and interphase, described in Materials and Methods, are referred to as phenol extraction and the phenol phase respectively.

Because of the possibility that a DNA - protein aggregate could be artefactually produced during the first sodium acetate-isopropanol precipitation, the supernatant in one experiment was phenol extracted before precipitation. The nucleic acids and proteins in the phenol phase were precipitated with ethanol and resuspended in 4 M Guanidine·HCl, and as before, more than 50% of the ^{3}H-labelled material was recovered in the phenol phase. In another control, deproteinized ^{32}P-labelled ϕX DNA was added to the infected culture before stopping the pulse; after extraction, 85% of the ^{32}P-labelled ϕX DNA was recovered in the aqueous phase when the nucleic acids were phenol extracted without Pronase digestion. Therefore, loss of ^{3}H-labelled DNA to the phenol phase is apparently the result of an association with protein that is not artefactually produced during phenol extraction or the first isopropanol precipitation.

The ^{3}P-labelled nucleic acids from the phenol phase were recovered by equilibrium density gradient centrifugation in 4 M Guandine·HCl-CsCl gradients. The material in the band in Figure 7(a) was sedimented on 4 M Guanidine·HCl sucrose gradients. A typical profile, depicted in Figure 7(b), reveals that the ^{3}H-labelled molecules recovered from the phenol phase are heterogenous in size and range from very short to longer than unit length.

The ^{3}H-labelled molecules shorter than unit length (fractions 10 to 20 of the gradient in Figure 7(b)) were centrifuged to equilibrium in neutral CsCl gradients with ^{32}P-labelled ϕX DNA of the same size obtained by sonicating ϕX viral strands. In the gradient in Figure 8(a), there is a shift of the ^{3}H-labelled nascent DNA to a lighter density relative to

the ^{32}P-labelled non-nascent DNA. The density shift of the ^{3}H-labelled nascent DNA is caused by an association with protein, since it is no longer observed after the DNA has been digested with Pronase (Figure 8(b)). The ^{3}H-labelled nascent DNA recovered from the aqueous phase when Pronase treatment is eliminated does not show any density shift relative to ^{32}P-labelled non-nascent DNA of the same size (data not shown).

Very little protein is associated with the ^{3}H-labelled short DNA molecules recovered from the phenol phase. There is not enough protein to be detected by optical density; the $OD_{260/280nm}$ is 2, as expected for deproteinized DNA. Also neither the sedimentation coefficient of the ^{3}H-labelled DNA in neutral sucrose nor the electrophoretic mobility of the ^{3}H-labelled DNA in agarose gels are altered by the Pronase treatment that abolishes the density shift in neutral CsCl (data not shown).

Figure 9 shows the effect of alkali on the association of the ^{3}H-labelled DNA with protein. The density shift of the ^{3}H-labelled DNA relative to ^{32}P-labelled DNA is much smaller after 6 hours in 0.25 M NaOH at 37°C, and disappears completely after 24 hours in 1 M NaOH at 37°C.

If the density shift were due to the association of one specific protein molecule with the DNA, then as the length of the DNA molecule decreased, the proportion by mass of protein to DNA should increase, and consequently, the density shift should become more pronounced. The ^{3}H-labelled DNA in the gradient depicted in Figure 7(b) was divided into 4 pools: very short (fractions 10 to 15); short (fractions 16 to 20); unit length (fractions 21 to 25); and longer than unit length (fractions 26 to 30). These were separately banded in neutral CsCl gradients with

^{32}P-labelled short or unit length molecules. In Figure 10, one can see that the density shift is smallest for the very short molecules, is greater for the short molecules, and is largest for the unit length and longer than unit length molecules. The density shift varies directly with the size of the ^{3}H-labelled DNA molecule. The longer the molecule, the more protein is associated with the molecule.

The nascent molecules analyzed earlier with spleen exonuclease were treated with pronase and showed no density shift in CsCl gradients. So this effect may not explain the resistance of the nascent molecules to spleen exonuclease.

4. DISCUSSION

That the apparent resistance to degradation by spleen exonuclease of ^{3}H-labelled nascent DNA relative to ^{32}P-labelled non-nascent DNA is not due to an uneven distribution of label in the nascent molecules, with the [^{3}H]thymidine located predominantly at the 3' ends, is suggested by two lines of evidence. When the uniformly ^{32}P-labelled DNA has been completely degraded by the 5' end specific exonuclease, a significant fraction of the ^{3}H-labelled nascent DNA is undegraded. And second, the release of [^{3}H] label by the 3' end specific snake venom phosphodiesterase initially exceeds the release of [^{32}P] label by only a very small amount, not enough to account for the much lower release of [^{3}H] label from the 5' end by spleen exonuclease.

The ^{3}H-labelled nascent DNA does not contain detectable regions of secondary structure which would impede the progress of spleen exonuclease. The *Neurospora crassa* nuclease, which is highly specific for single-stranded DNA, does not discriminate between the ^{3}H-labelled nascent and the ^{32}P-labelled non-nascent DNAs, but degrades both to completion at the same rate. And second, denaturing the partially degraded DNA and adding fresh enzyme does not result in an increase in the rate of the digestion as would be expected if internal regions of duplex structure obstructed the course of the enzyme.

These data do not allow us to conclusively eliminate the possibility that secondary structure is responsible for the resistance of the ^{3}H-labelled nascent DNA to spleen exonuclease. A very small duplex region which folds back on itself like a hairpin would renature instantaneously and so would not be destroyed by a second heating.

Also, if the duplex region were very small, perhaps only 10 to 20 nucleotides long, and comprised only 1 to 2% of the total length of the average DNA molecule, it might not readily be observed as material resistant to the *Neurospora crassa* nuclease.

Anderson (1978) reported that old preparations of [^{3}H]thymine can label non-DNA components in *E. coli*. That both the *Neurospora crassa* nuclease and snake venom phosphodiesterase degrade the ^{3}H-labelled material which is resistant to spleen exonuclease completely to acid-soluble nucleotides indicates that the [^{3}H]thymidine in our experiment labelled nucleic acids.

Pyrophosphatase was apparently unable to remove the block; therefore, a pyrophosphate linkage is most probably not joining an unusual structure to the 5' ends of the nascent molecules. Neither isoamyl nitrite nor hydroxylamine hydrochloride were able to eliminate the block, suggesting that a phosphoamide bond linking a few amino acid residues to the 5' termini was not responsible for the resistance to spleen exonuclease. The data are suggestive, not conclusive, because the difficulty in obtaining suitable substrates made it impossible to perform the necessary positive controls.

Phosphoester linkages between DNA and protein are alkali labile (Shabarova, 1979). It was found that mild alkali could not remove the block, but strong alkali followed by a second phosphatase treatment allowed the ^{3}H-labelled nascent and ^{32}P-labelled non-nascent DNAs to be degraded by spleen exonuclease to the same extent. Quantitative end-labelling with [γ-^{32}P]ATP and kinase, and electrophoresis on agarose gels, revealed that strong alkali resulted in considerable fragmentation of both nascent and non-nascent DNAs. This leads to some ambiguity in the

interpretation of the results of alkali treatment. On the one hand, it could be argued that if alkali simply reduced the size of the molecules without removing the block, one would still expect to find a portion of the ^{3}H-labelled nascent DNA resistant to spleen exonuclease, although the difference between the ^{3}H- and ^{32}P-labelled DNAs would be less pronounced. That this was not observed suggests that the block has been removed. On the other hand, if the ^{3}H- and ^{32}P-labelled DNAs are first cleaved to 2S fragments by pancreatic DNAse, spleen exonuclease degrades both the ^{3}H-labelled nascent and ^{32}P-labelled non-nascent DNAs at the same rate and to the same extent (data not shown). So it cannot be unambiguously concluded that strong alkali has removed the block.

There is protein associated with the ^{3}H-labelled nascent DNA isolated from the phenol phase when Pronase treatment is omitted from the purification procedure. The associated protein shifts the ^{3}H-labelled DNA to a lower buoyant density in neutral CsCl equilibrium density gradients; the shift can be abolished by treating the DNA with Pronase. The bound protein resists dissociation: heating at 100°C for 3 minutes in 4 M Guanidine·HCl and equilibrium centrifugation in 4 M Guanidine·HCl-CsCl gradients; heating at 100°C for 2 minutes in 4 M Guanindine·HCl and sedimentation velocity in 4 M Guanidine·HCl-sucrose gradients; heating for 2 minutes at 100°C in TE buffer containing 0.1% SDS and equilibrium centrifugation in neutral CsCl. This indicates a very tight, possibly covalent, association between the DNA and protein. If the crude suspension was heated at 100°C for 3 minutes before phenol extraction, the DNA-protein complex was isolated from the phenol phase. However, if the phenol extraction was performed at 65°C, the DNA which was recovered from the

phenol phase was no longer associated with protein. The ColE1 protein can be detached from the DNA by heating the cells at 70°C for 15 minutes before lysis (Clewell & Helinski, 1970). The φ29 DNA-protein complex can be dissociated by heating at 70°C in 2% SDS (Harding & Ito, 1976). The ^{3}H-labelled nascent φX DNA - protein complex we have isolated is labile in phenol at 65°C. It is also alkali labile. Incubation in 0.25 M NaOH for 6 hours at 37°C partially removed the protein; incubation in 1M NaOH for 24 hours at 37°C completely removed it.

Is the protein associated with the ^{3}H-labelled nascent DNA responsible for the resistance of the DNA to spleen exonuclease? Although it has been reported that the adenovirus protein (Carusi, 1977), the φ29 protein (Ito, 1978), and the φX gene A protein (Ikeda *et al.*, 1979) attached to the 5' ends of the DNA molecules render the molecules resistant to degradation by 5' end specific nucleases, we have no evidence to support the hypothesis that the protein in the complex we have isolated is located at the 5' ends of the nascent molecules.

That the protein which is associated with the ^{3}H-labelled nascent DNA is not responsible for the resistance of the DNA to degradation by spleen exonuclease is suggested by several observations. First, when the ^{3}H-labelled nascent DNA was recovered from a neutral CsCl gradient, and divided into less dense and more dense fractions, both were found to be equally resistant to spleen exonuclease (Data not shown). One would predict that if the associated protein were responsible for the resistance to spleen exonuclease, the less dense material should be enriched for DNA-protein complex and should show a greater resistance to spleen exonuclease. One reason why this could be true yet not observed

is because the two fractions were not well enough resolved. Second, the results with mild alkali suggest that the protein is not responsible for the blocked 5' ends because after 6 hours in 0.25 M NaOH at 37°C, the protein was partially removed, but the sensitivity of the ^{3}H-labelled nascent DNA to spleen exonuclease was not altered. Third and finally, if the protein were located only at the end of the DNA molecule, one would expect that the shorter the molecule, the more pronounced the density shift because the greater the mass ratio of protein to DNA. However, the reverse effect was observed, with more protein associated with the unit length molecules than with the shortest molecules. Increased binding of a protein to a longer single-stranded DNA molecule would be expected of a protein such as the *E. coli* DNA binding protein. If the proteins are not attached to the 5' ends of the DNA molecules, but are very tightly bound along the length of the DNA molecule, it is difficult to understand why such residual proteins would interfere only with the spleen exonuclease and not with the *Neurospora crassa* or snake venom phosphodiesterase reactions.

That this occurs, that the protein(s) are in fact responsible for the resistance of the ^{3}H-labelled DNA to spleen exonuclease is suggested, first, by the fact that the short molecules are more resistant to spleen exonuclease and are also associated with more protein than the very short molecules. And, second, resistance to spleen exonuclease and the size of the density shift vary from experiment to experiment and appear to correlate: the more pronounced the density shift and the greater the association with protein, the more resistant the ^{3}H-labelled DNA to degradation by spleen exonuclease (data not shown). But we have not been

able to unequivocably demonstrate that the protein(s) responsible for the density shift are causing the resistance of the ^{3}H-labelled nascent molecules to spleen exonuclease. There may be two types of protein(s) involved: one, constituting most of the total mass of the associated protein, bound along the length of the DNA molecules, and responsible for the density shift; and the other, representing only a small fraction of the total, and not sufficient to cause a density shift, located specifically at the 5' end of the DNA molecules and blocking the action of spleen exonuclease.

It is known that the synthesis of the ϕX viral strand is closely coupled to assembly of the phage capsid, both *in vivo* (Fujisawa & Hayashi, 1976) and *in vitro* (Sumida-Yasumoto *et al.*, 1979). It is reasonable that the proteins associated with the unit length viral strands are capsid proteins, but it is not certain whether the proteins associated with the shorter than unit length nascent molecules are capsid proteins or other proteins involved in the synthesis of the molecules. Ligase is known to go through an intermediate stage where it becomes covalently bound to an AMP residue (Gumport & Lehman, 1971). The mechanism responsible for joining the short molecules observed during ϕX viral strand synthesis has not yet been identified. That it is not the NMN-dependent *E. coli* DNA ligase is suggested by the finding that full length viral strands are synthesized in ligase defective hosts (McFadden & Denhardt, 1975). It is possible that whatever enzyme mediates the joining of the short molecules becomes bound to the DNA. Another possibility is binding of the enzyme which removes the primer. And finally, the protein itself may function as a primer by positioning a deoxynucleoside triphosphate

residue on the template DNA as was first suggested by Denhardt (1972). It has been proposed that the adenovirus 5' terminal protein plays an analogous role in priming adenovirus DNA synthesis (Rekosh *et al.*, 1977).

Confirmation of the role of the protein(s) associated with the ^{3}H-labelled nascent ϕX molecules awaits identification of the protein(s) and localization of the protein(s) on the DNA molecules. Experiments to label the protein(s) with ^{125}I are in progress and hopefully will reveal whether the protein(s) are specific. Localization of the protein(s) on the DNA molecules could possibly be achieved by electron microscopy.

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Legend to Table I:

Comparison of the degradation by spleen exonuclease of DNA molecules obtained by sonication or digestion with pancreatic DNase. Uniformly ^{32}P- or ^{3}H-labelled ϕX viral strand DNA was isolated as described in Chapter II, Section 2(d). The ^{3}H-labelled DNA was fragmented either by sonicating for 30 seconds at output 60 on an Artek sonic dismembrator, or by treatment with pancreatic DNase as described above. The ^{3}H and ^{32}P-labelled DNAs were fractionated into two size classes by sedimentation on neutral sucrose gradients in the SW27 rotor. The very short molecules are found in fractions 5 to 11 of the neutral sucrose gradient shown in Chapter II, Figure 8(c); the short molecules are found in fractions 12 to 18 of the same gradient. ^{3}H-labelled DNA obtained by sonication or DNase digestion was combined with ^{32}P-labelled DNA of the same size obtained by sonication. The samples were treated with bacterial alkaline phosphatase, then exposed to spleen exonuclease. After 2 hours the spleen exonuclease reaction was terminated, and aliquots were assayed for total and acid-soluble radioactivity as described in Materials and Methods, Section 2(d). Acid soluble cts/min ranged from 1000 to 6000; total cts/min ranged from 5000 to 10,000.

TABLE I

Sensitivity of Spleen Exonuclease of Non-nascent DNA

% acid-soluble DNA				
Sonicated ^{3}H	Sonicated ^{32}P	DNase-treated ^{3}H	Sonicated ^{32}P	size of DNA
58	56	60	59	very short
28	27	25	23	short

Legend to Table II:

Nascent ϕX DNA shorter than unit-length and pulse-labelled with [^{3}H]thymidine, and non-nascent ϕX DNA, uniformly labelled with [^{3}H]thymidine and sonicated to the same size as the nascent DNA, were separately treated with phosphatase. Each was divided into three samples which were incubated at 37°C for 24 hours. Two of the three contained 1 M NaOH. After neutralization, one of the NaOH-treated samples was again treated with phosphatase (BAP). Then all three samples were end-labelled with [γ-^{32}P]ATP and kinase. The unincorporated [γ-^{32}P]ATP was removed by three isopropanol precipitations and nitrocellulose chromatography. The ^{32}P/^{3}H-labelled DNA was hybridized to ϕX RF on nitrocellulose filters as described in Materials and Methods, Section 2(g). The numbers shown are the ratios of the radioactivity annealed to the filters; it ranged from 2500 to 20,000 cts/min.

TABLE II

Quantitation of Ends Exposed by Alkali and Phosphatase

$^{32}P/^{3}H$ ratios

	^{3}H-labelled nascent ϕX DNA		^{3}H-labelled non-nascent ϕX DNA	
	very short	short	very short	short
control	1.6	1.9	1.1	1.3
+NaOH	2.1	2.9	2.8	1.5
+NaOH + BAP	4.3	3.7	4.3	2.9

Legend to Table III:

Nascent ϕX DNA, shorter than unit length and pulse-labelled with [^{3}H]thymidine, was combined with non-nascent uniformly-labelled [^{32}P] ϕX viral strand DNA of the same size obtained by sonication. The very short and short molecules were analyzed separately. One half of the sample was treated with pyrophosphatase; the other half was untreated. Both samples were digested with bacterial alkaline phosphatase before being degraded with spleen exonuclease. Aliquots, removed 1 and 2 hours after the start of the spleen exonuclease reaction, were assayed for total and acid-soluble radioactivity as described in Materials and Methods, Section 2(d). Acid soluble cts/min ranged from 500 to 2000; total cts/min ranged from 3000 to 6000.

TABLE III

Sensitivity to Spleen Exonuclease after Exposure to Pyrophosphatase

	% acid-soluble DNA			
	+ Pyrophosphatase		Control	
size of DNA	^{3}H nascent	^{32}P non-nascent	^{3}H nascent	^{32}P non-nascent
very short	29	37	26	35
	42	53	38	45
short	17	24	16	23
	24	32	23	32

Legend to Table IV:

Nascent ϕX DNA, shorter than unit length and pulse-labelled with [^{3}H]thymidine, was combined with non-nascent uniformly -labelled [^{32}P] ϕX DNA of the same size, obtained by sonicating viral strands. The very short and short molecules were combined and analyzed together. The isoamyl nitrite-treated, hydroxylamine hydrochloride-treated, and appropriate control samples were prepared as described in Materials and Methods, Section 2(e). All samples were digested with bacterial alkaline phosphatase before being incubated with spleen exonuclease. Aliquots removed 1 and 3 hours after the start of the spleen exonuclease reaction were assayed for total and acid-soluble radioactivity as described in Materials and Methods, Section 2(d). Acid-soluble cts/min ranged from 100 to 500; total cts/min ranged from 500 to 1000.

TABLE IV

Sensitivity to Spleen Exonuclease After Exposure to Isoamyl Nitrite and Hydroxylamine Hydrochloride

% acid-soluble DNA			
+ Isoamyl Nitrite		Control	
^{3}H nascent	^{32}P non-nascent	^{3}H nascent	^{32}P non-nascent
17	28	16	27
42	52	42	54
+ Hydroxylamine Hydrochloride		Control	
^{3}H nascent	^{32}P non-nascent	^{3}H nascent	^{32}P non-nascent
23	31	20	27
39	48	36	45

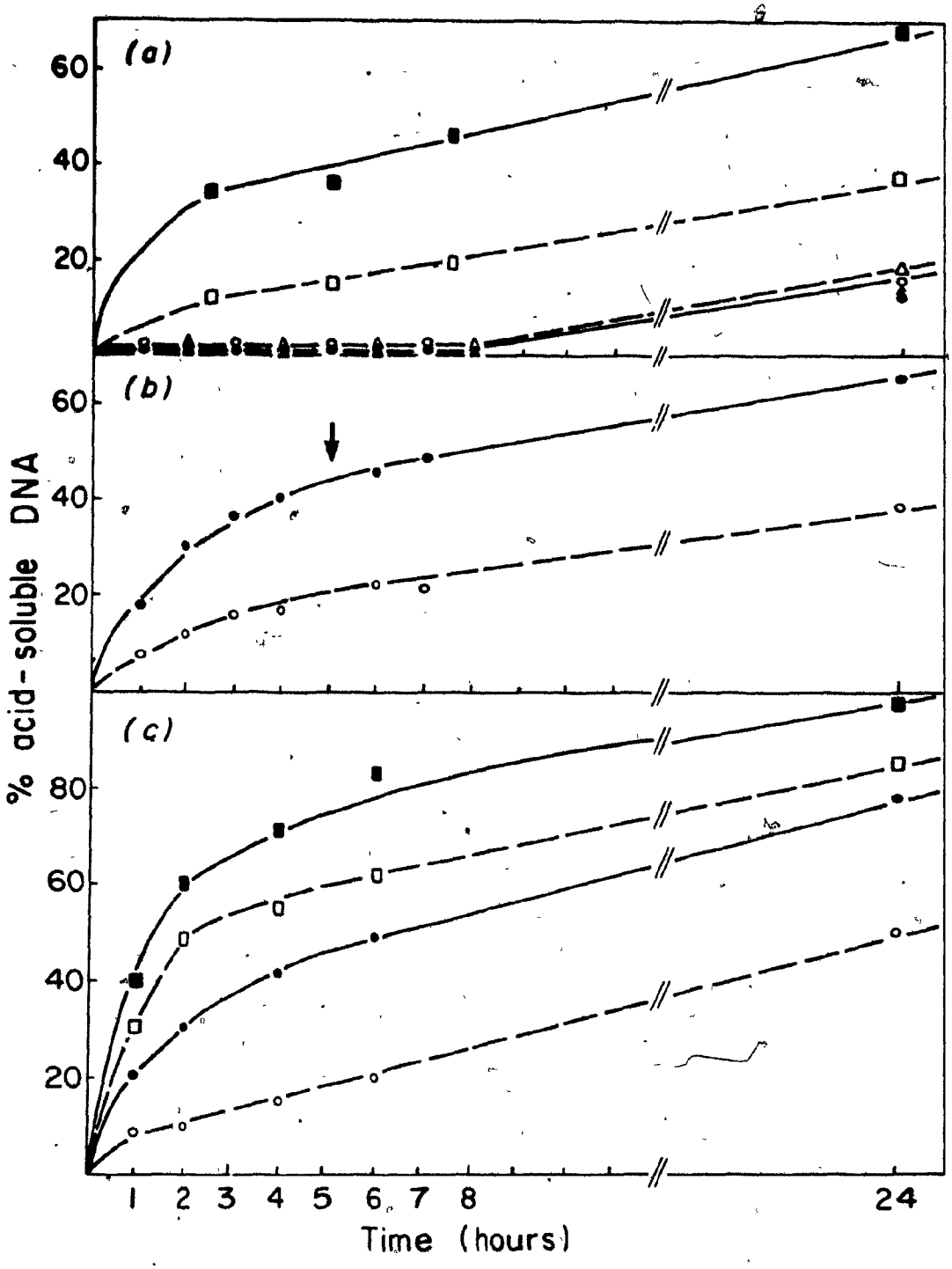
Legend to Figure 1:

Degradation of DNA by Spleen exonuclease. ^{3}H-labelled nascent DNA and ^{32}P-labelled non-nascent ϕX DNA of the same size, obtained by briefly sonicating viral strands, were combined and incubated with spleen exonuclease for the indicated times. Aliquots were assayed for total and acid-soluble radioactivity as described in Materials and Methods, Section 2(d). Acid-soluble cts/min ranged from 0 to 5000; total cts/min averaged about 5000. The open symbols (O, □ , Δ) denote ^{3}H-labelled nascent DNA. The closed symbols (●, ■ , ▲) denote ^{32}P-labelled non-nascent DNA. Short molecules are found in fractions 12 to 18 of the neutral sucrose gradients shown in Chapter II, Figure 8 (a,c). Very short molecules are found in fractions 5 to 11 of the same gradients.

(a) Short DNA molecules were digested with spleen exonuclease directly (O, ●); after phosphatase treatment (□, ■); after phosphatase and kinase treatment (Δ, ▲).

(b) Short DNA molecules were digested with spleen exonuclease after phosphatase treatment. The arrow indicates the point at which the sample was heat-denatured before fresh enzyme was added.

(c) Comparison of the degradation of short (O, ●) and very short (□, ■) DNA molecules by spleen exonuclease.

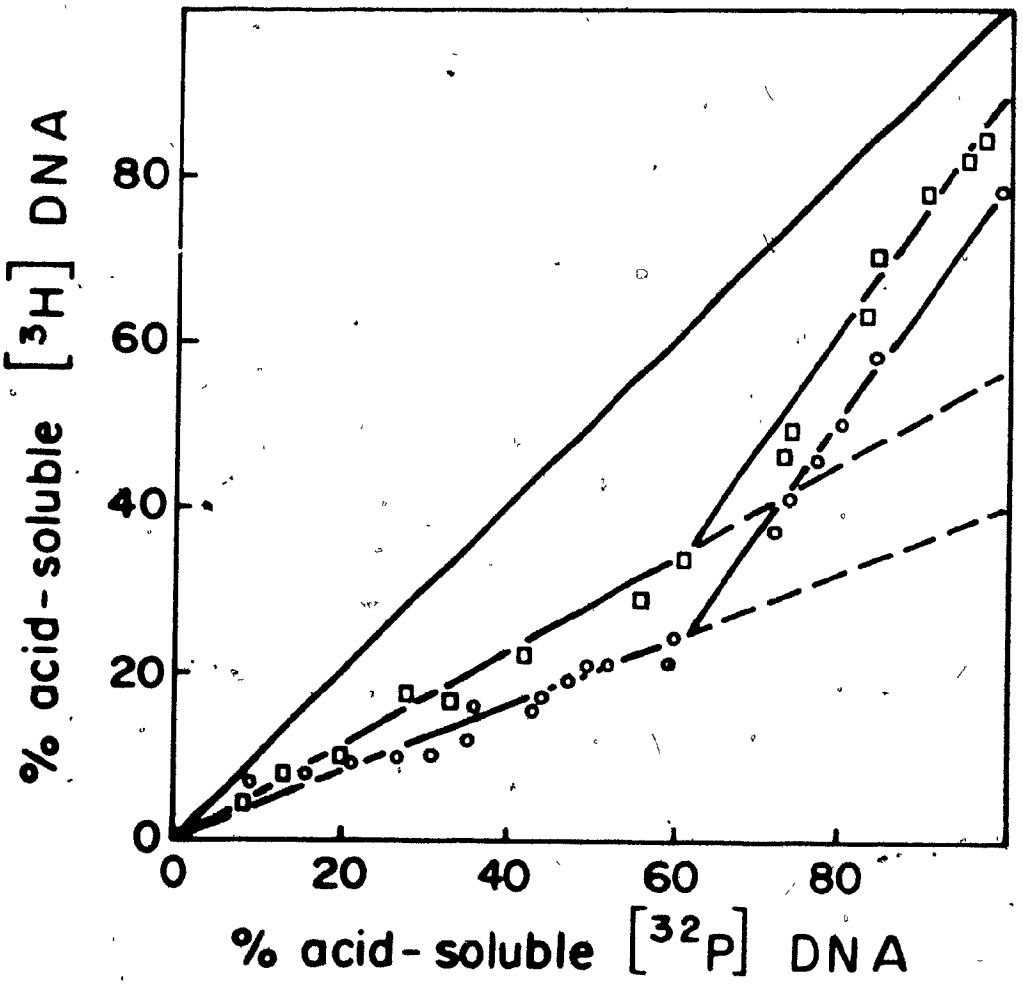

(a)
(b)
(c)
60
40
20
60
40
20
80
60
40
20
% acid-soluble DNA
1 2 3 4 5 6 7 8 24
Time (hours)

Legend to Figure 2:

Comparison of the degradation of nascent and non-nascent DNA by spleen exonuclease. ^{3}H-labelled nascent DNA and ^{32}P-labelled non-nascent DNA of the same size, obtaining by briefly sonicating viral strands, were combined, treated with phosphatase, and then with spleen exonuclease. Aliquots were removed at appropriate intervals, and assayed for acid-soluble and total radioactivity as described in Materials and Methods, Section 2(d). Acid soluble cts/min ranged from 100 to 5,000; total cts/min averaged about 5000. Very short and short DNA molecules were analyzed separately.

(-□-□-□-) very short DNA molecules

(o - o - o) short DNA molecules


% acid-soluble [3H] DNA
0
20
40
60
80
0
20
40
60
80
% acid-soluble [32P] DNA

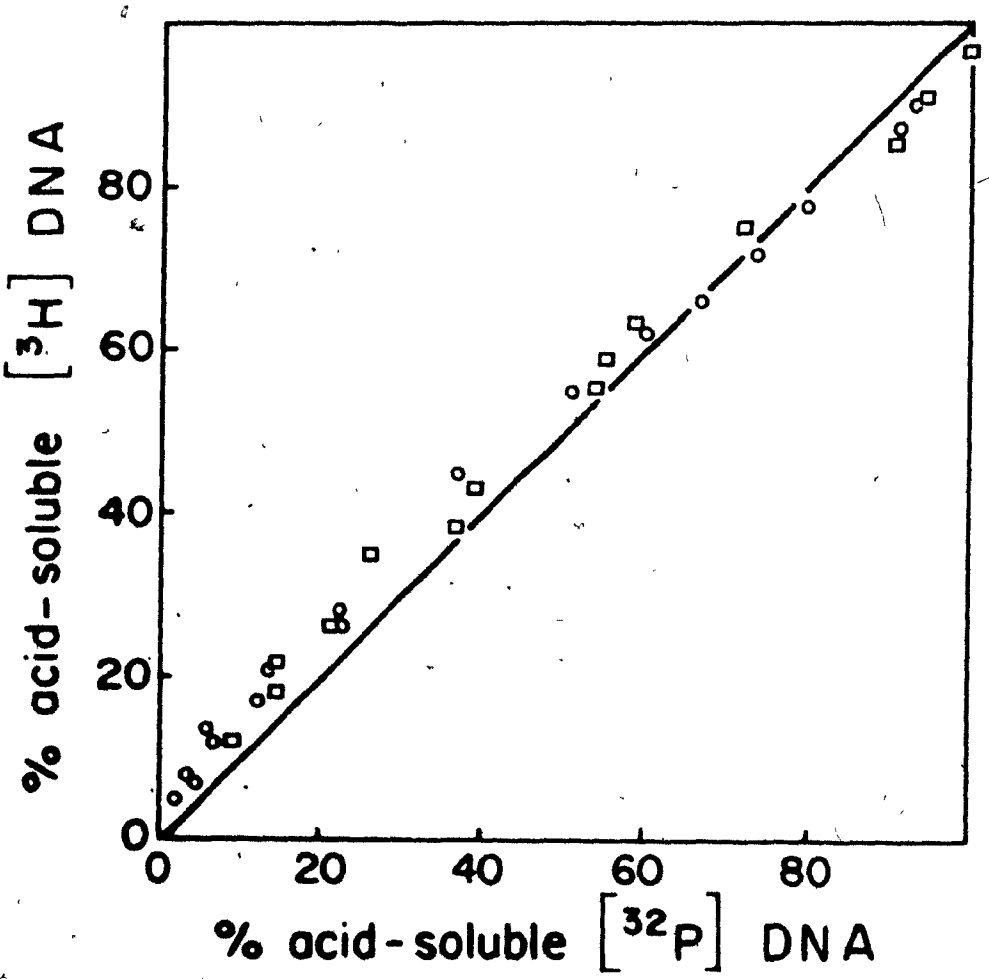
Legend to Figure 3:

Comparison of the degradation of nascent and non-nascent DNA by snake venom phosphodiesterase.

^{3}H-labelled nascent and ^{32}P-labelled non-nascent DNA of the same size, obtained by briefly sonicating viral strands, were combined, and incubated with snake venom phosphodiesterase after phosphatase treatment. Aliquots were removed at appropriate intervals and assayed for total and acid-soluble radioactivity as described in Materials and Methods, Section 2(d). Acid soluble cts/min ranged from 100 to 5000; total cts/min averaged about 5000. Very short and short DNA molecules were analyzed separately.

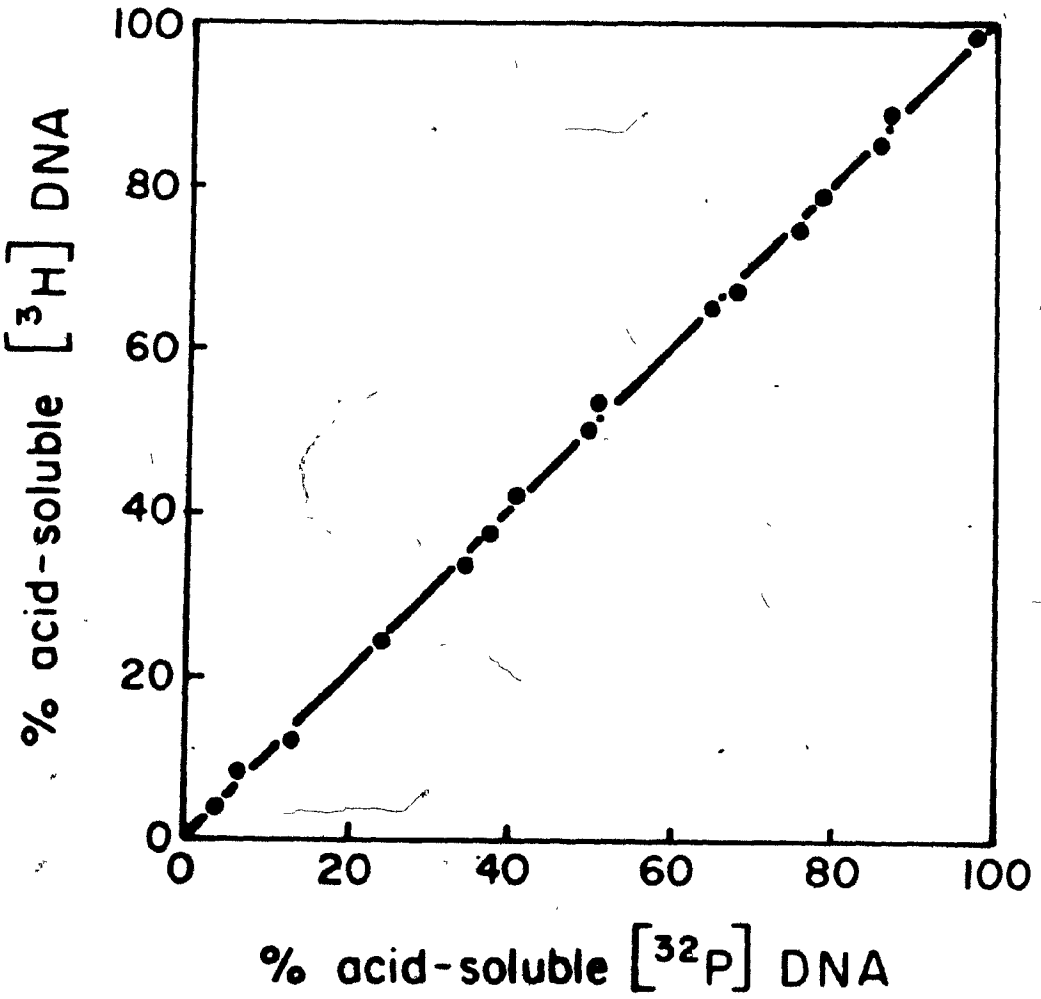
(□ - □ - □) very short DNA molecules

(o - o - o) short DNA molecules


% acid-soluble [3H] DNA
% acid-soluble [32P] DNA
0
20
40
60
80
0
20
40
60
80

Legend to Figure 4:

Comparison of the degradation of nascent and non-nascent DNA by *Neurospora crassa* nuclease. A mixture of very short and short, ^{3}H-labelled nascent and ^{32}P-labelled non-nascent DNA molecules of the same size were combined and incubated with *Neurospora crassa* nuclease. At intervals from the start of the reaction until 2 hours later, aliquots were removed and assayed for total and acid-soluble radioactivity as described in Materials and Methods, Section 2(d). Acid-soluble cts/min ranged from 100 to 5000; total cts/min averaged about 5000.


100
80
60
40
20
0
0
20
40
60
80
100
% acid-soluble [3H] DNA
% acid-soluble [32P] DNA

Legend to Figure 5:

Effect of alkali on the resistance of the nascent DNA to degradation by spleen exonuclease. ^{3}H-labelled nascent and ^{32}P-labelled non-nascent DNA of the same size were combined, treated with phosphatase and incubated at 37°C with or without alkali, then after neutralization, degraded with spleen exonuclease directly or after a second phosphatase treatment. Aliquots were removed at appropriate intervals and assayed for total and acid-soluble radioactivity as described in Materials and Methods, Section 2(d). Acid-soluble cts/min ranged from 200 to 2000; total cts/min averaged 4000.

(o - o - o) incubated at 37°C for 24 hours without alkali

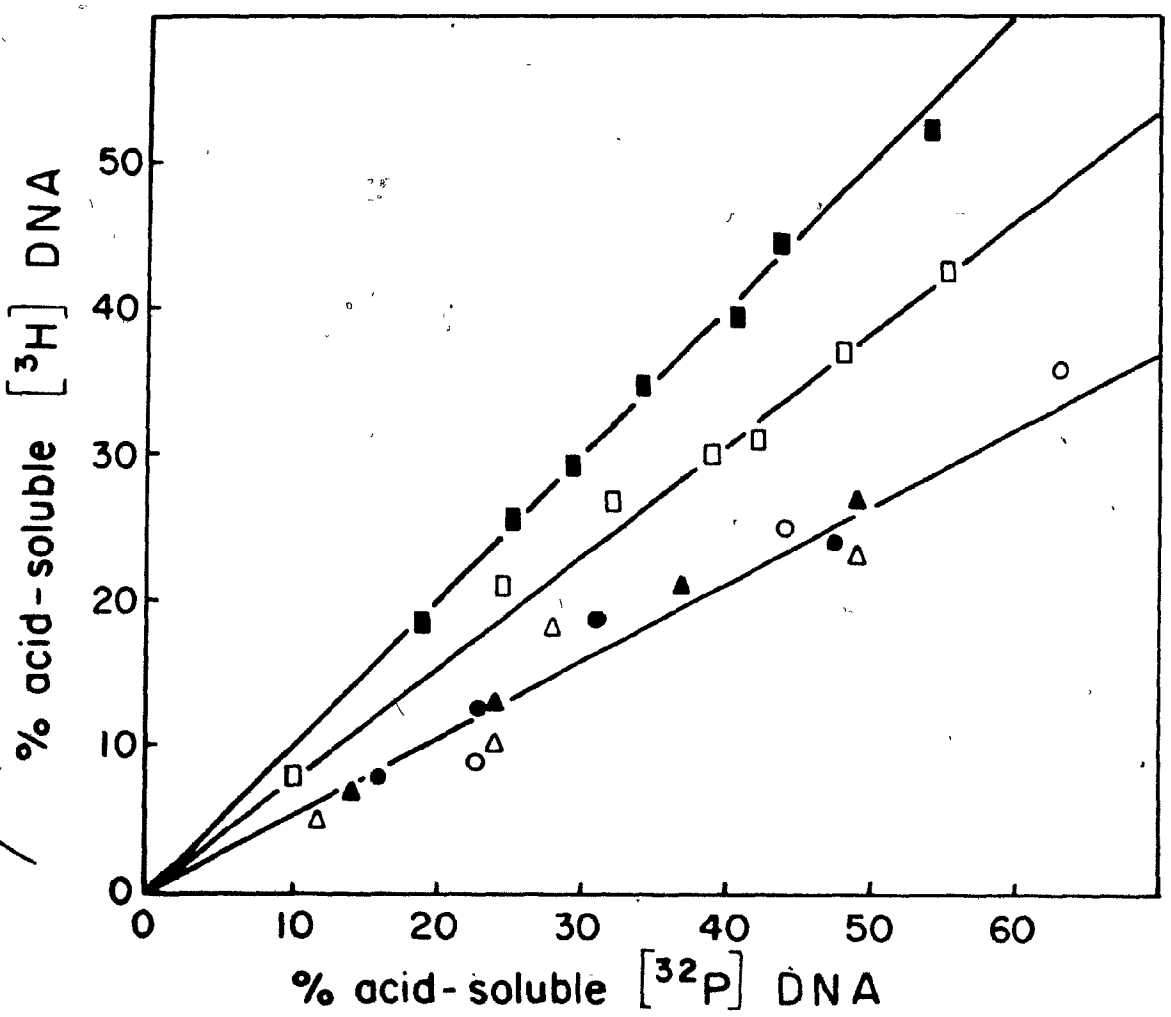
(● - ● - ●) above DNA after second phosphatase treatment

(Δ - Δ - Δ) incubated at 37°C for 6 hours in 0.25 M NaOH

(▲ - ▲ - ▲) above DNA after second phosphatase treatment

(□ - □ - □) incubated at 37°C for 24 hours in 1 M NaOH

(■ - ■ - ■) above DNA after second phosphatase treatment


% acid-soluble [3H] DNA
% acid-soluble [32P] DNA
0
10
20
30
40
50
60

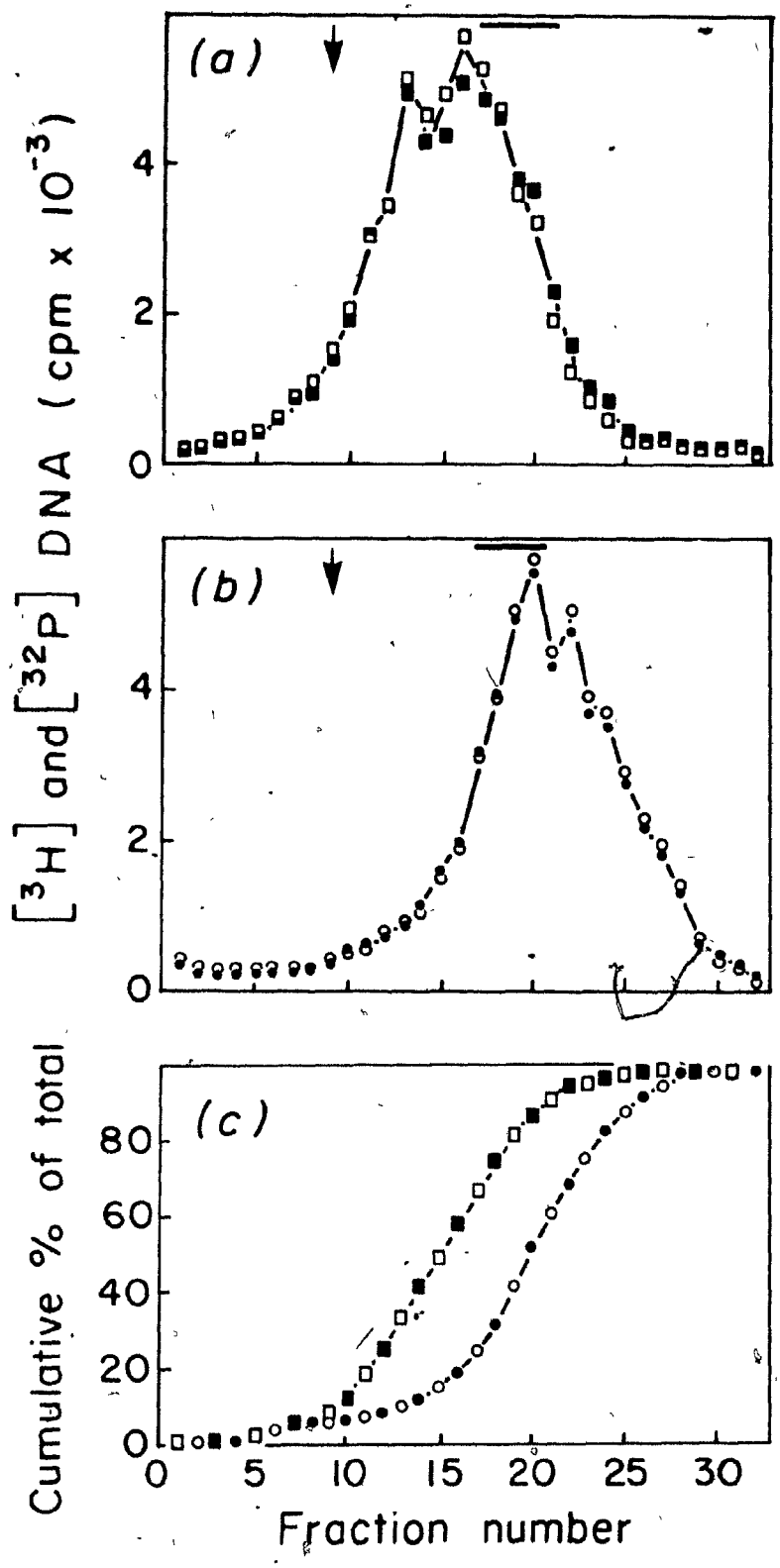
Legend to Figure 6:

Alkaline agarose gel electrophoresis of ^{3}H-labelled nascent ϕX DNA (open symbols) and ^{32}P-labelled non-nascent ϕX DNA of the same size obtained by sonication (closed symbols). The arrows in panels (a) and (b) indicate the position of circular ϕX viral strand DNA while the heavy horizontal line denotes the position of the bromophenol blue marker dye. Electrophoresis is from left to right; composition of the gel and conditions of electrophoresis are described in Materials and Methods, Section 2(h). Very short and short molecules were combined and analyzed together. ^{3}H-labelled nascent DNA (o , □); ^{32}P-labelled non-nascent DNA (● , ■).

(a) gel profile of DNA incubated at 37°C for 24 hours without alkali (□, ■)

(b) gel profile of DNA incubated at 37°C for 24 hours in 1 M NaOH (o, ●).

(c) data shown in panels (a) and (b) plotted as cumulative % of total; the sum of the radioactivity in all fractions upto and including fraction n, divided by the sum of the radioactivity in all the fractions of the gel, expressed as a %, is the cumulative % of the total for fraction n.

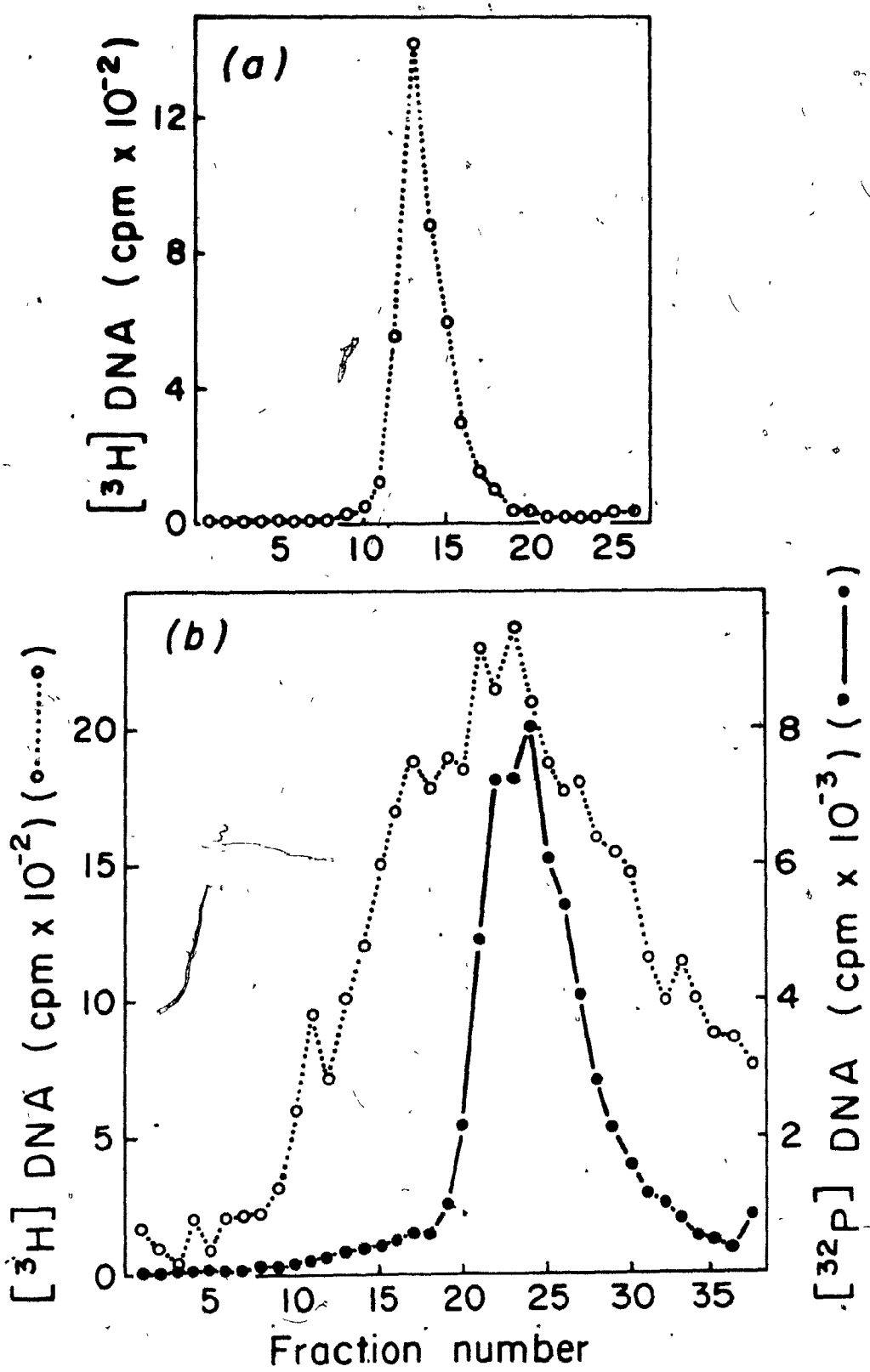

(a)
(b)
(c)
[3H] and [32P] DNA (cpm x 10-3)
Cumulative % of total
Fraction number
0
2
4
20
40
60
80
5
10
15
20
25
30

Legend to Figure 7:

Preparative centrifugation of ^{3}H-labelled nascent ϕX DNA recovered from the phenol phase if pronase treatment is omitted. Composition of the gradients and centrifugation conditions are described in Materials and Methods, Section 2(c).

(a) 4 M Guanidine·HCl-CsCl gradient; the density of the gradient decreases from left to right (o - o - o) ^{3}H-labelled nascent DNA.

(b) 4 M Guanidine·HCl-sucrose gradient; direction of sedimentation is from left to right. (o - o - o) ^{3}H-labelled nascent DNA; (● - ● - ●) ^{32}P-labelled ϕX viral strand DNA sedimented on a separate gradient. Two gradient profiles were superimposed to facilitate comparison.


(a)
[3H] DNA (cpm x 10-2)
0
4
8
12
5
10
15
20
25
(b)
[3H] DNA (cpm x 10-2) (o.....o)
[32P] DNA (cpm x 10-3) (•——•)
0
5
10
15
20
2
4
6
8
5
10
15
20
25
30
35
Fraction number

Legend to Figure 8:

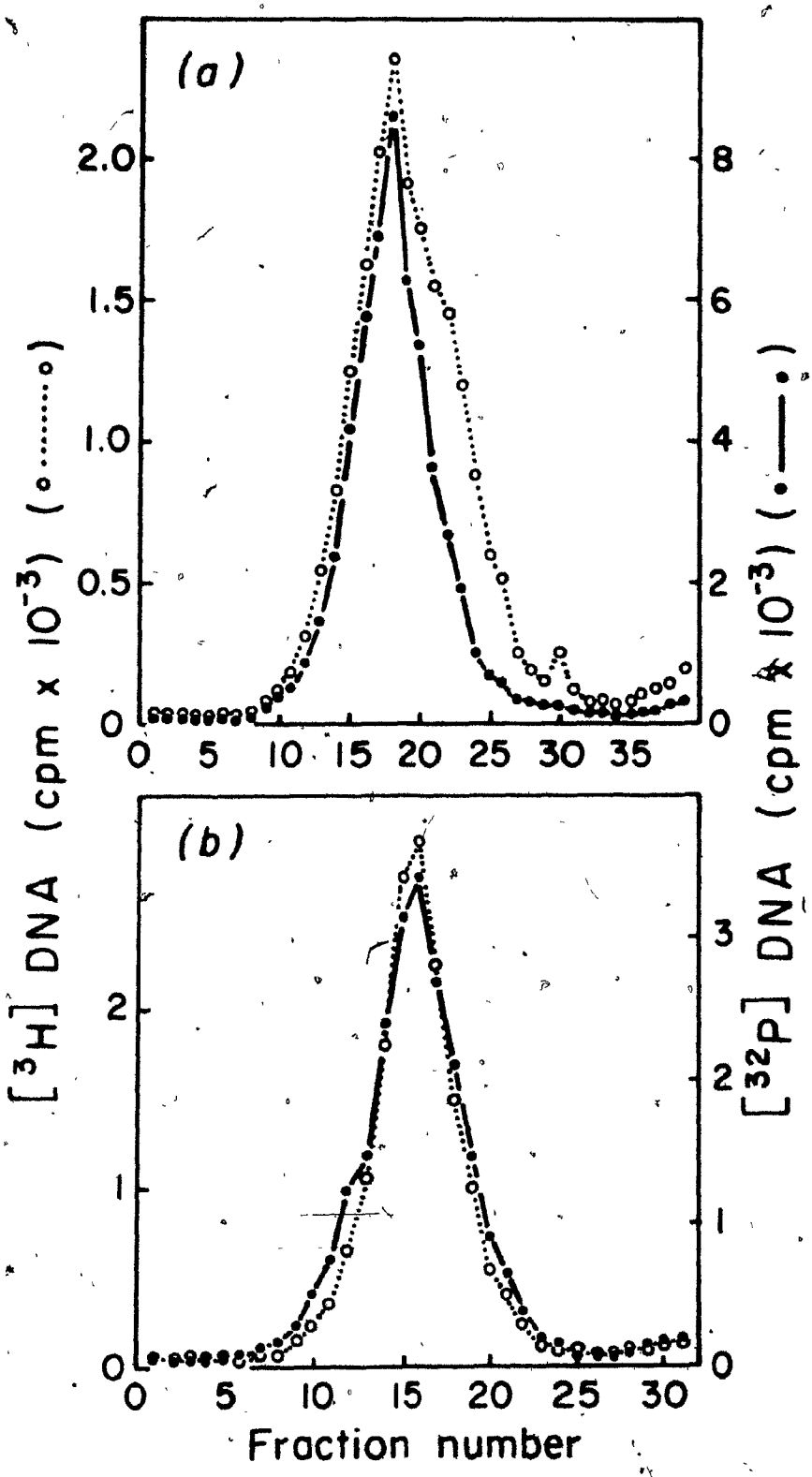
Analytical CsCl centrifugation of nascent ϕX DNA shorter than unit length recovered from the phenol phase when pronase treatment was omitted. The density of the gradients decreases from left to right. The composition of the gradients and centrifugation conditions are described in Materials and Methods, Section 2(h).

(o - o - o) ^{3}H-labelled nascent ϕX DNA

(● - ● - ●) ^{32}P-labelled non-nascent ϕX DNA of the same size obtained by sonicating viral strands

(a) without pronase treatment

(b) after pronase treatment


(a)
(b)
[3H] DNA (cpm x 10-3) (o.....o)
[32P] DNA (cpm x 10-3) (•——•)
Fraction number
0
0.5
1.0
1.5
2.0
2
4
6
8
1
3
5
10
15
20
25
30
35

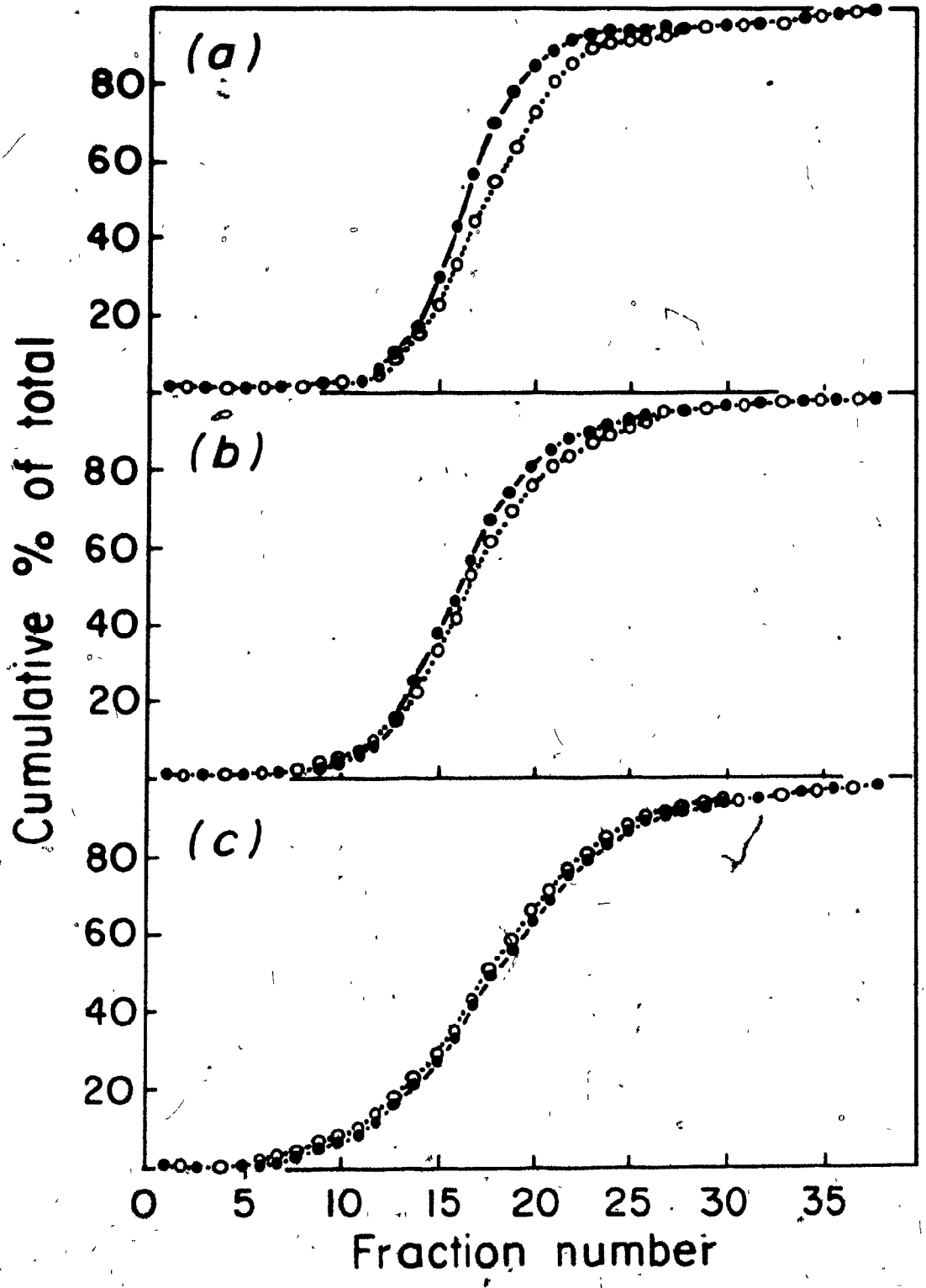
Legend to Figure 9:

Effect of alkali on the nascent DNA-protein complex recovered from the phenol phase when pronase treatment was omitted. ^{3}H-labelled nascent ϕX DNA shorter than unit length and ^{32}P-labelled non-nascent ϕX DNA of the same size obtained by briefly sonicating viral strands were combined and incubated at 37°C with or without alkali before centrifugation in CsCl gradients. The density decreases from left to right. The composition of the gradients and centrifugation conditions are described in Materials and Methods, Section 2(h). The data are expressed as cumulative % of total to facilitate comparison. The cumulative % of total for a fraction n is the sum of the radioactivity in the fractions upto and including n, divided by the sum of the radioactivity in all the fractions of the gradient, expressed as a %. (o - o - o) ^{3}H-labelled nascent ϕX DNA; (● - ● - ●) ^{32}P-labelled non-nascent ϕX DNA.

(a) incubated at 37°C for 24 hours without alkali

(b) incubated at 37°C for 6 hours in 0.25 M NaOH

(c) incubated at 37°C for 24 hours in 1 M NaOH


(a)
(b)
(c)
80
60
40
20
Cumulative % of total
0
5
10
15
20
25
30
35
Fraction number

Legend to Figure 10:

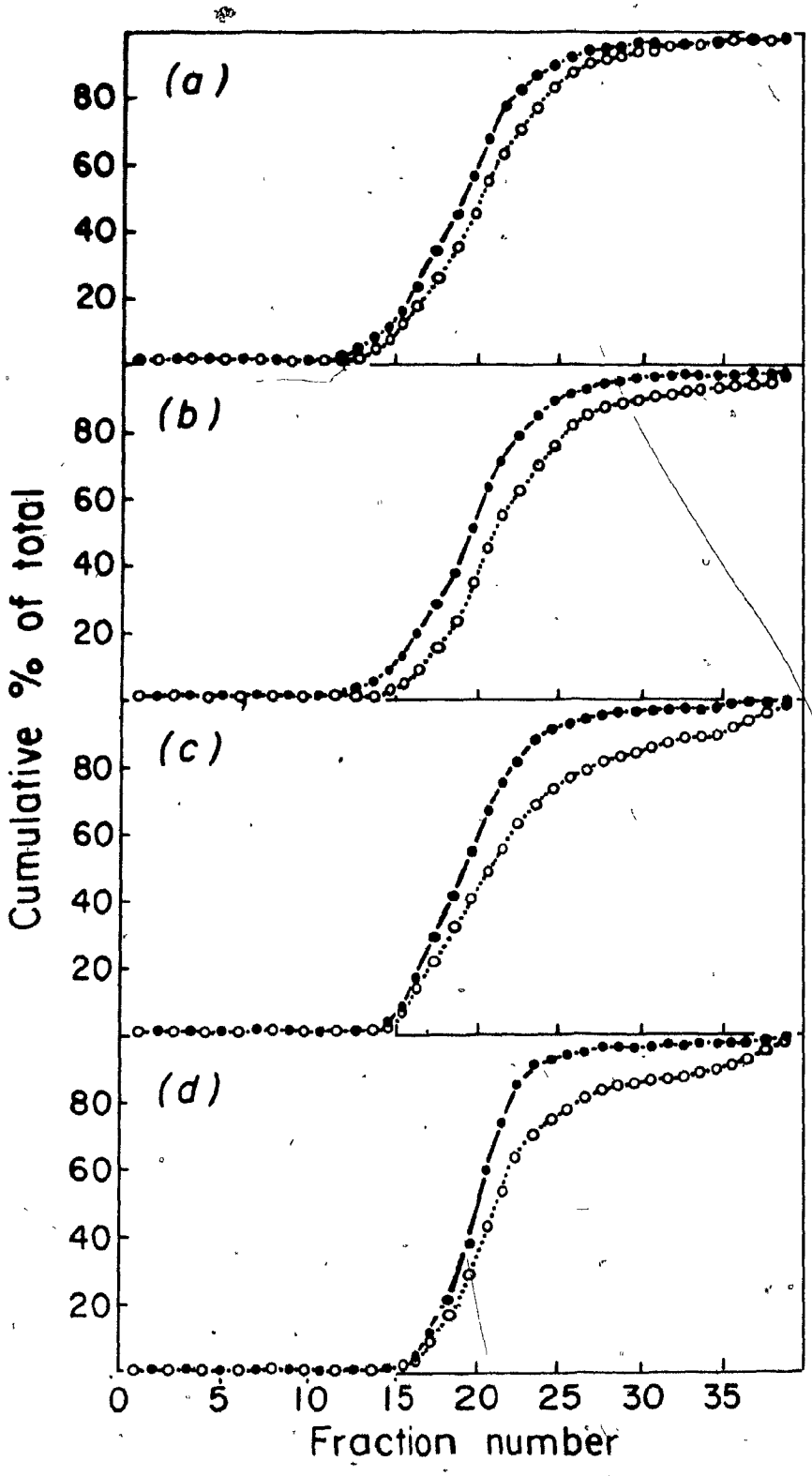
The ^{3}H-labelled nascent ϕX DNA recovered from the phenol phase when pronase treatment was omitted was divided into four size classes after sedimentation on the 4 M Guanidine·HCl-sucrose gradient. These were separately combined with short or unit-length ^{32}P-labelled non-nascent ϕX DNA and centrifuged to equilibrium in CsCl gradients. The composition of the gradients and centrifugation conditions are described in Materials and Methods, Section 2(h). The density of the gradients decreases from left to right. The data are expressed as cumulative % of total to facilitate comparison. The cumulative % of total for a fraction n is the sum of the radioactivity in the fractions upto and including n, divided by the total radioactivity in the gradient, expressed as a %. (o - o - o) ^{3}H-labelled nascent DNA; (● - ● - ●) ^{32}P-labelled non-nascent ϕX DNA.

(a) very short ^{3}H- and ^{32}P-labelled ϕX DNA

(b) short ^{3}H- and ^{32}P-labelled ϕX DNA

(c) unit length ^{3}H- and ^{32}P-labelled ϕX DNA

(d) longer-than-genome-length ^{3}H-labelled ϕX DNA and unit-length ^{32}P-labelled ϕX DNA.


(a)
(b)
(c)
(d)
80
60
40
20
Cumulative % of total
0
5
10
15
20
25
30
35
Fraction number

CHAPTER IV

A Model for the Synthesis of the ϕX174 Viral Strand

ϕX174 has provided an excellent system for the study of DNA replication. The 5000 nucleotide-long genome has been extensively characterized, and the entire base sequence has been determined (Sanger *et al.*, 1977). The small size of the genome allows it to be readily isolated intact and in large quantities. Furthermore, with the exception of the gene A protein, all of the enzymes ϕX174 uses for its replication are host enzymes, so ϕX174 provides a useful tool for the analysis of the function of the proteins used by *E. coli* for its own replication. And finally, since only one strand is replicated during the final stage of the ϕX cycle, the mechanism of synthesis of this strand can be studied without the need to separate intermediates derived from two strands, which would result if both strands were being simultaneously copied.

The discovery that a brief pulse of [^{3}H]thymidine labelled longer than genome length intermediates during the final stage of the ϕX replicative cycle (Dressler & Denhardt, 1968) led to the proposal that the ϕX viral strand was replicated by a rolling circle model (Gilbert & Dressler, 1969). This model suggested that the first step in replication is the introduction of a nick with 5' P and 3' OH termini at the origin in one of the two strands of the covalently closed circular duplex RF. The 5' P terminated parental strand is displaced, with the concommittant elongation of the 3' OH end by DNA polymerase. This results in an intermediate longer than genome length in a structure which has been called a rolling circle.

The rolling circle model is supported by the observation that *in vitro* only 4 proteins are required to synthesize the ϕX viral strand: the gene A protein, the *rep* protein, the *E. coli* DNA binding protein (DBP),

and DNA polymerase III holoenzyme (Eisenberg *et al.*, 1976; 1977). It was suggested that the gene A protein nicked at the origin, creating a 3' OH end and binding to the 5' P end; the *rep* protein formed a complex with the bound gene A protein, and moved around the duplex, hydrolyzing ATP, and displacing the parental strand; DBP bound to the displaced parental strand; and DNA polymerase III holoenzyme elongated the parental strand using the 3' OH at the origin as a primer. This gives rise to a "looped" rolling circle intermediate.

That this model is an oversimplification of the way in which the ϕX viral strand is replicated is suggested by several observations which it does not take into account. First, the *dnaG* protein, sometimes called primase, is required continuously *in vivo* for ϕX viral strand DNA synthesis (McFadden & Denhardt, 1975). The *dnaG* protein is a polymerase which is capable of the *de novo* synthesis of short oligonucleotides, ribo-, deoxyribo-, or mixed ribo- and deoxyribonucleotides, which serve as primers for DNA polymerase (Wickner, 1977; Rowen and Kornberg, 1978). Second, Sumida-Yasumoto *et al.* (1978) have not been able to replicate the ϕX viral strand *in vitro* using only these 4 proteins, and have found that other proteins, including the *dnaB*, *dnaC*, *dnaG*, and some not yet identified, are required as well. A recent report indicates that the DNA polymerase III holoenzyme used by Eisenberg *et al.* (1976, 1977) is a complex assembly of at least eight separate polypeptides which can be resolved on SDS polyacrylamide gels (Meyer *et al.*, 1979). Until the role and identity of each of the proteins in the complex is determined, it cannot be assumed that the only function of the DNA polymerase III holoenzyme in replication is the synthesis of a DNA chain from a 3' OH

end. Finally, the rolling circle model predicts and requires that most if not all intermediates be longer than unit length. This is inconsistent with reports of viral strand intermediates shorter than unit length, observed during ϕX viral strand synthesis *in vivo* (Hours *et al.*, 1978; Matthes & Denhardt, 1980), during RF replication *in vivo* (Machida *et al.*, 1977), and in both stages *in vitro* (Sumida-Yasumoto *et al.*, 1978; 1979).

In Figure 1, we present a more complex model of ϕX viral strand synthesis, which takes into account all these observations, and leaves room for the elucidation of aspects of the process which are not yet completely understood. As suggested by Eisenberg *et al.* (1977), the gene A protein nicks at the origin and becomes attached to the 5' end of the parental strand. The *rep* protein recognizes the bound gene A protein, and forms a complex which moves around the duplex with the replicating fork, displacing the parental viral strand and hydrolyzing ATP in the process. The displaced parental strand becomes associated with phage capsid proteins which prevent the synthesis of a complementary strand (Fujisawa & Hayashi, 1977). The *dnaG* protein or primase, aided by the *dnaB* protein (Arai & Kornberg, 1979) and perhaps other proteins as well, synthesizes short oligonucleotides on the exposed template strand (Wickner, 1977; Rowen & Kornberg, 1978). These can be elongated by DNA polymerase III holoenzyme, resulting in viral strand intermediates shorter than unit length. Failure to detect RNA primers on the majority (80%) of the short nascent molecules may be a consequence of rapid removal of the ribonucleotide primers, or initiation with deoxyribonucleotide primers synthesized by the *dnaG* protein, or utilization of another method

to prime DNA synthesis. Resistance of the nascent molecule to degradation from the 5' end with spleen exonuclease, coupled with the observation that protein is very tightly associated with the nascent DNA, leaves open the possibility that a protein may be functioning as a primer by positioning a deoxynucleoside triphosphate residue on the template as originally suggested by Denhardt (1972). The primers are removed and the short molecules are joined by mechanisms which have not yet been characterized. The combined polymerase and 5' to 3' exonuclease activity of DNA polymerase I could remove the ribonucleotide primers and simultaneously fill in the resulting gaps. That the NMN-sensitive ligase which has been identified in *E. coli* is not involved in joining the short molecules is suggested by the observation that defects in this enzyme do not block the formation of unit length ϕX viral strands (McFadden & Denhardt, 1975). A second NMN-resistant ligase activity, first described by Hess *et al.* (1973), and recently observed also by Sakakibara (1978), maybe responsible for joining the short ϕX viral strand intermediates. There is nothing to prevent the short molecules closest to the origin from being joined to the 3' OH end of the nicked parental viral strand at the origin. This would give rise to pulse-labelled longer than unit length intermediates which did not reflect the utilization of a rolling circle model, but rather the ligation of short molecules to unit length molecules. Nicking by the gene A protein at the regenerated origin when a round of replication is complete would result in the release of the parental viral strand.

Our studies have revealed that the ϕX viral strand is synthesized discontinuously, as short molecules, even though the potential for continuous synthesis exists. Differences in the size of the intermediates

from the two strands which were initially observed (see Chapter I, Section h) reflect differences in the rate of synthesis or the mechanism by which the short nascent intermediates of the two strands are joined. This may also be true of other replication systems which have not been as extensively characterized as ϕX174.

What would be the advantages to the cell of synthesizing as short molecules a stretch of DNA which could be made as one long piece? Not with ϕX, but with larger chromosomes, one obvious advantage is speed. Several polymerases could simultaneously synthesize short molecules which are rapidly joined, as opposed to one polymerase slowly elongating a long molecule. Another advantage is the synthesis of multiple primers. An error in the synthesis of one of many primers could easily be obviated by the presence of an adjacent primer. By contrast, the failure to synthesize a primer responsible for the replication of a long stretch of the chromosome could be severely detrimental to the cell.

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Sumida-Yasumoto, C., Ikeda, J.-E., Yudelevich, A., Marians, K.J., Schlagman, S., and Hurwitz, J. (1978). In *The Single-Stranded DNA Phages* (Denhardt, D.T., Dressler, D.H., and Ray, D.S. Eds.) pp. 303-324, Cold Spring Harbor Press, New York.

Sumida-Yasumoto, C., Ikeda, J.-E., Murians, K.J., Vicuna, R., Sugrue, S., Zipursky, S.L., and Hurwitz, J. (1979). Cold Spring Harbor Symp. Quant. Biol. 43, 311-329.

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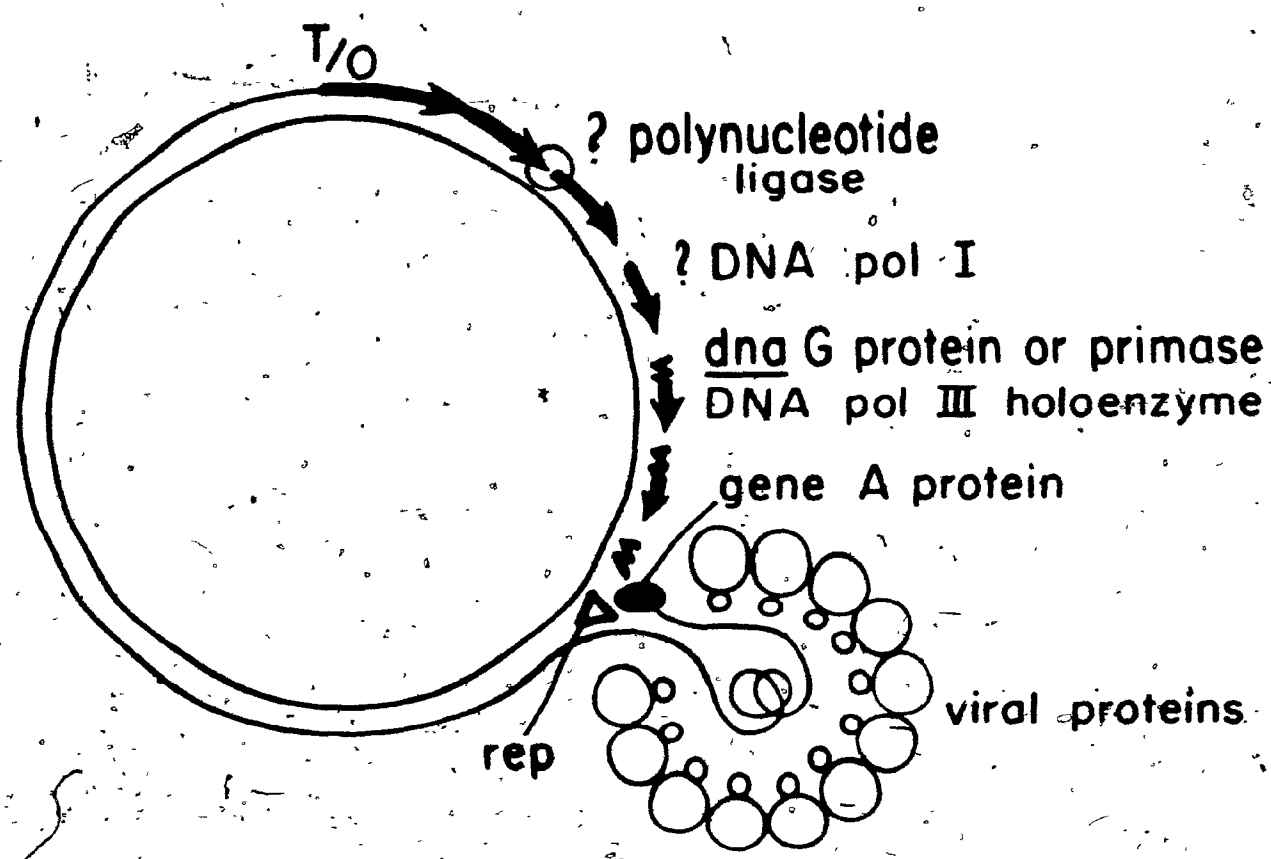
Legend to Figure 1:

A model for the discontinuous synthesis of ϕX viral strand DNA. The thin lines represent parental DNA; the heavy lines denote newly synthesized DNA while the heavy zig-zag lines denote primers synthesized by the *dna*G protein or primase. T/O indicates the terminus/origin of a round of replication, and the arrows show the direction of replication.

The gene A protein nicks at the origin, then binds to the 5' end of the parental strand. The *rep* protein forms a complex with the gene A protein, which moves around the genome separating the two strands. Viral proteins bind to the displaced strand. The *dna* G protein synthesizes short oligonucleotides on the exposed template strand. These primers are extended by the DNA polymerase III holoenzyme. Removal of the primers and joining of the short molecules occurs rapidly by mechanisms which are not yet characterized. The short molecules closest to the origin may be joined to the 3' OH end of the parental strand, resulting in intermediates longer than genome length.

DISCONTINUOUS SYNTHESIS OF ϕX VIRAL STRAND DNA

(Matthes and Denhardt, 1979)



CHAPTER V

Contribution to Original Knowledge

In the course of my studies on the final stage of replication of bacteriophage ϕX174, when predominantly viral strands are synthesized, the following observations were made for the first time:

(1) The molecules shorter than unit length, observed when a brief pulse of [^{3}H]thymidine is stopped by pouring the infected pulse-labelled culture into a boiling SDS-phenol solution, are nascent intermediates. The short molecules are not generated by the excision of misincorporated uracil. Approximately 20% of the short molecules have at least one ribonucleotide at the 5' terminus as determined by the spleen exonuclease assay.

(2) The relative abundance of short pulse-labelled nascent intermediates depends on the method used to stop the pulse, on the aeration level of the infected culture, and on the host strain. The short molecules account for a significant fraction of the total pulse-labelled DNA in the wild-type host only when the pulse is stopped by our rapid heating procedure, but not by the rapid cooling procedures used by other investigators. The short molecules account for a larger fraction of the total pulse-labelled DNA in slowly aerated cultures, and in thymine-requiring strains.

(3) The short pulse-labelled molecules hybridize to all regions of the ϕX genome, but preferentially to the origin and terminus region. The enrichment for pulse-labelled DNA in the terminus region may result from initiation of DNA synthesis at a site which resembles the origin in the center of the genome. An excess of short ϕX molecules whose 5' termini map in this region was detected by end-labelling and hybridization.

(4) A variable proportion of the short pulse-labelled molecules are resistant to degradation from the 5' end by spleen exonuclease. This

resistance, which is only removed by prolonged incubation of the DNA in strong alkali, cannot be accounted for by an uneven distribution of label in the nascent molecules, or by a detectable enrichment for secondary structure.

(5) When proteolytic digestion is omitted from the purification procedure, some of the short pulse-labelled ϕX molecules are recovered from the phenol phase in DNA-protein complexes which resist dissociation, but are alkali-labile.

On the basis of my studies of the short nascent intermediates found during the final stage of ϕX174 replication, I conclude that the ϕX viral strand is synthesized discontinuously, not continuously, as originally proposed by the rolling circle model.

ABBREVIATIONS

A	adenine
ADP, ATP	adenosine 5' di- and triphosphate
am	amber mutation
BAP	bacterial alkaline phosphatase
BSA	bovine serum albumin
B. subtilis	*Bacillus subtilis*
mCi, μCi	millicurie, microcurie
C	cytosine
CTP	cytidine 5' triphosphate
cts/min; cpm	counts per minute
dCTP	deoxycytidine 5' triphosphate
dGTP	deoxyguanosine 5' triphosphate
dNMPs, dNTPs	deoxynucleotide 5' mono- and triphosphates
dTMP, dTTP	deoxythymidine 5' mono- and triphosphate
dUMP, dUDP, dUTP	deoxyuridine 5' mono-, di-, and triphosphate
DBP	DNA binding protein
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dut	dUTPase mutant
dUTPase	deoxyuridine triphosphate diphosphohydrolase
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetate
EGTA	ethylene-bis-(β-amino-ethyl ether)-N,N-; tetracetic acid
G	guanine
GTP	guanosine 5' triphosphate

Hae	*Haemophilus aegyptius*
Hind	*Haemophilus influenzae*
KCN	potassium cyanide
lig	ligase mutant
NMN	nicotinamide mononucleotide
OH	hydroxyl
P	phosphate
pfu	plaque-forming unit
pNp	2'(3'),5' ribonucleoside diphosphate
pol	polymerase mutant
RF	replicative form
RFI	covalently closed supercoiled RF
RFII	RF possessing at least one nick
RNA	ribonucleic acid
RNase	ribonuclease
rNMPs, rNTPs	ribonucleoside 5' mono- and triphosphates
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
T	thymine
TCA	trichloracetic acid
TMP	thymidine 5' monophosphate
Tris	Tris (hydroxymethyl) aminomethane
ts	temperature-sensitive mutant
ung	uracil-DNA glycosylase mutant