STUDIES ON PLASMID AND CHROMOSOME REPLICATION IN ESCHERICHIA COLI

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Declaration.

Except where otherwise stated, this thesis is my own work and has not been submitted for any other degree.

Abstract.

Two aspects of DNA replication have been examined, the timing of F'lac replication in the cell cycle and the effect of the presence of F-primes carrying the origin segment from the chromosome, on the host cell.

The cells of an exponential culture of B/r E/F'lac were separated according to size on a sucrose gradient in a zonal rotor. The cells had prior to separation been pulse-labelled with ^{3}H thymidine and pulse-induced with IPTG. Plasmid replication was measured in each size fraction by three methods: The rate at which β-galactosidase could be induced, the amount of label incorporated into CCC plasmid DNA which had been separated from chromosomal DNA by ultracentrifugation through CsCl-EtBr gradients, and the amount of label incorporat into CCC plasmid DNA which had been separated from chromosomal DNA in agarose gels. All these methods gave the same result, that F'lac which had been replicated during the pulse-labelling were found in cells of all sizes. This would sugges that F'lac replication is not confined to a part of the cell cycle, but additional experiments to clarify this furhter are suggested.

F-primes, which carry the origin region of the chromosome were isolated. These can be divided into two distinct classes those which include the region between bgl and ilv, in which oriC is, and those which don't. The plasmid strains which do carry this region show abnormal cell division, are able to replicate in Hfr strains, and integrate into the chromosome of a recA^{-} host at a very high rate. Plasmid strains

which do not contain this region of the chromosome do not have these characteristics. The recA independent integration has been shown to occur very near to the origin of replication and it is suggested that some event occurs near the origin of replication which obviates the need for, and may thus be analogous to the action of the recA protein. The possibility that the abnormal growth properties of the strains diploid for oriC might reveal something about the control of DNA replication is discussed.

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Contents.

1. Introduction.

The plasmids of E.coli are circular DNA molecules which are dispensable for cell growth. For reviews see Falkow (1975), Clowes (1972), Helinski (1973).

Because of their small size (6 - 60x10^6daltons), and because they are not essential for the survival of the bacteria, the bacterial plasmids have been considered to be suitable tools for studying DNA replication, and the control of DNA replication in particular. However, there may be differences in the way in which these replicons are replicated, and they may in fact represent special cases of replicative control. In this Introduction I shall review some aspects of the mode and control of initiation of replication in the E.coli chromosome and some of the plasmids which have been used for studies of control of DNA replication.

Involvement of chromosome-coded replication functions in plasmid replication.

Replication of the E.coli chromosome is a complex process, requiring at least 13 proteins (Wickner, 1978).

The products of the genes dnaA, dnaB, dnaC(D) dnaI, dnaP and rpoB are needed for the initiation of chromosome replication. The products of dnaB and dnaC(D) are also required for elongation, as are those of dnaG, dnaZ, dnaE(polC), polAex, lig, cou and nal. The proteins of many of these genes have been identified (table 1).

Table 1 *E. coli* functions required for DNA replication[a]

Locus	Characterization of protein	References
Required for initiation of chromosome replication		
dnaA	?	
dnaB	~250,000 dalton active protein; 48,000 dalton subunit; ~10 molecules/cell; rNTPase stimulated by single-stranded DNA	64–67
dnaC(D)	~25,000 dalton active protein; interacts physically and functionally with *dnaB* protein	44, 68
dnaI	?	
dnaP	?	
rpoB	RNA polymerase β subunit	
Required for chromosome elongation		
dnaB	see above	
dnaC(D)	see above	
dnaG	~65,000 dalton active protein; priming protein which synthesizes ribo- deoxy- and mixed oligonucleotides; ~10 molecules/cell	69–72
dnaZ[b]	~125,000 dalton active protein; interacts with DNA EF III; functions in DNA elongation with DNA EF I, DNA EF III, and DNA polymerase III	74, 75
polC (dnaE)	DNA polymerase III; ~180,000 dalton active protein; ~10 molecules/cell	76–80
lig	DNA ligase	81
*polA*ex	5'–3' exonuclease of DNA polymerase I	82
cou	component of DNA gyrase sensitive to novobiocin and coumermycin	54
nalA	component of DNA gyrase sensitive to nalidixic acid and oxolinic acid	55, 56
rep[c]	~67,000 dalton active protein; involved in φX174 RFI replication	83, 84

Table 1-1 List of proteins required for DNA replication after Wickner, 1978.

The replication of a few plasmids has been studied in mutants of *E. coli* which produce temperature sensitive products of some of these genes, in order to find out whether these proteins play a part in plasmid replication. The enzyme requirement *in vitro* has been determined by complementing *E. coli* mutant extracts in soluble membrane free systems.

DNA polymerase I is required for the replication of small, multicopy plasmids like ColE1, RSF1030 and CloDF 13 both *in vivo* (Kingsbury and Helinski, 1973b; Veltkamp and Nijkamp, 1973) and *in vitro* (Staudenbauer, 1976; Staudenbauer et al., 1978), but large and medium-sized plasmids that exist in fewer copies per cell, like F, R1, R100, R6K and pSC101 can replicate in *polA* cells.

DNA polymerase III seems to be required for the replication of all the above mentioned plasmids (Thompson and Broda, 1973; Nordström et al., 1974, Mayer et al., 1977, Arai and Clowes, 1974; Collins et al., 1975; Staudenbauer, 1976) although contradictory results have been reported for the replication of the small multicopy plasmids *in vivo* (Goebel, 1972; Veltkamp and Nijkamp, 1973).

The *dnaG* protein has been shown to synthesize both ribo- and deoxyribo- oligonucleotides, and is used for synthesizing the RNA primer in M13, G4 and ØX 174 (Wickner, 1978; Alberts and Sternglanz, 1977). It has been suggested that this protein plays a part in priming DNA replication at the origin in phages lambda (Lusky and Hobom, 1979) and G4

(Zechel et al., 1975). The dnaG protein is needed for the replication of all plasmids where this has been tested, it seems however, that it is not used for priming the initiation of ColE1 replication (Staudenbauer et al., 1978).

The product of dnaB is an rNTPase, stimulated by single stranded DNA. This protein seems to be required for the replication of most plasmids (Goebel, 1970; Arai and Clowes, 1974; Staudenbauer et al., 1978; Mayer et al., 1977), the replication of the small multicopy plasmid CloDF13 in vivo is one exception (Veltkamp and Nijkamp, 1974). A dnaB mutant dnaB252 is defective in chromosome initiation (Zyskind and Smith, 1977; Lanka et al., 1978), and a role in DNA initiation has therefore been assigned to this protein as well as a role in DNA elongation. The replication of the early replicative intermediates of ColE1 has however been shown to be independent of the dnaB gene product (Staudenbauer 1976). Wang and Iyer (1977, 1978) have found that a number of wild type, low copy number, conjugative R factors either suppress or enhance the thermosensitivity of DNA synthesis in dnaB ts strains. The phage P1, which is maintained in E. coli K12 lysogens as a plasmid, codes for an analog of dnaB (D'Ari et al., 1975). Wang and Iyer have suggested, that the conjugative R-factors either code for dnaB analogs or proteins that interact with the dnaB protein.

The dnaC(D) protein is a 25000 dalton protein which interacts physically and functionally with dnaB protein.

It is required both for the initiation and elongation of the chromosome replication. It is needed for the replication of the plasmids where it has been looked for, but as dnaB it is not needed for the initiation of ColE1 replication *in vitro* (Staudenbauer, 1976).

The dnaA gene product is required for the initiation of chromosome replication, but pSC101 is the only other replicon that has been shown conclusively to need this gene product for its replication.

It is evident, that while all plasmids whose replication has been studied use some of the host functions for their replication, they vary in their use of these functions. The diversity may be mostly in the way these replicons initiate replication, and may reflect different ways of controlling initiation of replication.

Plasmid coded replication proteins.

Extracts of cells that do not carry ColE1 are able to replicate added exogenous ColE1 DNA. Previous addition of chloramphenicol, which inhibits protein synthesis, has little effect on ColE1 DNA synthesis; plasmid coded protein synthesis is not required for ColE1 DNA synthesis *in vitro* (Tomizawa et al.1975).

Recently Kahn et al.(1978) and Donoghue et al.(1978) have provided evidence that ColE1 DNA can be replicated *in vivo* without plasmed-coded protein synthesis. Phage-ColE1 hybrids were constructed which could replicate

either via plasmid or phage replication systems and be packaged into infective bacteriophage particles. These phage hybrids were used to infect *E. coli* cells that had previously been treated with chloramphenicol to block protein synthesis. The phage-plasmid hybrids replicated normally as ColE1 plasmids, dependent on DNA polymerase I and sensitive to rifampicin. A protein, which regulates plasmid copy number negatively is of course not excluded by these results.

In vitro manipulation of the ColE1 plasmid has resulted in a reduction of its size to about 4-500 nucleotide pairs without loss of its ability to replicate. (Oka et al. 1979; Ohmori and Tomizawa, 1979).

Large plasmids, however, seem to code for some of their replication functions, and these genes are usually clustered around an origin of replication. Kollek et al. (1978) have shown that a segment of not more but also not much less than 1,8 kb is required for the replication of the copy mutant pKN102 of R1. This segment of DNA carries the origin of replication, and at least two proteins, with molecular weights of about 11.000 and 20.000 dalton, are synthesized in minicells containing this piece of DNA. The function of these proteins is unknown.

Yoshikawa (1974) has mapped by P1 transduction a plasmid locus, *repA*, necessary for autonomous replication of R100.

The replication functions of R6-5 have been located

on a 2,6 kb segment of the plasmid. These replication functions are : The incompatibility function, the repA function and ori V. Three RNA polymerase binding sites have been found on this segment (Timmis et al., 1979).

The plasmids R1, R100 and R6-5 have extensive homologies in common (Sharp et al., 1973), in all three R factors the replication functions are clustered in a region on the RTF unit proximal to the IS sequence which separates RTF from the r-determinant.

Recently it was demonstrated that all functions essential for R6K replication are clustered in a 2,1 kb DNA segment between the two origins of replication that have been found on R6K (Kolter and Helinski, 1978a). Inuzuka and Helinski (1978a) have developed an in vitro system for the replication of R6K DNA. It has been demonstrated that a plasmid-coded protein is required for this replication. This protein is involved in the initiation of R6K DNA replication (Kolter et al.,1978).

Plasmid mutants.

A number of temperature-sensitive mutants of plasmids have been described, which seems to suggest that these plasmids use plasmid-coded protein(s) for their replication, or at least that there is a plasmid-specified function involved in replication which can be affected by the raised temperature.

Jacob et al (1963) reported t.s. mutants of F'lac in

support of their replicon hypothesis. These mutant sex factors, which did not multiply in the autonomous state at high temperature were replicated normally at high temperature, when integrated into the bacterial chromosome. Recently t.s. mutants have been found of the mini-F plasmid pML31 (Eichenlaub, 1979). These plasmid-coded functions are thought to be involved both in initiation and in a later step of pML31 replication.

Kingsburg et al. (1973a; 1973b) reported the isolation of t.s. mutants of ColE. These mutations fell into two phenotypic classes on the basis of ability of F'lac to complement the mutation in the ColE1 plasmid. ColE1 DNA synthesis does not require any plasmid-coded protein synthesis (Donoghue et al., 1978; Kahn et al., 1978). These plasmid mutants must therefore be temperature sensitive either in regulatory proteins or some structure other than a protein, RNA or secondary structure at the origin ?

F is specifically sensitive to acridine dyes, acridine orange etc. (Hirota, 1960; Yamagata et al., 1969), which points to a different mode or control of chromosome and F replication, although there is evidence to suggest that acridine dyes affect the membrane rather than F replication per se (Nakamura, 1974).

Host chromosome mutations which affect plasmid maintenance.

Mutants, defective in plasmid maintenance, which map

on the host chromosome have been found in addition to those which map on the plasmid. Jacob et al.(1963) found a number of these when they were looking for their plasmid mutants. These chromosomal mutants were mapped by Hirota et al. (1968) and were all found to map very close to the str locus. Other chromosomal mutants defective in F maintenance, isolated by Stadler and Adelberg (1972) and Hathaway and Bergquist (1973) were mapped by Jamieson et al.(1976) and all mapped near thr-serB. This gene was termed seq, and the role of the protein is not clear. Only F factors and phage lambda are prevented from replicating at 43°C in cells carrying seq mutations while all colicin- and R-factors tested replicated normally. An other set of host mutants affecting plasmid maintenance, called maf, map in the vicinity of thr as well (Wada et al. 1976 ; Wada and Yura, 1979).

Host mutants defective in ColE1 replication have also been described (Kinsbury et al. 1973a, 1973b). The majority of these mutations exhibited temperature sensitivity to growth in deoxycholate in addition to the inhibition of plasmid DNA replication, suggesting a membrane alteration in the mutants when grown at the restrictive temperature.

Yamagata et al. (1972) found chromosomal mutations mapping near spc, which suppressed F'lac ts, and the AO sensitivities of the wild-type sexfactors were affected by the mutation. Yamagata et al. suggest that this is a ribosomal mutation which affects the episomal replicative

process selectively, since it does not suppress any known T4 nonsense or missense mutations, and since it renders the plasmid more resistant to acridine orange. It is, however, difficult to envisage specific ribosome participation in plasmid replication. Yamagata et al. mention two possibilities. One is that the episome produces its own specific initiation factor needed for the synthesis of episomal proteins. Another possibility is to assume that the ribosomes or ribosomal proteins interact with the bacterial membrane and affect its functions by changing its conformation. Acridine orange sensitivity can also be changed by a change in the membrane.

Relaxation complexes.

A number of plasmids can be isolated from E.coli in the form of supercoiled DNA-protein complexes, as first described for ColE1 by Clewell and Helinski (1969) (Kline and Helinski, 1971; Morris et al., 1973; Kuperstoch-Portnoy et al., 1974). Clewell and Helinski found that when a ColE1 containing strain was lysed gently, plasmid molecules could be obtained that sedimented at 24S, whereas supercoiled ColE1 DNA isolated from a dye-CsCl gradient had a sedimentation value of 23S. Treatment of the 24S ColE1 DNA with pronase, trypsin, SDS, Sarcosyl or heat resulted in its conversion to a slower sedimenting form of 17S or 18S, but noncomplexed supercoiled ColE1 DNA was resistant to conversion to the slower-sedimenting form. Electron

microscope examination indicated that the slow sedimenting peak consisted of open circular molecules.

When the strands of the 18S DNA separated, one linear molecule and one circle are obtained (Clewell et al. 1970), and centrifugation to equilibrium in CsCl in the presence of an excess of poly (U,G) indicates that the linear nicked strand is predominently the heavy strand both in ColE1 and ColE2 (Blair et al., 1971) and in F1 (Kline et al., 1971).

A 60.000 dalton protein is found covalently bound to the 5'end of the nicked strand (Lovett et al., 1975b; Guiney et al., 1975). It has been demonstrated for a number of plasmids, that the single strand nick that results from the relaxation of these plasmids is at a unique position. The site of the nick produced by induced relaxation in ColE1 and R6K is very near to the origin of replication (Lovett et al., 1974; Lovett et al., 1975a). This proximity to the origin of replication has led many people to suggest that relaxation complexes had a role in vegetative plasmid replication (Helinski et al., 1971). Recently evidence has however been obtained, which suggests that relaxation complexes are involved in transfer replication rather than vegetative replication (Inselburg, 1977; Warren et al., 1978; Guiney and Helinski, 1979), and it has turned out that the relaxation complex is dispensable for the maintenance of a number of miniplasmids.

The nucleotide sequence surrounding the relaxation

site of ColE1 has now been determined, the nick produced by relaxation is separated from the origin of replication by 300 nucleotides (Tomizawa et al., 1977; Bastia, 1978).

The origin and direction of DNA replication.

The initiation of DNA replication usually occurs at a unique site on the DNA molecule, although a few replicons have been reported to carry more than one origin.

The E.coli chromosome has been shown to replicate bidirectionally from a unique origin (Masters and Broda, 1971; Bird et al.,1972; Prescott and Kuempel, 1972; Rodrigues et al.,1973; Hohlfield and Vielmetter, 1973; Jonasson, 1973). This origin of replication, for which the symbol oriC has been introduced (Hiraga, 1976), has been mapped between the unc and asn genes at 83,5 minutes on the E.coli map (Messer er al., 1978; von Meyenburg et al., 1978; Marsh, 1978; Fayet and Louarn, 1978; Sugimoto et al., 1979; Meijer et al., 1979). This map position of the chromosomal origin was obtained by making maps of restriction endonuclease fragments in the origin region, which had previously been located between the mtl and ilv genes (Louarn et al., 1974). The origin was identified by various methods: Marsh (1978) labelled the DNA origin in a dnaC mutant which initiated DNA replication synchronously upon a shift from the non-permissive temperature to the permissive temperature. Von Meyenburg et al.(1978) used specialized transducing phages, lambda asn, which were

able to replicate via the chromosomal origin. Messer et al.(1978) linked DNA from the origin region to a DNA fragment coding for penicillin resistance, and these plasmids were able to replicate autonomously. By deleting these plasmids using restriction endonucleases the replication origin has in fact been located within a 232-245 base pair segment (Oka et al.1980). Recently Diaz et al. (1978) reported a second origin of DNA replication on the E. coli chromosome. They generated a selfreplicating plasmid from an EcoR1 chromosomal fragment, different from the one that contains the origin of replication (oriC) normally active in chromosome replication. It is however likely that this is an origin of a cryptic lambda-like plasmid (Kaiser and Murray, 1979).

The site of the origin and the direction of replication have been determined for a number of plasmids by employing the method of Fareed et al.(1972), who determined the origin and direction of SV40 DNA replication. They made use of the fact that the restriction enzyme EcoR1 makes a cut at one specific site in the molecule. Replicating bubbles were isolated, cut with EcoR1, and examined in the electron microscope, and the distance from the EcoR1 cut ends to the replicating bubble were measured.

It turns out that both uni- and bidirectional replication can be found among plasmids, and some plasmids even seem to use both modes under the same growth condi-

tions.

Eichenlaub et al. (1977) examined replicating molecules of the mini-F plasmid pML31. They found that replication was predominently bidirectional from an origin which mapped at 42.6 kb on the F map, but about 20% of the molecules replicated unidirectionally in either direction. A second potential origin has been found located 1.8 kb from the previously identified origin (Lane and Gardner, 1979).

Perlman and Rownd (1976) concluded from their denaturation mapping of replicating R100 molecules derived from a Proteus strain, that replication proceeds either uni- or bidirectionally from either of two replication origins which are located on the RTF and r-determinant regions respectively. Now additional origins of replication have been found.(Warren et al., 1978b).

Three small plasmids derived from a copy mutant of R100 were found to replicate unidirectionally from a fixed origin (Ohtsubo et al., 1977). Silver et al.(1977) also concluded that R100 replicates mostly unidirectionally from a single origin. They were able to synchronize the plasmid replication by sequential amino acid- and thymine starvation, and they did both partial denaturation mapping of replicative intermediates and electron microscope autoradiography of partially denatured molecules. R100 and its transfer factor derivative pAR132 were studied in this way and were both found to replicate predominently

unidirectionally from a single origin.

R6-5 replicates unidirectionally form a single origin of replication, but two additional regions of replication activity, themselves incapable of autonomous replication, but able to support the replication of ColE1 in polA strains, are believed to be potential DNA replication origins (Timmis et al., 1978b).

The conjugative, multicopy R factor R6K, has been shown to replicate from a fixed origin first in one direction to a unique replication terminus and then from the origin in the other direction (Lovett et al., 1975a). The terminus is placed asymmetrically 20% of the genome size from the origin. In a deletion mutant of R6K, RSF1040, two origins of replication have been observed, and molecules have been found in which both origins seem to be operating simultaneously. The replication of RSF1040 is asymmetrically bidirectional and proceeds to the termination site. One of the origins seems to be predominantly used (Crosa et al., 1975a, 1976). Crosa et al. speculate that the reason for there being an extra functional origin on the deletion mutant may be that this origin is normally silent, but has become activated as a consequence of the deletion.

The nonconjugative plasmid RSF 1010 replicates either unidirectionally or bidirectionally with equal frequency from a fixed origin (DeGraaf et al., 1978).

ColE1 has been shown to replicate unidirectionally

from a single origin both *in vivo* (Lovett et al., 1974) and *in vitro* (Tomizawa et al., 1974). However, a mini-ColE1 plasmid pVH51 has been shown to replicate mostly bidirectionally from the same origin as ColE1 (Eichenlaub et al., 1979). It therefore seems as if other factors than the nucleotide sequence at the origin of replication can affect directionality of replication.

The nucleotide sequence of the origin of DNA replication has been determined for a number of replicons. These include the plasmids ColE1 (Tomizawa et al., 1977), R1 (Oertel et al., 1979), R100 (Rosen et al., 1979), pBR 345 (Bolivar et al., 1977), R6K (Stalker et al., 1979), the phages f1 (Ravetch et al., 1977), fd (Gray et al., 1978), G4 (Fiddes et al., 1978), ØX174 (Sanger et al., 1977), lambda (Denniston-Thompson et al., 1977) and the *E. coli* chromosome (Sugimoto et al., 1979; Meijer et al., 1979).

The nuclotide sequence does not reveal much about the initiation event, but there are a few characteristic features which give scope for speculation.

Common to all these origins are a number of repeats that allow for extensive secondary structures with hairpin loops, and there is in fact evidence for such hairpin structures in fd (Gray et al., 1978). These structures may represent binding sites for initiation proteins, or the opening of double stranded DNA into hairpin structures may represent the mechanism for activating the origin.

There is some homology between parts of the E.coli replication origin and the replication origins of lambda and G4, but these are different from those of ColE1 and f1. ColE1 and f1 use RNA polymerase to prime DNA synthesis, whereas G4 and lambda use dnaG protein (Fiddes et al.1978). Messer et al. (1979) suggested that this homology between the origins of E.coli and replicons which use dnaG protein for making origin-RNA might mean that dnaG protein is used to prime DNA synthesis at the chromosome origin. RNA polymerase is required for initiation of chromosomal DNA synthesis (Lark, 1972; Messer, 1972), but Messer (1979) points out that the role of RNA polymerase could just be to open the double helix as it apparently does in lambda DNA replication (Dove et al., 1971).

Involvement of RNA in the initiation of DNA replication.

A requirement for an RNA primer for the initiation of DNA replication was first suggested by Brutlag et al. (1971) to explain the requirement for RNA synthesis in the conversion of the single stranded M13 bacteriophage DNA to the RF form. The initiation of DNA replication of a number of replicons has since been found to be sensitive to rifampicin, an inhibitor of RNA polymerase, whereas elongation is not.(Lark,1972; Messer, 1972; Kline, 1973; Clewell et al., 1973; Sakakibara et al., 1974; Tomizawa, 1975). All known DNA polymerases require a 3'OH group to which to add deoxynucleotides. This can be acquired by

making an RNA primer, and it is considered likely that the dnaG protein primes the Okazaki fragments during elongation (Alberts and Sternglanz, 1977). The priming at the origin may be different in that it is carried out by RNA polymerase, although, as noted earlier, in some instances the role of RNA polymerase may be to open up the double helix.

Messer et al. (1975) isolated an RNA species that is covalently bound to high molecular weight DNA. This RNA, termed o-RNA, seems to be present on both strands of the DNA molecules, and is quite stable (Womack and Messer, 1978). The existence of RNA covalently bound to DNA in the origin region has been demonstrated for ColE1 (Blair et al., 1972, Williams et al., 1973, Tomizawa et al., 1977). The region surrounding the transition point from RNA to DNA has been sequenced (Tomizawa et al., 1977).

Recently the large plasmids R1 and R100 have been found to code for low molecular weight RNA (Kricek et al., 1978; DeWilde et al., 1978). R100 plasmid RNA was obtained from minicells and hybridized to endonuclease generated fragments of R100 DNA. Three RNA species hybridized with a small fragment of DNA, which has been shown to contain an origin of replication. It is tempting to speculate that these RNA species may be involved in the priming of the initiation of DNA replication. An additional RNA species turned out to be coded for by a piece of DNA on which the origin of transfer is thought to be located (DeWilde et al., 1978).

DNA initiation mutants.

A number of mutants defective in initiation have been found, these include dnaA (Wechsler and Gross, 1971), dnaC (Hirota et al., 1970), dnaI (Beyersmann et al., 1974), dnaP (Wada and Yura, 1974) and dnaB (Zyskind and Smith, 1977). Very little is known about the biochemical action of these gene products.

DnaA mutants differ a great deal; some produce products that become inactive at 42°C, but will be reactivated at the permissive temperature, while others become permanently inactive at the nonpermissive temperature. Both dominant (Beyersmann et al., 1974; Zahn et al., 1977) and recessive (Wechsler and Gross, 1971; Gotfried and Wechsler, 1977) dnaA mutants have been reported.

It has been observed that initiation capacity is accumulated at the non-permissive (or intermediate) temperatures and released at the permissive temperature in dnaA ts mutants (Blau and Mordoh, 1972; Messer et al, 1975; Zyskind et al., 1977; Hansen and Rasmussen, 1977; Fralick, 1978; Tippe-Schindler et al., 1979).

These results have prompted many people to suggest a regulatory role for the dnaA gene product. Fralick (1978) suggested that the dnaA gene product is a repressor protein which prevents transcription of o-RNA. The temperature sensitivity is in a site that binds to an antirepressor; at 42°C the re-

pressor cannot be inactivated, and hence no DNA initiation; at 30°C the antirepressor site is renatured again and since initiation proteins have accumulated, multiple initations take place. Hansen and Rasmussen (1977) suggested that the dnaA gene product has a dual role; a positive function as an initiator of replication and a negative control function in its own synthesis. Both functions would have to be t.s. at the same time to fit the experimental data.

A similar positive regulatory role was postulated by Zahn and Messer (1979). Their proposal was based on the behaviour of strains diploid for dnaA mutation. These strains showed increased DNA per cell and an increased rate of DNA synthesis due to multinucleate cells in the population. The DNA replication was judged to be only at half the normal rate, and this was suggested to be due to over-initiation. Thus it was concluded that the dnaA protein must be a positive control protein: twice the amount resulting in enhanched initiation. It had to be assumed that autoregulation of the dnaA protein was impaired in this particular mutant to explain why all strains diploid for the dnaA gene did not behave in the same way. It seems that all these models are highly speculative and need additional experimental evidence to substantiate them.

Messer et al. (1975) found that the dnaA product is required for synthesis of o-RNA, and additional evidence for the relation of dnaA protein and RNA polymerase comes from

the finding that a suppressor of dnaA maps in the rpoB gene (Bagdasarian et al., 1978).

DnaA ts mutations can be suppressed by integration of various plasmids into the chromosome. Among these are F (Nishimura et al., 1973), R100-1 (Bird et al., 1976), R1 (Goebel, 1974), ColV (Nishimura et al., 1973) and P2 (Lindahl et al., 1971). Initiation takes place at the site of the integrated plasmid (Bird et al., 1976; Chandler et al., 1976; Louarn et al., 1977) and the replication is characteristic of that of the plasmid. It can be concluded that these plasmids do not need the dnaA gene product for their replication. Indeed, it seems that very few replicons other than the chromosome do. pSC101 has been shown to require the dnaA product for initiation of replication (Hasunuma and Sekiguchi, 1979; Frey et al., 1979; Felton and Wright, 1979), and contradictory results have been reported for ColE1. Goebel (1970, 1973) found that ColE1 replication was dependent on the dnaA gene product, but Collins et al. (1975) and Frey et al. (1979) reported that the replication of ColE1 was independent of the dnaA gene product.

The role of the dnaA gene product is thus obscure, but the fact that pSC101 and miniplasmids that use oriC for replication require this product for their replication (von Meyenburg et al., 1979) seems to offer useful tools for studying the action of the dnaA product.

The dnaC protein is required both for elongation and initiation of chromosome replication (Wechsler, 1978).

In initiation the dnaC gene product acts at a stage after o-RNA has been synthesized (Messer et al., 1975; Zyskind et al., 1977) probably in the first polymerization event (Zyskind et al., 1977). The dnaC protein also seems to play a part in the attachment of the folded chromosome to the membrane (Ryder et al., 1975; Ryder and Smith, 1975). The dnaC gene product interacts with the dnaB protein both physically and functionally (Wickner, 1978), and one dnaB mutant has indeed been found that affects DNA initiation (Zyskind and Smith, 1977; Lanka et al., 1978). It has been suggested that the function of the dnaB protein is to direct priming at the origin, and that it thereafter serves as a "mobile promoter" for primase dependent synthesis of the Okazaki fragments (McMacken et al., 1977).

Termination of replication.

The termination of DNA replication usually occurs at a site opposite the origin if replication is bidirectional, or at the origin if replication proceeds unidirectionally. It is generally not known whether a specific termination signal is required or whether termination occurs where the replication forks happen to collide.

Lai and Nathans (1975) have shown that in deletion mutants of SV40 where the original termination site has been displaced asymmetrically from the origin, replication proceeds symmetrically to a new position opposite the origin. It thus appears that SV40 does not need a specific termi-

nation sequence. Similar results have been obtained with the phage lambda (Valenzuela et al.,1976).

On the other hand, Kuempel et al. (1977) and Louarn et al. (1977) have reported that the replication of the E. coli chromosome slows down in the termination region, when replication starts from an integrated episome, which suppresses dnaA mutations. This termination region is located between 27 and 43 minutes on the E. coli map, opposite the E. coli origin of replication, even in strains where the site of initiation had been displaced by 26 min.

For one plasmid, the R factor R6K, a specific termination site has been found, that is located asymmetrically 20% of the genome from one of the two origins on the plasmid (Lovett et al.,1975a;Crosa et al.,1975; 1976). Kolter et al. (1978a) have constructed in vitro hybrids of R6K and a derivative of ColE1, and analysed the activity of the R6K terminus in the hybrid plasmids in the presence and absence of a functional R6K origin of replication. Replication of this hybrid molecule starts at the ColE1 origin in the presence of chloramphenicol and proceeds to the R6K terminus, where it slows down considerably, but passes the terminus and continues in the same direction to the ColE1 origin/terminus. The same happens if the R6K origins are deleted from the hybrid and replication is studied in a culture which has not been treated with antibiotic. Furthermore, it makes no difference on which side of the R6K terminus the ColE1 origin is; the

terminus is functional in slowing down replication in both orientations when replication is initiated from the ColE1 origin.

The R6K termination region is thus not a complete block, and it turns out not to be essential for R6K replication, since a number of derivatives of R6K exist, in which the terminus has been deleted (Kolter and Helinski, 1978a; Crosa et al. 1978).

It can be concluded that a specific termination site is not obligatory for termination to occur. The mechanism of termination and of subsequent segregation of daughter molecules remains obscure.

Incompatibility.

Incompatibility is the inability of two related plasmids to coexist in the same cell. This phenomenon was first observed with F in that F'lac multiplication was inhibited in Hfr cells (Scaife and Gross, 1962; Maas and Maas, 1962).

Plasmids which can not coexist in the same cell are said to belong to the same incompatibility group, and so far 26 incompatibility groups have been described in the enteric bacteria (Novick and Hoppensteadt, 1978).

Copy number.

Each plasmid species has a specific number of copies per cell. The number of these varies from 1 to 5 for large,

conjugative plasmids like F, R1 and R100, e.g.. Small, nonconjugative plasmids, on the other hand are maintained at many - 10-50 - copies per cell. The number of plasmids per cell has been estimated by measuring the ratio of supercoiled DNA to chromosomal DNA in CsCl-EtBr gradients, by measuring the amount of enzyme production coded for by the plasmid or by DNA-DNA hybridization. None of these methods is necessarily very accurate, the recovery of supercoiled DNA is in particular thought to underestimate the actual amount of plasmid DNA in the cell (Cress et al., 1976).

Frame and Bishop (1971) and Collins and Pritchard (1973) have estimated the copy number of F'gal and F'lac respectively by hybridization, and found that the copy number is approximately 1,2 - 1,4 copies of the plasmid per genome equivalent at one doubling per hour. It was also found that the number of copies of the plasmid per chromosome varied with growth rate, while there were 1,2 plasmid copies per genome equivalent at one doubling per hour, there were 0,84 copies of the plasmid per genome equivalent at 2,86 doublings per hour. Similarly, Engberg et al. (1975) found that the copy number of R1 decreased with increasing growth rate.

Control models.

Plasmids like F, of which there are few copies per cell, are nevertheless maintained very stably in the

population. It is obvious that for these plasmids, as for the chromosome, there must exist a mechanism that ensures that the plasmid replicates before the cell divides, and a mechanism that controls the partition of plasmids into daughter cells.

Two main control models have been proposed to account for this. Jacob et al. (1963) proposed a positive control model, which assumes the production of a specific initiator that acts positively in a recognition site (the replicator). Furthermore, it was proposed that there were a limited number of membrane sites, to which the replicons in the cells became attached and replicated. This could explain the incompatibility phenomenon in that the incoming plasmid was excluded from replication because all the attachment sites were occupied.

A simple interpretation of this model does not, however, explain the inability of F to replicate in an Hfr strain, as observed by Scaife et al. (1963) and Maas et al. (1963). If it is assumed that the integrated F is replicated passively with the chromosome, it should leave its site at the membrane vacant for an incoming autonomous F to replicate, but if the membrane site were occupied by the integrated F, replication from the F site should not be inhibited, as has been reported (Masters and Broda, 1972). Pritchard et al. (1969) proposed an alternative model, a negative control model. According to this model a repressor-like protein molecule is made just after

initiation of replication. This protein acts at a site at or near the origin of replication, and inhibits any further initiations of replication until the concentration of the repressor, which has been diluted out by increase in the volume of the cell, falls below a certain threshold. According to this model, incompatibility can be explained by a common repressor, which does not differentiate between the incompatible plasmids. This model, however, does not account for the partition of plasmids into daughter cells. It has been calculated (Novick et al., 1978; Ishii et al.,1978) that with random segregation (and equipartition), sooner or later the cellular population comes to carry only one or the other of two incompatible plasmid types. This happens after 18 generations if the copy number is 10, and in 77 generations if the copy number is 45 (Ishii et al., 1978). A negative control model could explain the inability of F to replicate in an Hfr strain; if it is assumed that there are normally fewer copies of F in a cell than there are chromosomes, then F would be replicated passively with the chromosome , F repressor would be produced at a higher rate than normal, and the concentration of the repressor would never fall below the threshold that allows replication.

A third model, the so-called autorepressor model has been proposed (Sompyrac and Maaløe, 1972). According to this model there is a repressor that is under the

control of the operator on which it acts, and an initiator protein that acts on the origin of replication is controlled by the same operator-repressor. The operon is transcribed when the level of repressor in the cell is low, and then the repressor and initiator are made. When enough repressor has been made, the operator is switched off, but initiator has also been made and probably accumulates at the origin until replication can start. The repressor is then diluted out by the growth of the cell.

The replication of the plasmid lambda dv seems to be controlled by an autorepressor. Lambda dv is an autonomously replicating DNA fragment of phage lambda, and consists of an origin of replication, a promoter-operator region and three genes; tof, which codes for a negatively controlling protein, and O and P which code for positive control proteins. Lambda dv is maintained at 60-100 copies per cell and expresses incompatibility (Matsubara et al.,1978). It has been shown (Matsubara 1976) that the tof gene codes for an autorepressor which acts on the promoter-operator (pRoR) region, and the O and P genes code for DNA replication initiation proteins. Matsubara et al.(1978) have shown that the incompatibility function is determined by the region on the lambda dv genome that carries the autorepressor (tof) gene and the promoter-operator (pRoR). The site that carries the initiator genes and the origin of replication does not

affect incompatibility. The autorepressor system is also thought to be responsible for controlling the plasmid copy number.

Copy number- and incompatibility mutants.

Both a fixed copy number of a plasmid and the incompatibility phenomenon have generally been thought to be a consequence of the control mechanism of plasmid replication and/or partition. Therefore, mutants in copy number and incompatibility have been much searched for in the hope that they might cast some light on the control mechanism of plasmid replication.

Conditional copy mutants have been found of the plasmid R1drd-19 (Gustafsson and Nordström, 1978). Both temperature dependent and amber suppressible mutants were found, and these showed three to four fold copy number at the elevated temperature or in the absence of suppression. These results indicate that there is a negatively acting protein involved in the regulation of copy number, since the copy number becomes higher when the protein is knocked out. Uhlin and Nordström (1975), who isolated and studied copy mutants of R1drd-19, found that the incompatibility of the mutants was altered as well as the copy number, which indicates that there is a connection between incompatibility and copy number control. If a repressor protein is responsible for incompatibility, it should be possible to isolate two kinds of incompatibility mutants,

one in the repressor protein, that should be recessive, and an other in the target site for the repressor, that should be cis-dominant. A copy mutant of R1drd-19 has indeed been found that could belong to the latter class. This mutant neither segregates from cells containing R1 nor from cells carrying the incompatibility function on multicopy plasmids (Uhlin and Nordström, 1975). A second mutation in the temperature dependent copy mutant of R1drd-19, that seems to replicate without control of copy number, has been isolated (Uhlin and Nordström, 1978). This is a temperature dependent mutant that produces plasmid DNA at a rate twice that of the chromosome until about 75% of the total DNA of the cell is plasmid DNA and cell growth stops. The normal copy number could be restored by lowering the temperature before the host cell growth was inhibited. It is not known whether the two mutations occurred in the same gene or in different genes, but it looks as if copy number is solely controlled by the plasmid.

Plasmid-borne copy mutants have also been isolated from other plasmids, like ClodF13 (Kool and Nijkamp, 1974), R100 (Morris et al., 1974) and lambda dv (Matsubara and Takeda, 1975).

Chromosomal mutants, affecting copy number have been found for F (Cress and Kline, 1976) and R6K (Macrina et al., 1974).

Incompatibility mutants in F have been searched for, and only been found in integrated Fs (DeVries et al., 1974).

San Blas et al. (1974) found a chromosomal mutation, that allowed the coexistence of F'his and F'gal, but the loss of incompatibility applied only for this pair of plasmids, and it is difficult to tell what kind of a mechanism was underlying this.

Palchaudhuri and Maas (1977, 1978), have mapped by electron michroscope heteroduplex analysis a DNA sequence common to the four plasmids of incompatibility group FI: F, R386, ColV-K94 and EntP307 on the one hand, and plasmids R1, R100, R6 of incompatibility group FII on the other. In both cases they found that there was a short region of homology (2 and 4 kb respectively) common to all the plasmids in each group in the region of autonomous replication, copy control and incompatibility. There is no homology between the replication regions of the plasmids of the two incompatibility groups. This suggests that incompatibility is in fact due to a common mechanism, and that this mechanism is related to plasmid maintenance functions.

Manis and Kline (1978) have divided the replication region of F (40,3 - 49,3F) into three fragments containing the sequences 41-43, 43-46 and 46-49F. They have cloned each of these fragments in pSC101 and tested incompatibility with F'lac. The incompatibility region of F, mapped by Palchaudhuri and Maas (1977) had the coordinates 46.4-48.6 F . As expected, Manis and Kline found that the 46-49F fragment exerted incompatibility with F'lac,

but unexpectedly they also found that the 43-46F fragment expressed incompatibility, so there seems that there are at least two genes for incompatibility. Copy mutants of mini-F plasmids were isolated, and the mutation mapped in the 43-46F region. These plasmids had also lost the incompatibility function of the 43-46F fragment, but not that of the 46-49F fragment. Again this strongly indicates a relationship between incompatibility and copy number control, although another function may well cause incompatibility as well, for example the partition mechanism of plasmids into daughter cells, as proposed by Novick et al., (1976, 1978) and Timmis et al. (1978). Incompatibility mutants of pSC101 have been found to affect plasmid replication as well as incompatibility, but mutants defective in replication did not necessarily affect incompatibility (Meacock and Cohen, 1979).

The clustering of replication genes, copy control and incompatibility in a small region has been found in most plasmids where it has been looked for, it has also been found that incompatibility is separable from the origin of replication. Molin and Nordström (1980) found that a very small ($0,35 \times 10^6$ dalton) piece of DNA of the R factor R1 coded for copy control and incompatibility. Since this DNA sequence was so small that it could hardly code for more than one gene, it was suggested that incompatibility and copy control are governed by the same gene. Their method was to suppress a dnaA ts strain by

integration of a derivative of R1. Various fragments from the replication region of R1 were then cloned in the multicopy plasmid pBR322, and introduced into the integratively suppressed strain by transformation. It was found that some of the plasmids did prevent the suppression of the strain at the high temperature, and all these plasmids had only the before mentioned $0,35x10^6$ dalton piece of DNA in common. It is thus concluded that this piece of DNA codes for a negative control protein that is produced in large amounts by the multicopy plasmid and prevents the replication of the integrated R1.

As mentioned earlier, the plasmid R6K has two origins of DNA replication, called alpha and beta, about 3900kb apart. The alpha origin is normally used, and then about 20 copies are produced per cell. If, on the other hand, the alpha origin is deleted, the beta is used, and the copy number is 10 copies per cell (Crosa et al.,1978). This is in accordance with the results that have been obtained with plasmid cointegrates, e.g. pSC101-ColE1 and F'lac-ColE1 (Cabello et al.,1976). When two compatible replicons are fused together, it seems to be the rule that the origin of the plasmid with the higher copy number is used in the DNA replication. These results can be explained by the negative control model in the same way as the replication of the chromosome - F cointegrate is explained, in that the level of repressor of the low copy number replicon is maintained too high for replication to take place.

Crosa et al. (1978) cloned different restriction fragments from the replication region of R6K on the ColE1 derivative pBR313, and looked at the incompatibility towards an R6K replicon. They found that the incompatibility function resided in the region between the two origins of replication, this region also contains the gene for the protein that has been shown to be necessary for initiation of R6K replication (Inuzuka and Helinski, 1978b).

The plasmid pBR313 exists in 40-50 copies per cell, but R6K in 20 or 10 copies per cell depending on which origin is used. It would therefore be expected, if incompatibility and copy number are controlled by same simple repressor, that there would be a preferential loss of the replicon with the lowest copy number (i.e. R6K), but in fact the composite plasmid is lost 100% in most cases. This seems to indicate that incompatibility may be a more complex process than just a function of a repressor.

Kolter et al. (1978) have assigned a positive control function to the TT protein. They found that cell extracts from strains which have an elevated copy number of R6K have increased DNA replication activity <u>in vitro</u> assays. This seems to indicate that the TT protein controls copy number. They also found that when the gene for the TT protein was inserted into the chromosome and complemented in trans the replication of a non-selfrepli-

cating R6K derivative, the same copy number was maintained as that of the parent plasmid. It thus seems that the level of TT protein in the cell is autoregulated.

There is evidence for a negative control element in the replication of relaxed plasmids as well as stringent plasmids. Grindley et al. (1978) have found copy mutants of the nonconjugative plasmid NTP1. When these mutant strains were shifted to 42°C from 28°C, the copy number of the plasmid increased from 8% of the chromosomal DNA or about 40 copies per cell to 150% or about 750 copies per chromosome. The high copy number at the elevated temperature suggests that there is a negative element controlling the initiation of DNA replication of NTP1.

Mini-plasmids of ColE1 are present at a higher copy number per cell than the wild type plasmid (Hershfield et al., 1976). This could be explained by a negative control element missing in the mini-plasmid, but the alternative explanation has been put forward by Mayer et al. (1977) in accordance with their results with mini-R1drd-1982, that the copy number of miniplasmids is elevated because the copy number is determined by the total amount of plasmid DNA. It has been pointed out, however, (Pritchard, 1978) that it is possible that although copy number is normally controlled by other means, conditions can be created where components of DNA synthesis become rate limiting.

Attachment of replicons to the cell envelope.

a) The chromosome.

In the replicon model of Jacob et al. (1963) it was proposed that the replicons of the cell were attached to the cell membrane. There are indeed some lines of evidence that suggest an attachment of the chromosome to the membrane.

Electron microscopic studies have shown an apparent association of DNA with the mesosome in B. subtilis (Ryter, 1968). The mesosome is an invagination of the membrane, and similar structures have been looked for in E.coli, but with ambiguous results (Ryter et al., 1968, Pontefract et al., 1969).

The chromosome of E. coli is packaged into a nucleoid, ca 0,5u in diameter (Woldringh and Nanninga, 1976). Worcel and Burgi (1972) suggested on the basis of the behaviour of the folded chromosome in sucrose gradients in the presence of varying concentrations of EtBr and the effect of DNase and RNase that the folded chromosome consists of supercoiled loops, and that it is stabilized by an RNA core.

When the cells are lysed carefully (Stonington and Pettijohn, 1971), the chromosome can be isolated in the folded conformation attached to membrane fragments. The nature of this attachment to the membrane is not clear. Portalier et al. (1976) found two inner membrane proteins that could be cross-bridged with DNA, and it was postu-

lated that these were specific membrane attachment proteins. Wolf-Watz and Nordquist (1979) have provided evidence for the association of chromosomal DNA with a protein in the outer mambrane. This protein-associated DNA has been shown to be enriched in DNA from the origin region (Wolf-Watz and Masters, 1979). It was found that 5-10% of the protein-associated DNA was homologous with the origin DNA carried by a lambda phage, although this DNA only comprised about 1% of total chromosomal DNA. An enrichment of origin-DNA in membrane bound DNA has been suggested by other workers as well (Parker and Glaser, 1974; Craine and Rupert, 1978; Nicolaides and Holland, 1978). Association has also been found between the membrane and DNA sequences at the origin and terminus of DNA replication in B. subtilis (O'Sullivan and Sueoka, 1972; Sueoka and Quinn, 1968; Sueoka and Hammers, 1974; Yamaguchi and Yoshikawa, 1975, 1977). It seems, however, that the membrane-chromosome association is not static, since it has been shown that the DNA remaining associated with the membrane after digestion with endonucleases is indistinguishable from the total DNA released from the membrane (Drlica et al., 1978).

Fuchs and Hanawalt (1970) and Parker and Glaser (1974) found that membrane bound DNA was much enriched in labelled DNA after a short pulse of radioactive precursors, whereas Drlica et al. (1978) did not find any preferential binding of labelled DNA to the membrane,

leaving the attachment of the replication forks to the mambrane in doubt.

There have been contradictory reports about the attachment of the folded chromosome to the membrane during the cell cycle. Worcel and Burgi (1974) were not able to isolate the folded chromosome associated with membrane from amino acid starved cells that had finished replication, but as soon as replication had initiated, membrane bound chromosome could be readily isolated. It was concluded that the membrane-chromosome attachment was associated with the initiation of replication, and that chromosomes having completed rounds of replication were released from the membrane. Others have found on the contrary that the nucleoid is attached to the cell envelope throughout the cell cycle (Ryder and Smith, 1974; 1975; Korch et al.,1976; Craine and Rupert, 1978; Yaffe et al., 1979).

Growth of the cell envelope in a central zone which is predicted by the replicon model has not been conclusively confirmed. The cell envelope of E.coli consists of three layers: An inner lipoprotein membrane,then a murein layer and an outer lipoprotein membrane. Various groups of workers have studied the distribution of components of the cell envelope with growth, and both random and localized growth of the cell envelope has been reported. Beachey and Cole (1966) followed the distribution of fluorescent antibodies in the cell envelope through a

few generations and found that the antibodies were evenly distributed. Lin et al. (1971) and Green and Schlechter (1972) also provided evidence for random growth, based on autoradiography of labelled membrane components. Hirota et al. (1974), who studied the incorporation of tritiated diaminopimelic acid into the murein layer of the cell envelope, came to the conclusion that initially there was growth of a narrow band in the center, but after a while the molecules were randomly dispersed. Kapes and Autissier (1974) concluded on the other hand that the growth of the cell envelope was localized. Their conclusions were based on studies on the distribution of inducible permeases in the inner membrane after growth without inducer. Donachie and Begg (1970 ; Begg and Donachie, 1973; 1977) proposed a model of cell growth that assumes localized growth at the cell poles. This model was based on the results these workers got from studies on the position at which penicillin induced autolysis of the murein layer occurred and the distribution of T6 receptor sites in the outer membrane after growth in conditions where such sites were not made.

Begg and Donachie (1977) suggest, that these discrepancies can be explained by some proteins and lipids being mobile in the membrane, while some proteins are permanently bound to a proposed framework.

b) Plasmids.

Although the replication of some plasmids seems to be controlled, at least in part, by a negative element, the segregation of plasmids may well be controlled by another mechanism. The finding that F'gal cosegregates with the host chromosome during curing by acridine orange (Hohn and Korn, 1969) supports this hypothesis.

Kline and Miller (1975) found that when they lysed cells under conditions that preserved the folded chromosome, much of the F DNA sedimented in association with the folded chromosome, while exogenously added plasmid DNA did not. This suggests that the observed association of plasmid and the folded chromosome is not due to an artificial trapping of plasmid DNA in the folded chromosome during the isolation procedure. It has been suggested that the association of F with the chromosome is stabilized by RNA (Miller and Kline, 1979).

Kline et al. (1976) examined over 25 distinct plasmids of E.coli and Salmonella typhimurium, and in every case they found that the plasmid was complexed to the folded chromosome. There seems to be a difference between stringently controlled F-like plasmids and relaxed ColE1-like plasmids in that a much higher proportion of F-like plasmids is associated with the folded chromosome than ColE1-like plasmids. It has been suggested that these plasmid-chromosome complexes are related to plasmid replication, since it has been found both for colE1 (Miller

et al.,1978) and R6K (Archibold et al.,1978) that mostly replicating forms are associated with the folded chromosome, and evidence has been obtained for the association of the origin/terminus region of ColE1 DNA with the cell membrane (Sparks and Helinski,1979). Miller et al.(1978) found that when the copy number of a plasmid is raised because of chromosomal or plasmid mutations, the frequency of plasmid bound to the folded chromosome remained constant, and the actual number of copies bound raised, but if the copy number of the plasmid increased because it was linked to another replicon that was replicated in more copies per cell (i.e. a mini-F-ColE1 integrate, where replication is under the control of ColE1), the binding was characteristic of that of ColE1. This was taken as a further indication of a relationship between replication and complexing to the folded chromosome. Furthermore, Miller et al. (1978) found that unique insertions and deletions of plasmid DNA altered plasmid complexing frequencies, which could suggest that the complexing is determined by plasmid sequences.

Timing of plasmid replication in the cell cycle.

Stringently controlled plasmids use DNA polymerase III for replication, as does the chromosome, and very likely replicate at the same rate as the chromosome (Gustafsson et al.,1978). Plasmid molecules are therefore idle with respect to DNA replication for most of the

cell cycle. The question then arises whether plasmid replication occurs at a particular time in the cell cycle, and whether all plasmids in the cell are replicated at the same time.

Several groups have studied the timing of F in the cell cycle, and most of them come to the conclusion that F is replicated at a particular time in the cell cycle (Donachie and Masters, 1966; Nishi and Moriuchi, 1966; Zeuthen and Pato, 1971; Cooper, 1972; Davis and Helmstetter, 1973; Finkelstein and Helmstetter, 1977), although they don't agree when in the cell cycle it is replicated. These groups have used either synchronized cultures or exponentially growing cultures separated into age classes by the membrane elution technique of Helmstetter (1967). In all cases F'lac was used, and the amount of inducible β-galactosidase was used as a measure of plasmid DNA content. Pritchard et al. (1975), working with exponentially growing cultures and using β-galactosidase as a measure of plasmid content, measured β-galactosidase at different chromosome replication velocities and different growth rates, and came to the conclusion that F replication was not coupled to any stage of the DNA replication or cell division cycle. Recently it has been questioned whether F'lac in fact does replicate at a discrete time in the cell cycle (Gustafsson et al., 1978). Evidence is presented in this Thesis which is consistent with the F'lac replicating throughout the cell cycle.

Concluding remarks.

It can be concluded that while the biochemistry of DNA replication in E.coli is relatively clear, at least in some aspects, its control is still obscure.

The timing of the initiation of chromosomal replication in the cell cycle is rather exact, starting at a constant initiation mass over a wide range of growth rates. It thus seems as if a critical mass somehow triggers off initiation of chromosome replication. This could be acquired through a positive control system as proposed by Donachie (1968), in that there is an accumulation of some initiation substance that is synthesized at a rate proportional to the increase in mass, or it could be obtained by a negative control, as suggested by Pritchard et al. (1969).

While there is no conclusive evidence for or against a positive control model, it seems difficult to explain the simultaneous initiations of all replication origins in fast growing cells in terms of the inhibitor dilution model, where it is assumed that a burst of repressor synthesis occurs just after initiation of DNA replication. If one origin started a little ahead of the others, it would result in repressor synthesis and prevent the others from initiating. If the negative control model were to apply here, additional assumptions would have to be made to account for this.

Studies with copy number- and incompatibility mutants

of plasmids seem on the other hand to favour a negative control model, and the fact that plasmids like ColE1 which depend on DNA polymerase I for replication continue to initiate DNA replication in the absence of protein synthesis speaks against there being a positive control element involved in its replication. However, a positive control protein appears to be involved in the replication of the plasmid R6K, and indeed there is no reason to believe that the same way of control is operating for all the replicons in the cell.

In the following chapters two aspects of DNA replication will be examined : The timing of F'*lac* replication in the cell cycle, and properties of strains diploid for *oriC*, which may elucidate events at the initiation of chromosome replication.

2. Materials and Methods.

Strains.

The strains that were used are listed in the Results chapters.

Media used routinely.

L broth.

Difco Bacto Tryptone, 10g ; Difco Bacto yeast extract, 5g ; NaCl, 5g ; Glucose, 1g per litre adjusted to pH 7,2.

L agar.

Difco Bacto Tryptone, 10g ; Difco Bacto yeast extract, 5g ; NaCl, 10g ; Difco agar, 15g per litre adjusted to pH 7,2.

Bacterial buffer.

KH_2PO_4, 3g ; Na_2HPO_4, 7g ; $MgSO_4 \cdot 7H_2O$, 0,2g ; NaCl, 4g per litre.

M9 x 4.

Na_2HPO_4 (anhydrous), 28g ; KH_2PO_4, 12g ; NaCl, 2g ; NH_4Cl, 4g ; H_2O, 1 litre adjusted to pH 7,0.

VB salts x 20.

$MgSO_4 \cdot 7H_2O$, 4g ; Citric acid, 40g ; $NH_4NaHPO_4 \cdot H_2O$, 70g ; K_2HPO_4, 200g ; H_2O, 1 litre.

McConkey agar.

Peptone, 20g ; Bile Salts = 3, 1,5g ; NaCl, 5g ; Neutral Red 0,03g ; Agar, 15g ; H_2O to 1 litre.

To this a relevant carbon source was added to a final concentration of 1%.

Amino acids

were prepared as 2 mg/ml solutions in H_2O, and used at 20 µg/ml.

Sugars

were used at 0,2%.

Tetrazolium plates.

To 500 ml L agar, 1 ml 1% 2,3,5-Triphenyl-2H-tetrazolium chloride (Sigma) and 25 ml 20% lactose (or other sugar) were added. Bacteria that can ferment the sugar turn out white, negative ones are red.

Giemsa plates.

For finding out sensitivity of cells to male specific phages, about 10^9 phages were spread on Giemsa plates, which had been made in the following way : 10g tryptone, 10g yeast extract, 8g NaCl, 15g Difco agar, 1 l H_2O. This was autoclaved, and then 4 ml 0,5 M $CaCl_2$, 50 ml 20% glucose and 12,5 ml Giemsa (Gurr R66) were added. Bacteria which had been growing for ca 6 hours on nutrient plates were replicated on the Giemsa plates. Males lyse and turn purple, the females are blue.

Tna plates.

The following method only works for trp B mutants (Yudkin, 1976) : 25 mg DL-5-methyltryptophan (Sigma) was added to 500 ml water agar and autoclaved. Then VB salts,

Some abbreviations:

IPTG isopropyl- -D-thiogalactoside

BCIG 5-bromo-4-chloro-3-indolyl- -D-galactoside

t-ONPG o-nitrophenyl- -D-thiogalactoside

EDTA Diaminoethanetetraacetic acid disodium salt

tris tris(hydroxymethyl)aminomethane

glycerol to a final concentration of 0,2%, amino acids, except tryptophan, and indole (2 µg/ml final concentration) were added. Tna^+ cells can be identified by their ability to convert indole to tryptophan.

Trimethoprim selection of thy^- cells.

A fresh overnight culture (0,02 ml) was inoculated into 1 ml trimethoprim medium (M9 salts, 0,2% glucose, 0,5 µg/ml B_1, 10^{-3}M Mg^{++}, 0,2% CAA, 20 µg/ml trimethoprim, 50 µg/ml thymine) and grown at 37°C for 2 days. The bacteria were streaked on L broth plates, and individual colonies tested for thymine requirements.

Construction of a $recA^-$ strain.

The strain was made thy^- (see above) and mated with Hfr JC5088, which transfers thy and $recA^-$ early. Recombinant colonies (thy^+) were tested for MMS sensitivity.

Testing for a recA mutation by MMS sensitivity.

A 10% solution of MMS (Methyl methanesulfonate, Eastman) in ethanol was added to minimal or broth agar at ca 45°C, 0,06 ml MMS solution/20 ml agar. $RecA^-$ colonies cannot grow on these plates, the plates last for 24 hours only.

Selection of a lac^- mutant. (Smith&Sadler, (1971))

Lac^- colonies were selected on o-nitrophenyl-β-D-thiogalactoside selective medium plates, which were made as follows : D buffer (Davis&Mingioli, 1950) plus $2 \cdot 10^{-3}$M t-ONPG (Cyclo), 2g sodium succinate/l 20 mg BCIG/l

(Sigma), 11g purified agar/l and 4.10^{-6}M IPTG. The plates were incubated in plastic bags (the selection is best in 100% humidity) at 30°C for 6 days. The resulting colonies were tested on McConkey lactose plates.

Selection of bgl^+ strains.

Spontanious revertants were selected on a McConkey plate, containing salicin (1%) as a sole carbon source.

Monitoring cell growth.

The cell growth was followed by measuring optical density of the culture at 540 nm, using a Hilger-Watts spectrophotometer (Hilger-Watts Ltd., London).

Cell number and volume were determined by using a Coulter Counter and Channelizer Model 28 (Coulter Electronics, Ltd., Harpenden, England). Samples of cells were taken into 10 ml of filtered azide-saline solution (36g NaCl, 2g NaN_3 in 4 l H_2O). An aliquot of 0,05 ml was counted, using a probe with a 30 µm orfice. A Coulter Channelizer C-1000 was fitted to an X-Y recorder, which plotted the cell volume distribution on a graph. The machine was calibrated by using latex spheres of known diameters. The actual volume of the cells was calculated by using the formula :

$$V=KxAxIxT$$

where V=volume, I = aperture current, A = amplification, K = 0,064, T = threshold (the threshold scale is thus linear with volume, and gives a relative scale of indi-

vidual particle volume).

When percentage of dividing cells in the culture was estimated, the cells were counted microscopically, using a Petroff-Hauser counter. At least 200 cells were counted, and only those cells, that clearly had formed a septum were scored as doubles.

Isolation of plasmid carrying strains.

Fresh overnight cultures were diluted 1 in 10 into L broth and grown for 2 hours. Equal volumes of male and female were mixed and incubated without shaking at 30°C (for ts plasmids) for 30 minutes. The cells were then harvested, resuspended in buffer, plated on selective medium and incubated at 30°C for 2-3 days. Progeny were patched on the same medium and then tested for nutritional markers.

Interrupted mating. (Zipkas & Riley, 1976)

The recipient was made resistant to 20 µg/ml nalidixic acid. The mating was interrupted by diluting the mating mix 10 fold into buffer containing 50 µg/ml nalidixic acid and mixed with a whirlmixer for 30 seconds. The mating mix was then spread on selective minimal plates with 20 µg/ml nalidixic acid.

P_1 transduction.

The recipient culture was grown to stationary phase in L broth plus 10^{-3}M Ca^{++}. The cells were harvested and resuspended in 1/10 volume of supernatant. 0,1 ml of the

10x concentrated cells were mixed with $3x10^7$ phage in 0,1 ml phage buffer. The mix was incubated at 37°C for 10 minutes, 0,5 ml phage buffer added, and 0,1 ml spread on selective plates.

Curing of F with acridine orange.

A loopful of overnight culture was inoculated into each of 4 tubes, containing 1 ml of L broth, pH 7,6 and 0, 50, 100, 200 µg/ml acridine orange and grown overnight in the dark. If the strain was recA⁻, lower concentrations of acridine orange were used. The bacteria were then streaked on nutrient plates and individual colonies were tested either for sensitivity to male specific phage (e.g. MS2) or loss of any marker on the F′.

Isolation of plasmid DNA.

Cleared lysate. (Clewell and Helinski (1969), modified).

10 ml cells, grown in the minimal medium, were harvested by centrifugation and the pellet resuspended in 0,4 ml 25% sucrose in 0,05 M tris pH 8,0. Lysozyme (0,024 ml of a freshly prepared solution at 10 mg/ml in 0,25 M tris, pH 8,0) was added and the mixture kept on ice for 5 minutes. Then 0,18 ml EDTA (0,25 M pH 8,0) were added and after a further 5 minutes the cells were lysed by adding 0,6 ml lytic mix (0,05 M tris, pH 8,0, 0,062 M EDTA, pH 8,0, 2% Triton X-100). After cellular lysis (5-20 minutes), the lysate was centrifuged at 15 krpm

at 4°C for 15 minutes in a Sorvall SS34 rotor and the supernatant was carefully decanted. This is the "cleared lysate" .

Neutral sucrose gradients.

Samples of 100 µl of the cleared lysates were mixed with 200 µl of TES buffer (0.05 M tris, 0.05 M NaCl, 0.005 M EDTA pH 8.0) to bring the sucrose concentration below 5%, and layered onto 5-20% (wt/vol) linear sucrose gradients, containing 0.5 M NaCl, 0.01 M potassium phosphate buffer, pH 7.0. ^{35}S labelled phage R17 was added as a sedimentation marker (80,5S). The gradients were centrifuged at 49.000 rpm at 4°C for 45-60 minutes in an SW 50.1 rotor of a Beckman L 2-50 centrifuge. After centrifugation 10 drop fractions were collected from a hole punctured in the bottom of the tube and the fractions were counted in a Packard Scintillation counter as described elsewhere.

CsCl-EtBr gradients.

Ethidium bromide was added to the cleared lysate to a final concentration of 500 µg/ml, and then caesium chloride was added to give a final density of 1.580 g/cm^3. The density was usually adjusted either by weighing 100 µl samples in Eppendorf pipette tips or by checking the refractive index of the solution. The gradients (7 ml) were then centrifuged for 48-60 hours in a 50Ti rotor of a Beckman centrifuge at 36.000 rpm at 18°C. Ten drop

fractions were collected from the bottom of the tube, either by puncturing the tube or by pumping with a peristaltic pump.

When crude lysates were run on CsCl-EtBr gradients, the following protocol was used for the lysis :

A culture of 20 ml in minimal medium was grown in the presence of 1 µCi/ml ^{3}H thymidine (50Ci/mmol specific activity) and 150 µg/ml deoxyadenosine. The cells were harvested in late logarithmic phase and resuspended in 2 ml of TES buffer, containing 4 mg/ml lysozyme. After 15-30 minutes incubation at 37°C, 10% SDS was added dropwise until lysis was completed. To 1.5 ml of this lysate 3.3 ml H_2O, 2.0 ml EtBr (700 µg/ml in 0.1 M Na phosphate buffer pH 7.0) and 6.55g CsCl were added. Gradients of 7 ml were centrifuged and fractions collected as described above.

Removal of EtBr.

Ethidium bromide was removed either by dialysis against Dowex or by isoamyl alcohol extraction.

a) Dowex.

The DNA solution was dialysed against 20g Dowex Na^{+} (50W-X8, 20-50 U.S. mesh) in 100 ml buffer (0.8 ml NaCl, 0.05 M tris-HCl, 0.01 M EDTA, pH 8.0). The resin was prewashed with 2 M HCl, distilled H_2O, 1 M NaOH to convert to the Na^{+} form.

b) ISOamyl alcohol was saturated with water and added to equal volume of DNA solution and mixed gently. The isoamyl alcohol rapidly becomes red, and was pipetted off. This was repeated at least 5 times.

Partial purification of plasmid DNA for gel analysis. (Meyers et al., 1976).

A cleared lysate was prepared as described elsewhere, from 10-20 ml cells in late logarithmic phase. The volume of the lysate was doubled by adding distilled H_2O. One volume of tris (50mM) saturated phenol was added to remove protein. The tube was inverted gently several times and centrifuged at 10 krpm in a Sorvall SM-24 rotor for 30 minutes at 20°C. The aqueous phase was brought to 0.3 M sodium acetate, and twice the volume of cold (-20°C) ethanol was added. This was kept at -20°C for 2-3 hrs (or overnight, if more convenient). The precipitated DNA was recovered by centrifugation at 10 krpm in the Sorvall SM-24 rotor at -10°C for 20 minutes. The ethanol was thoroughly drained from the tube and the DNA was suspended in 50-100 µl TES buffer.

DNA electrophoresis on agarose gels.

a) Slab gels.

DNA samples (0.5-2 µg) were mixed with 5 µl loading buffer (50% glycerol, 50% gel buffer (0.04 M tris, 0.0 2 M NaAcetate, 1mM EDTA ; pH 8.2, using Acetic acid), 0.04% Bromophenol Blue) and desiccated under vacuum to ca

10-20 µl before loading on the gel, which was made as follows : One of the two clean 20 cm x 20 cm glass plates was coated with 0,1% agarose solution and allowed to dry. Plastic spacers and a slotforming comb were greased with soft paraffin and placed between the two glass plates, which were kept in place with crocodile clips. 0,7% agarose was refluxed in gel buffer for 10 minutes and cooled to 50°C before it was poured between the two glass plates and allowed to set with the slotforming comb down. When set, the gel was placed between the buffer reservoirs, which were filled with gel buffer. 3 mm Watman filter paper was used to connect the gel and the upper reservoir. The DNA samples were now loaded on, and the gel was run at 20 mA for 16-18 hours. The gel was then stained in 0,5 µg/ml EtBr in gel buffer for 30 minutes, rinsed once in water, and viewed under short wave ultraviolet light.

b) Tube gels.

If small amounts of DNA (0.1-0,5 µg) and short running times were required, then tube gels were used.

Glass tubes (15 cm x 0.5 cm) with parafilm covering one end were filled with 0.7% agarose. When the gel had set, small holes were punched in the parafilm. The sample well was made by inverting the tube until the gel glided out of the open end, and cutting ca 1 cm off the gel with a razor blade.

The tubes were now loaded with the DNA samples

and run at up to 5 mA per tube for 2 hours or more. If run overnight, then 0.75 mA were applied to each tube. The gels were then stained and viewed as slab gels.

The intensity of the bands was scanned in a Joyce-Loebl densitometer.

Radioactive labelling and counting.

All radioactive chemicals were obtained from the Radiochemical Centre, Amersham, England.

DNA was labelled by adding ^{3}H thymine, ^{3}H thymidine or ^{14}C thymidine to the growth medium, sometimes in the presence of deoxyadenosine. The specific activities of the label are indicated in the descriptions of the individual experiments. Samples were taken onto 3MM Watman filter paper disks, washed twice with 10% TCA (trichloracetic acid, BDH, Poole, Dorset), containing 100 µg/ml thymine or thymidine, and once with 80% ethanol and dried. They were then counted in 5 ml of a 0.5% solution of Butyl-PBD (2-(4'- tert-butylphenyl)-5-(4'-biphenyl)-1,3,4, oxadiazole) (Ciba, Cambridge, England) in a Packard Tri-Carb liquid scintillation counter. For discrimination counting between ^{3}H and ^{14}C, the scintillation counter was adjusted such that less than 0.1% of tritium spilled over into the ^{14}C channel. The spillover of ^{14}C into the ^{3}H channel was 20%.

When bands were cut out of a gel for counting, they were dissolved by shaking overnight in scintillation fluid

which was made up as follows : 720 ml toluene, 80 ml soluene, 2,88g PPO (2,5 Diphenyloxasole, (Packard)), 80,8 mg dimethyl POPOP (1,4-bis-(2-(4-Methyl-5-Phenyl-oxazolyl))-Benzene, (Packard)), 0.72 ml 10% SDS (sodium lauryl sulphate (BDH)). The vials were then cooled and counted in the scintillation counter.

Fractionation of cells in the zonal rotor.

a) Growth conditions.

A litre of cells was grown in M9 minimal medium, supplemented with 0.2% glycerol, 20 µg/ml histidine, leucine, cysteine and 20 µg/ml thymine, to a cell density of 10^8 cells/ml. The cells were then pulse labelled for 1 minute with ^3H thymidine, (0,5 µCi/ml, specific activity 50 µCi/mmol.). The label was chased for 5 minutes with 100 ug/ml unlabelled thymidine. During the chase the cells were also induced with 10^{-3}M IPTG for β-galactosidase production.

b) Termination of DNA and protein synthesis.

DNA synthesis was terminated by adding 20 µg/ml nalidixic acid. Protein synthesis was stopped by adding 200 µg/ml chloramphenicol, and penicillin was also added at 60 units/ml to prevent large cells from dividing after they had been applied to the rotor (Moore, 1976). The cells were then rapidly chilled by pouring the culture into centrifuge tubes containing ice.

c) The sucrose gradient.

A 5-20% sucrose gradient was made in the minimal salts medium with the relevant antibiotics added. The sucrose was pumped into the edge of the zonal rotor, which was spinning at 600 rpm, the light sucrose being pumped in first and gradually displaced towards the centre by heavier sucrose. As 5% sucrose was pumped into the rotor, 20% sucrose was drawn into the flask containing 5% sucrose, thus creating a gradient that was linear with respect to the radius of the rotor.

d) The zonal run.

The cells were suspended in 10 ml 3% sucrose in the minimal salts medium containing the antibiotics. The rotor was kept spinning at 600 rpm, and the cells were applied through the central feed and sealed with 50 ml overlay of distilled water. The gradient was then centrifuged at 4°C at 2500 rpm for 20-40 minutes, at which time it was decelerated to 600 rpm without braking. Fractions of 20 ml were collected from the centre by pumping 30% sucrose into the edge of the rotor. This was done slowly to avoid turbulence and viscous drag. The cells usually came into 15-20 fractions.

Preparation of synchronous cultures.

Synchronous cultures were made by fractionating life cells in the zonal rotor at 15°C and starting a culture from a fraction with small cells. The cells were suspended

in 100 ml prewarmed medium to a final concentration of $3x10^7$ cells/ml.

At 5-10 min. intervals 1 ml aliquots of cells were withdrawn from the culture and pulse labelled with ^3H thymidine (0.5 μCi/ml, specific activity 50 Ci/mmol) for 1 minute. The incorporation of label was stopped by adding 1 ml of 10% TCA with 100 μg/ml unlabelled thymidine on ice.

β-galactosidase was pulse-induced by withdrawing 1 ml from the culture at given time intervals and inducing for 5 minutes with 10^{-3} IPTG. The induction was terminated by adding 250 μg/ml chloramphenicol.

200 μl cells were put into equal amount of 20% formaldehyde in the minimal salts medium. This was kept for later counting.

β-galactosidase assay.

Synthesis of the enzyme was induced with IPTG (Sigma: isopropyl-β-D-thiogalactopyranoside) at 10^{-3}M final concentration.

The enzyme was assayed essentially according to Abbo and Pardee (1960).

Suitable aliquots of cells, diluted to 1 ml total volume, were added to a mixture of 0,8 ml of PMZ reducing buffer (below), 0.2 ml of CETAB (BHD, Poole, Dorset: Cetyltrimethyl-ammonium bromide) at 1 mg/ml and 10 μl of a 1% solution of sodium deoxycholate.

The PMZ reducing buffer was prepared as follows : Na_2HPO_4, 5,1g ; NaH_2PO_4, 16,8g ; H_2O, 950 ml ; 10^{-1} M $MgSO_4$, 10 ml ; 10^{-1}M $MnSO_4$, 2 ml ; B-mercaptoethanol, 6,8 ml ; H_2O to a final volume of 1 litre.

Samples were kept on ice for at least 30 minutes, or overnight at 4°C without loss of enzyme activity. For assay, samples were preincubated at 28°C for 5 minutes, and the reaction commenced by timed additions of 0,6 ml of ONPG (Sigma: O-nitrophenyl-β-D-galactopyranoside) prepared at 1/75 M in the minimal salts medium. When sufficient yellow colour had developed, the reaction was terminated with 1,3 ml of 1 M Na_2CO_3, and the time of incubation noted. The colour was stable for several hours, or overnight at 4°C. Cell debris was centrifuged off and the extinction at 420 nm was measured. Enzyme units were expressed in terms of E_{420}/ml of cells/minute of incubation.

3. Timing of F′lac replication during the cell cycle.

Introduction.

In the following experiments the timing of F′lac replication in the cell cycle was examined. Several groups have studied the timing of F in order to see whether it could be correlated to any other events of the cell cycle. This was done by measuring the rate at which a plasmid-coded enzyme could be induced, and assuming that this rate was proportional to the number of copies of the plasmid. Both synchronously dividing (Donachie and Masters, 1966; Nishi and Horiuchi, 1966) and asynchronous cultures (Zeuthen and Pato, 1971; Cooper, 1972; Davis and Helmstetter, 1973; Finkelstein and Helmstetter, 1977) have been used. The exponentially growing asynchronous cultures were separated into size classes by the membrane elution technique (Helmstetter, 1967). All these groups concluded that F′lac was replicated at a particular time in the cell cycle, but they disagreed as to when that time was. Recently Gustafsson et al. (1978), using exponentially growing cultures and a density transfer technique to measure F replication, concluded that the replication of F is not confined to cells of any particular age.

We pulse induced and pulse labelled exponential populations and separated them for analysis into age classes by centrifugation through a sucrose gradient in a zonal rotor (Moore, 1976). Using this technique we measured the rate of synthesis of plasmid coded β-galactosidase and also the amount of pulse label incorporated into covalently closed circular plasmid DNA.

The bacterial strain.

a) Construction of the strain.

The B/r strain used in these experiments was originally obtained from S. Glover and had had *leu*$^-$, *cys* B$^-$, *thy*$^-$ and *his*$^-$ mutations introduced by UV mutagenesis (Masters, 1970). Strains from this line of descent will be referred to as B/r E. This strain was made *lac*$^-$ by using o-nitrophenyl-β-D-thiogalactoside as a selective agent as described in Methods. The *lac* mutation was judged to be in the z gene by the inability of the strain to be induced by isopropylthiogalactoside (IPTG) to form β-galactosidase (see fig. 3-13). No band of covalently closed circular molecules was observed when DNA from a lysate of this strain was centrifuged in a CsCl-EtBr gradient and it was therefore judged to be free of plasmids (fig. 3-1). The strain was made resistant to T_6 phage, and an F′*lac* was transferred into it from a K12 strain using T_6 as counterselection. This *lac*$^+$ strain was shown to contain covalently closed circular DNA (fig. 3-1).

b) Molecular weight of F′*lac*.

Figure 3-2 shows a neutral sucrose gradient of B/r E *lac*$^-$/F′*lac*. ^{35}S labelled phage R17 with a sedimentation value of 80,5 S (J.Maule, pers. comm.) was used as a sedimentation marker. The S value of the F′*lac* is 90 S, from which the molecular weight can be calculated, using

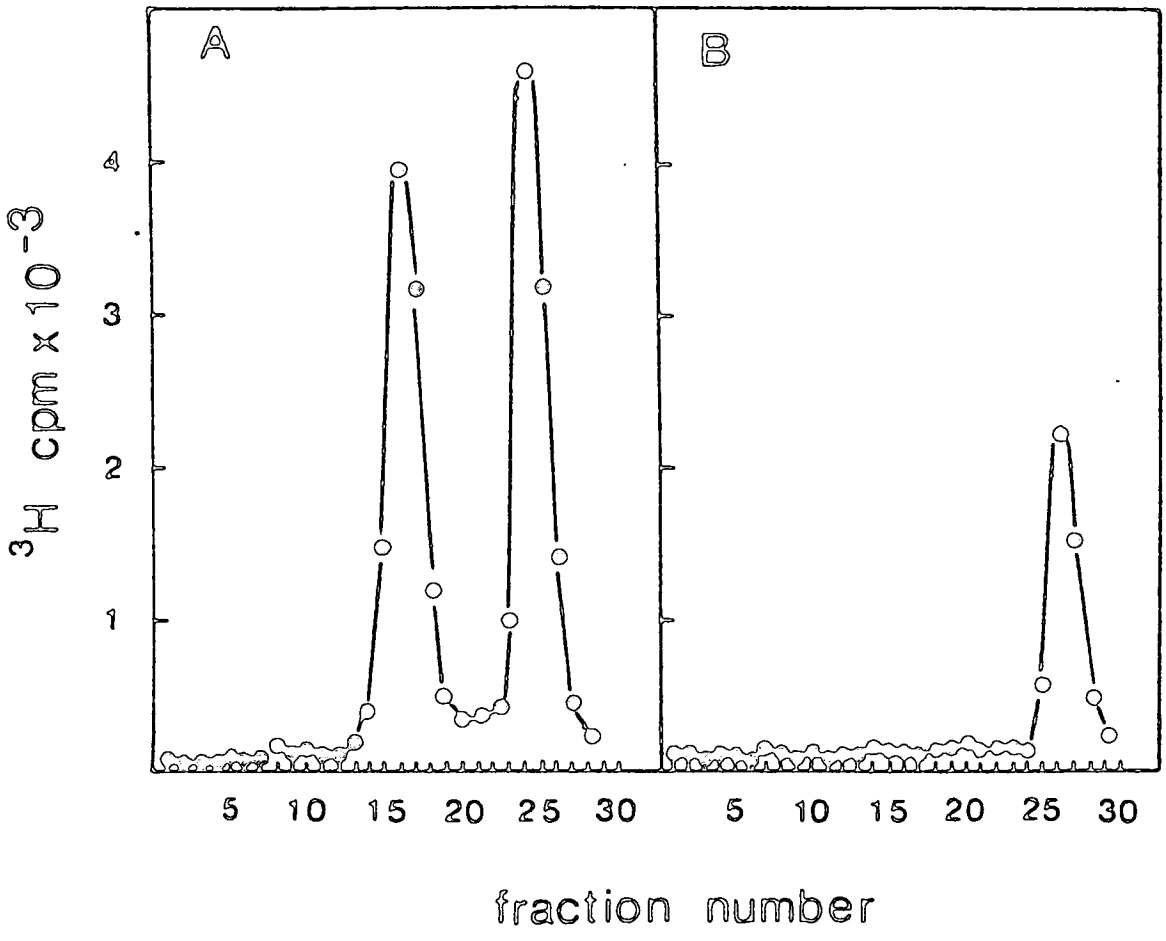


Fig. 3-1 Cleared lysates of B/r E lac$^-$/F$'$lac (A), and B/r E lac$^+$ (B) centrifuged in CsCl-EtBr gradients. The cells were labelled with ^{3}H thymidine (0.5 μCi/ml, 50 Ci/mmol), cleared lysates were prepared as described in Methods, and centrifuged in CsCl-EtBr in a 50Ti rotor of a Beckman L2-50 centrifuge at 34 krpm and 18°C for 48 hours. Ten drop fractions were collected and counted.

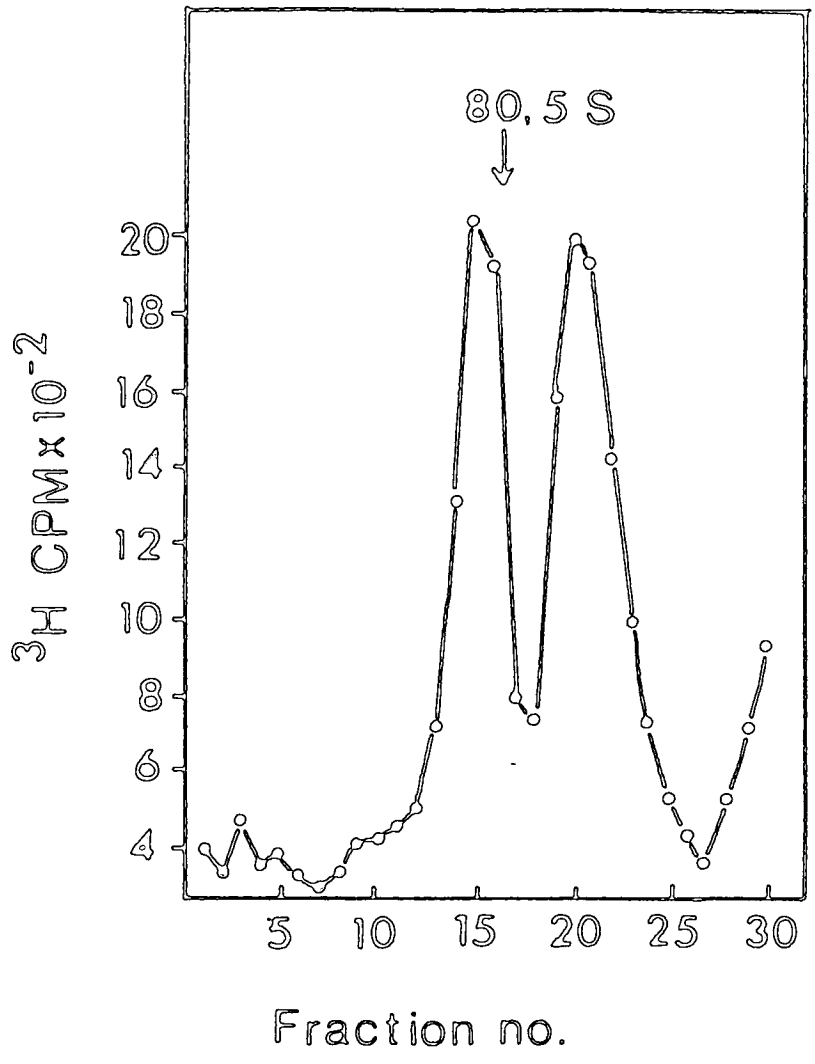


Fig. 3-2 Lysate of B/r E lac$^-$ / F′lac centrifuged on a neutral sucrose gradient. The cells were grown in the presence of 10 μCi/ml ^{3}H thymine for 3 generations. A cleared lysate was prepared and 0,3 ml put on top of a 5-20% neutral sucrose gradient. ^{35}S labelled phage R17 was added as a sedimentation marker (80,5S). The gradient was centrifuged at 49 krpm at 4°C for 60 minutes in an SW50.1 rotor of a Beckman L 2-50 centrifuge. Ten drop fractions were collected from the bottom of the tube and counted.

the following formula :

$$S_{lin} = 0,082M^{0,346}$$

where S_{lin} is the sedimentation value of linear molecules and the sedimentation value of covalently closed molecules is $S_{ccc} = S_{lin} \times 1,8$, M=molecular weight . (N.Willetts, pers.comm.). This calculation gives a molecular weight of ca. 90×10^6 daltons for F'lac.

Growth conditions.

A litre of cells was grown in M9 minimal medium supplemented as described until a cell density of 10^8 cells/ml was reached. The generation time of the cells was 58 minutes in this medium. . The cells were harvested at a cell density of 10^8 cells/ml. DNA synthesis was terminated by adding 20 µg/ml of nalidixic acid to the culture. Fig. 3-3 shows CsCl-EtBr gradients of cell lysates ; one aliquot of cells had been treated with 20 µg/ml nalidixic acid prior to labelling, while the other had not. It can clearly be seen that both chromosomal and plasmid replication is inhibited by this concentration of nalidixic acid.

Protein synthesis was stopped by adding 200 µg/ml of chloramphenicol to the culture, and 60 µg/ml of penicillin was also added to prevent large cells from dividing after they had been applied to the rotor.

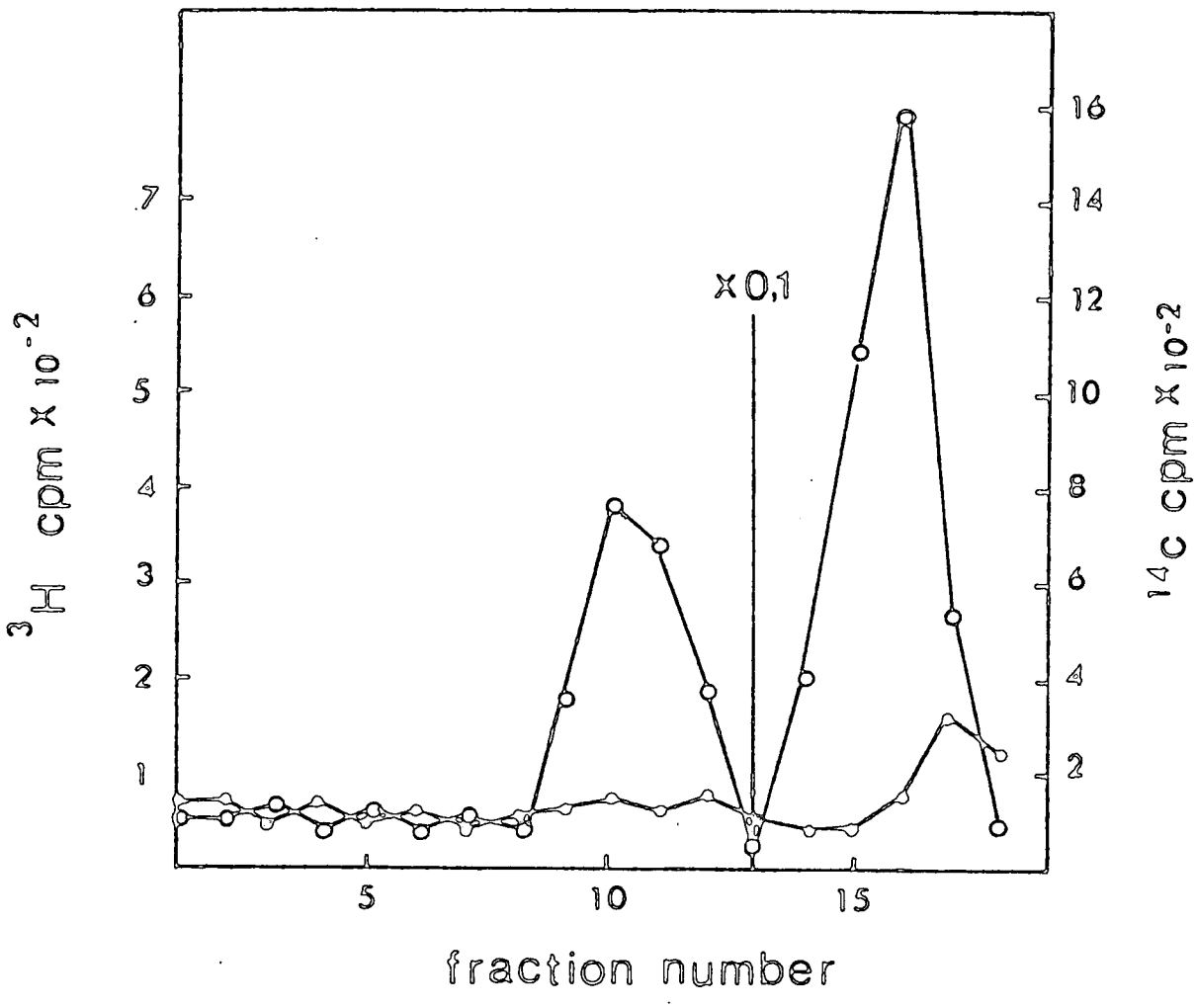


Fig. 3-3 Effect of nalidixic acid on plasmid and chromosome synthesis. A 20 ml culture of B/r E lac$^-$/ F'lac was grown to OD_{540} 0,1. An aliquot of 10 ml was pulse labelled with 0,1 µCi/ml ^{14}C thymidine for 5 minutes, 10 ml 20 µg/ml nalidixic acid was added to another aliquot of 10 ml prior to pulse labelling it with ^{3}H thymidine (1µg/ml, 50Ci/mmol) for 5 minutes. The cells were then lysed with lysozyme-SDS and the lysates centrifuged in CsCl-EtBr for 48 hours in a 50Ti rotor of a Beckman L2-50 centrifuge at 36 krpm and 18°C. Open circles (o) are ^{14}C counts from the lysate which had not been treated with nalidixic acid, closed circles (o) are ^{3}H counts from the lysate which had been treated with nalidixic acid prior to labelling.

Residual DNA synthesis in B/r E $lac^-/F'lac$.

The time it takes the chromosome to replicate was measured as illustrated in fig. 3-4 by adding rifampicin to a culture of B/r E $lac^-/F'lac$ which had been growing exponentially in the presence of ^3H thymine for 3 generations, and the uptake of thymine into TCA precipitable material was followed. DNA synthesis continued for about 40 minutes, and the total increment, ΔG, was about 30%.

The increment of DNA synthesis after blocking protein synthesis with rifampicin is assumed to be due to the completion of rounds of replication that were initiated before the addition of rifampicin, since protein synthesis is needed for the initiation of replication (Maaloe and Hanawalt, 1961). It can be seen in fig. 3-4 that the cells stop growing immediately after the addition of rifampicin. The cells go on dividing for a while, as expected (Jones and Donachie, 1973).

The C time can be calculated from ΔG by using the following relation (see D. Martin, 1970):

$$\Delta G = \left(\frac{2^n \times n \times \ln 2}{2^{n-1}} - 1\right) \times 100$$

where $n = C/T$, and T is the generation time.
This gives a C time of around 45 minutes

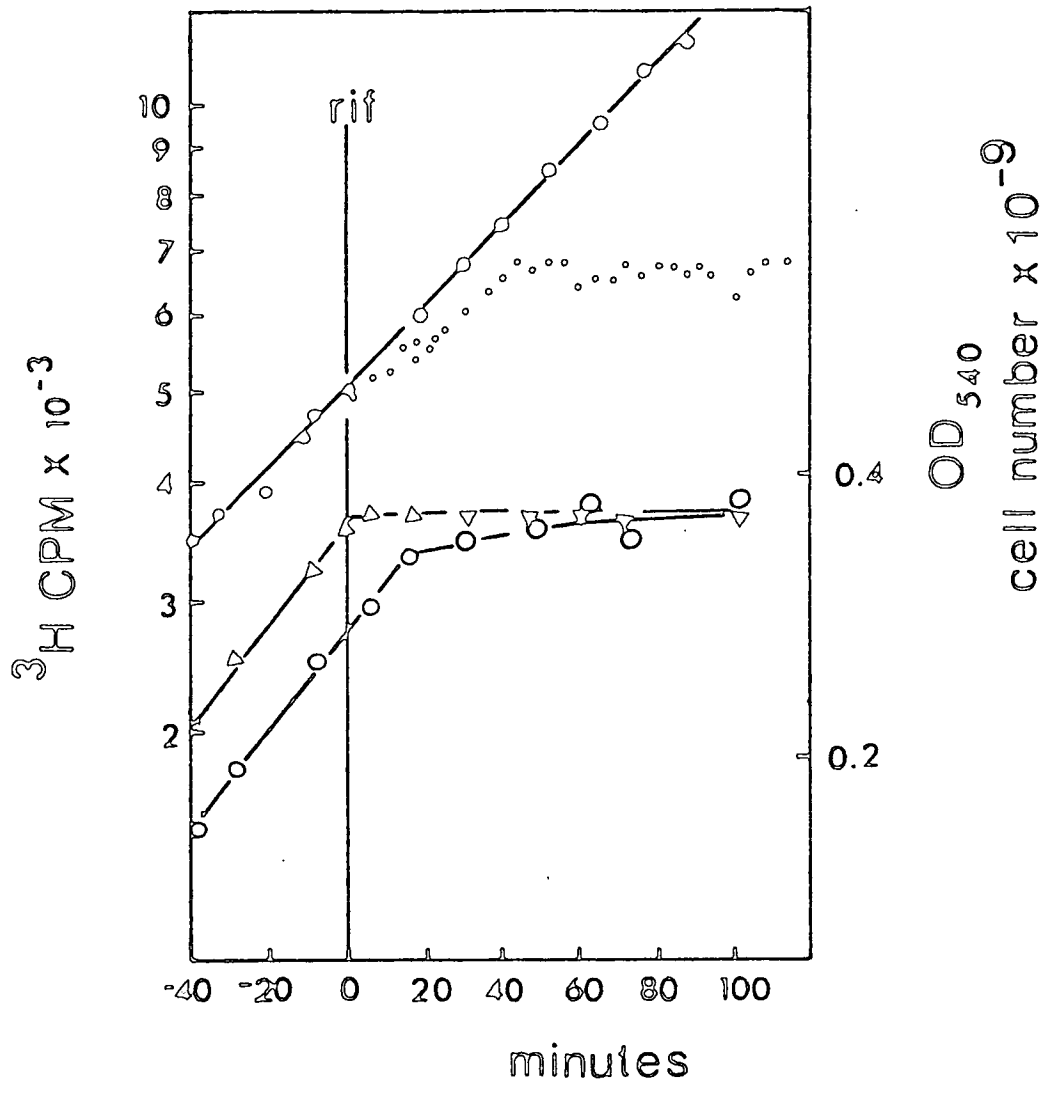


Fig. 3-4 Residual DNA synthesis in B/r E lac$^-$/F′lac.
A culture of exponentially growing cells was labelled with 10 μCi/ml ^{3}H thymine for 3 generations, and incorporation of label into TCA precipitable material (○), OD_{540} (▽) and cell number (O) were measured at intervals. At OD_{540} = 0,35, the culture was divided into two, and 150 μg/ml rifampicin was added to one aliquot. OD_{540} (▽), cell number (○) and incorporation of ^{3}H into TCA precipitable material (◦) was measured after addition of rifampicin, and the incorporation of ^{3}H into TCA precipitable material was measured in the culture that had not had rifampicin added.

Separation of cells into size classes.

The cells were separated according to size on a sucrose gradient in a zonal rotor. The cells generally appeared in twelve to twenty of the 65x20 ml fractions collected (see Methods).

The size of the cells from each fraction was analysed both in the Coulter Counter and microscopically. Fig. 3-5 shows the size distribution in exponentially growing cultures of B/r E *lac*$^+$ and B/r E *lac*$^-$/F'*lac* , and fig. 3-6 shows the size distribution in a number of fractions from the gradient after fractionating an exponentially growing culture of B/r E *lac*$^-$/F'*lac* in the zonal rotor. The growth of the cells is generally assumed to occur lengthwise (see Donachie et al., 1976), increase in the volume of the cells as measured in the Coulter Counter can therefore be regarded as increase in length. The size of the cells will here be referred to as units measured by the Coulter Counter. The smallest cells were 9 units, which corresponds to 1,15 μ^3 . The size classes differ therefore by 1/9 of the minimal cell length. It can be seen in fig. 3-6 that the small cells are in the top fractions and the large cells in the bottom fractions. The small cells are quite homogenous in the top fractions, but there is more heterogeneity in the cell size in the bottom fractions. In fig. 3-7 every cell size from 9 to 33 is taken separately and its proportion in the fractions from the gradient calculated. It can be seen

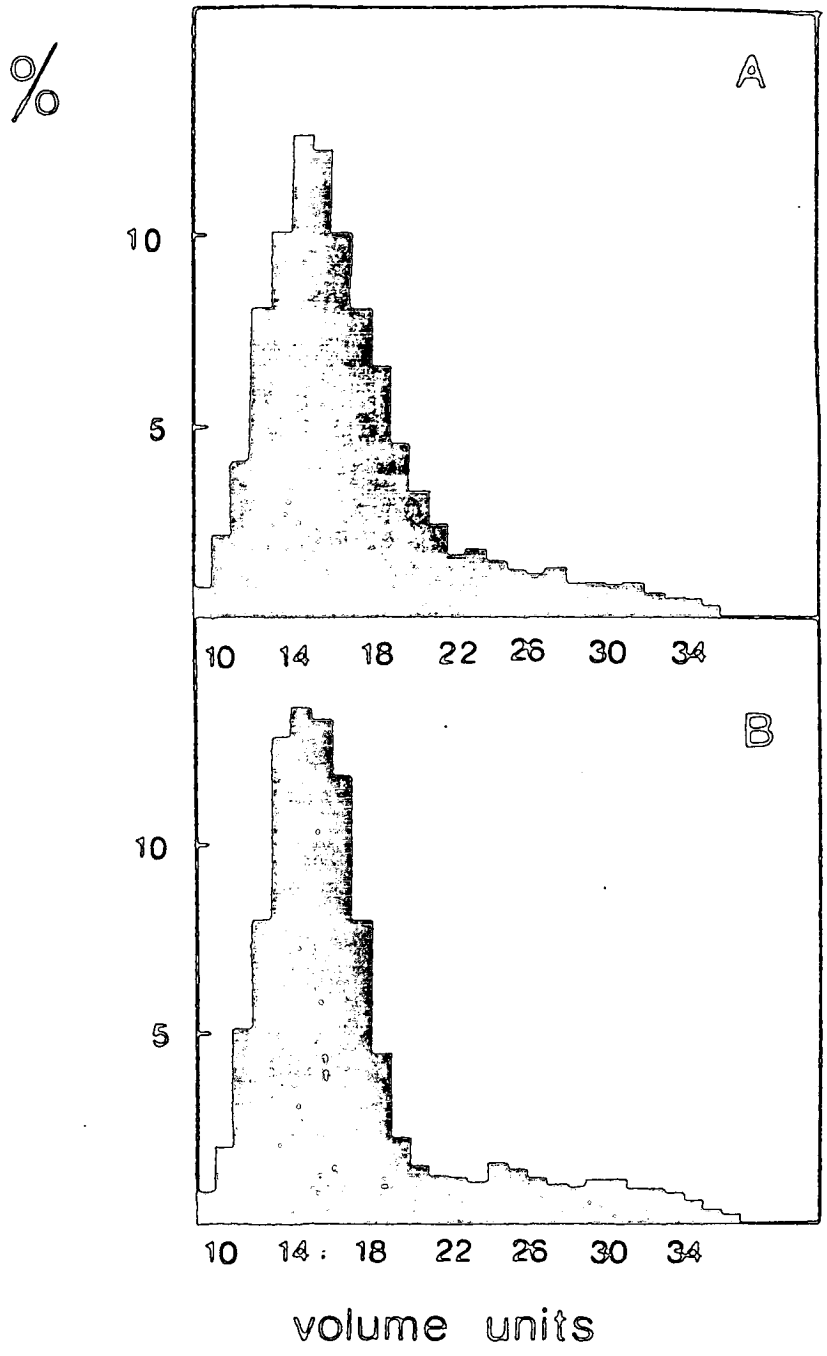


Fig.3-5 Histograms showing cell volume distributions in exponentially growing cultures of B/r E *lac*$^-$/F'*lac* (A), and B/r E *lac*$^+$ (B). The cell volume was determined using a Coulter Counter (Model 2B) and Channelizer. The volumes are expressed in relative units.

Fig. 3-6 Histograms showing cell volume distributions in six different fractions from the zonal rotor (fractions number 3, 5, 8, 12, 15 and 17), after fractionation of an exponentially growing culture of B/r F lac$^-$/ F'lac. The volumes were determined as in fig. 5.

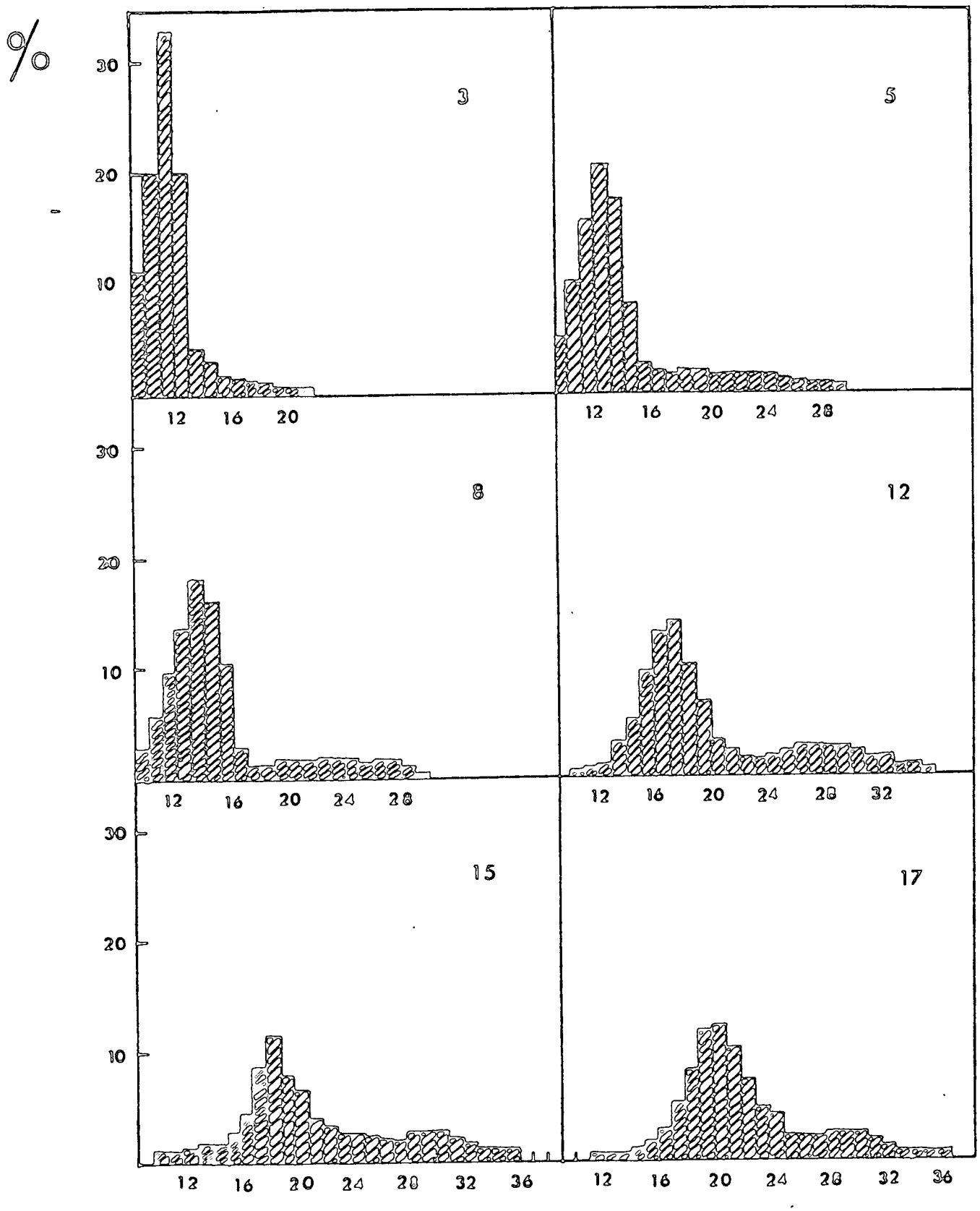
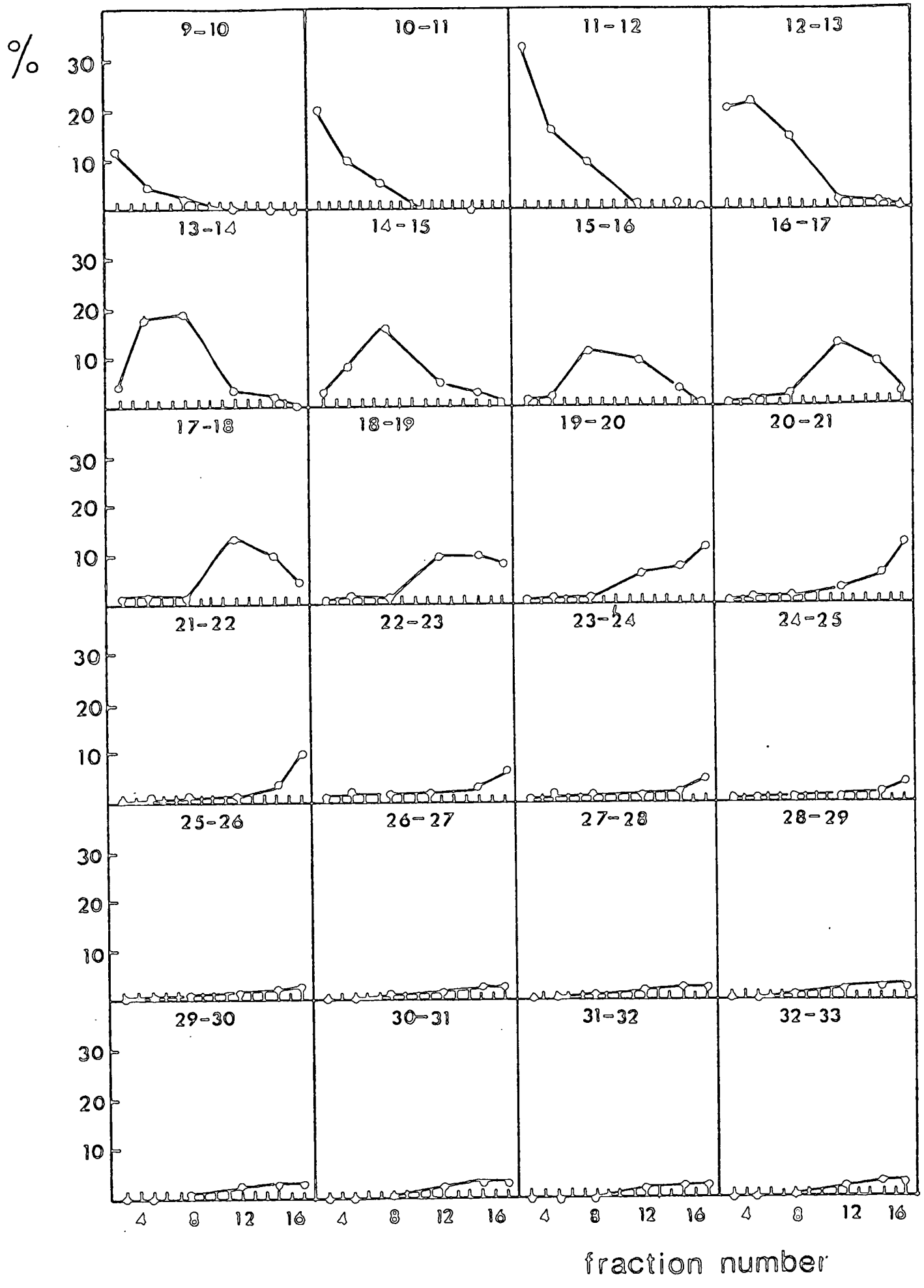

%
30
20
10
3
12 16 20
5
12 16 20 24 28
8
12 16 20 24 28
12
12 16 20 24 28 32
15
12 16 20 24 28 32 36
17
12 16 20 24 28 32 36
volume units

Fig. 3-7 The percentage of a given cell size in the fractions from the gradient. This is plotted for every cell size class from 9 to 33.


%
9-10
10-11
11-12
12-13
13-14
14-15
15-16
16-17
17-18
18-19
19-20
20-21
21-22
22-23
23-24
24-25
25-26
26-27
27-28
28-29
29-30
30-31
31-32
32-33
30
20
10
4
8
12
16
fraction number

that while the smallest cells are mostly confined to two or three fractions, as the cells get larger, they are more dispersed over the gradient. However, there is never less than a ten fold difference between the proportion of a given size in the fraction where it is most frequent and in the fraction where it is least frequent. Fig. 3-8 illustrates how the median volume of the cells varies across the gradient. The median volume increased continuously, but the increase was never more than 60 - 80%. The fraction of cells with septa was estimated microscopically for each fraction and it can be seen that the septa appear in fractions 10-13. Fig. 3-9 shows the steps that can be expected to be seen as a result of doublings in e. g. enzyme inducibility in a given size class, when the heterogeneity in cell size in each fraction is taken into account. This is plotted for every size class from 11 to 22. It is evident that doublings which occur very early or late in the cell cycle are not as prominent as those which occur in other cell sizes. It can be concluded that while the size separation is not perfect, it should be good enough for temporal events in the cell cycle to be detected.

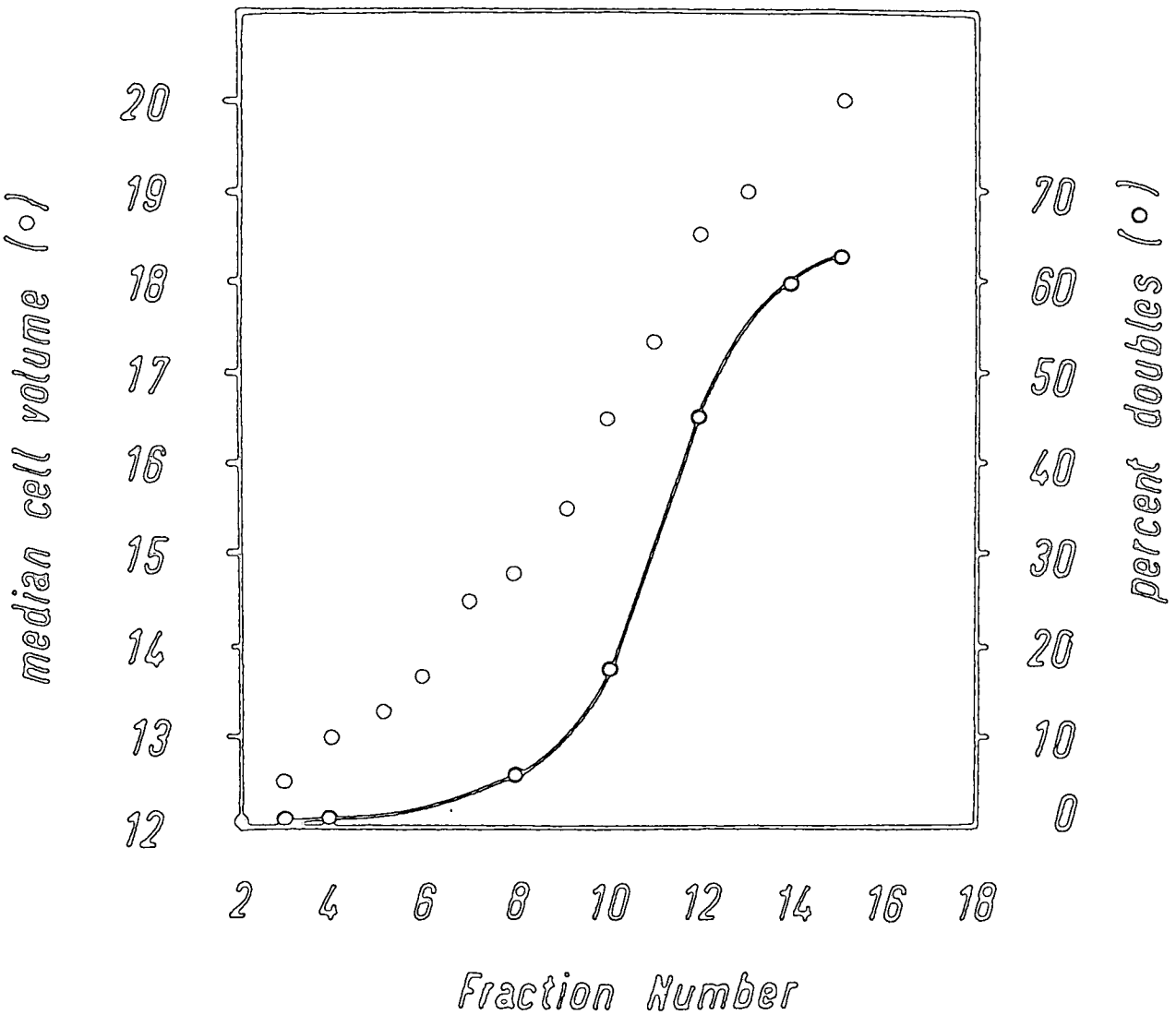
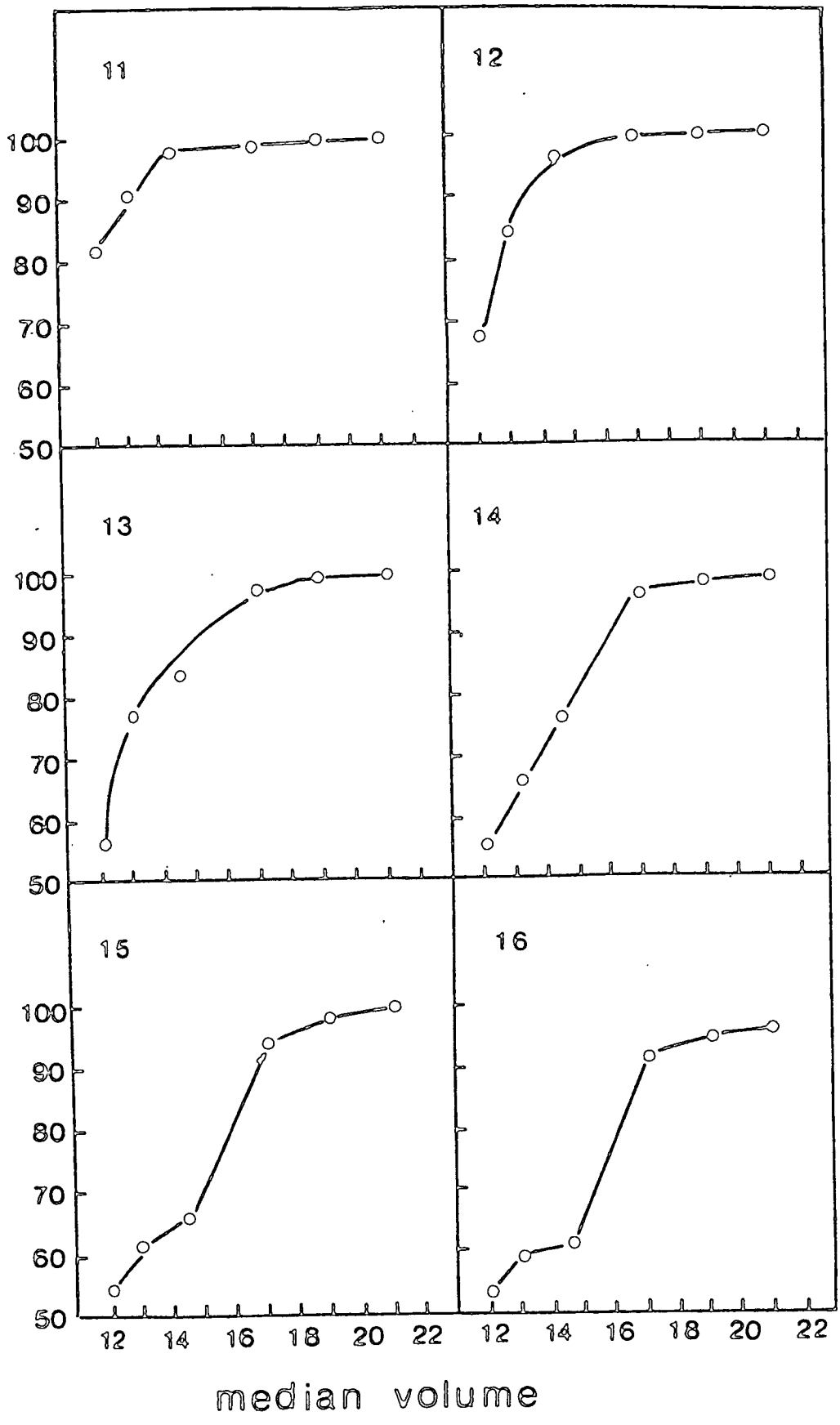
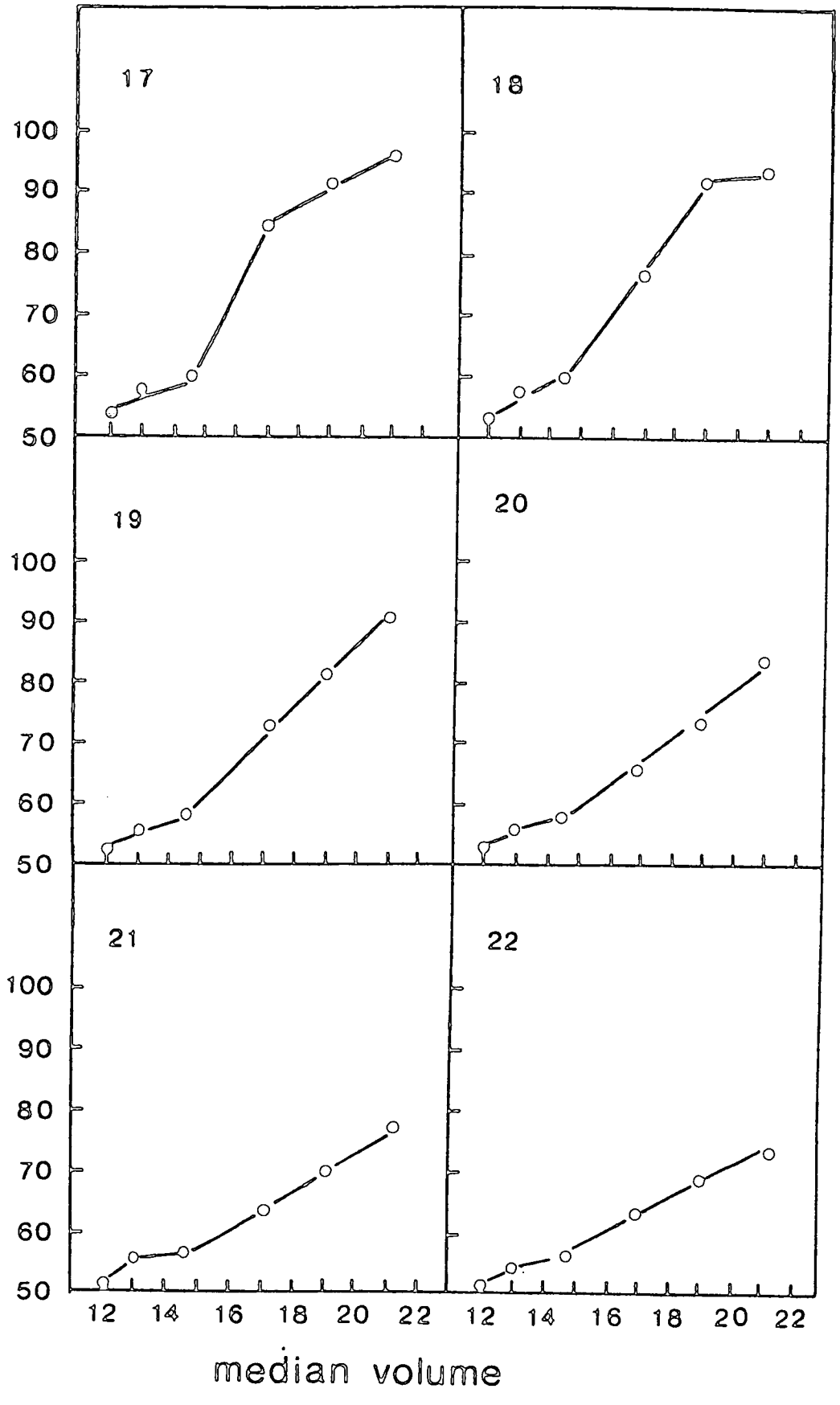


Fig. 3-8 Median cell volumes (○) and percent double cells (○) in the fractions from the zonal rotor. The cell volumes are expressed in relative units measured by the Coulter Counter and Channelizer. The fraction of cells in each sample exhibiting a septum was determined microscopically using a Petroff - Hauser Cell Counting Chamber.

Fig. 3-9 Steps which can be expected to be seen as a result of doublings in e.g. enzyme inducibility in a given size class when the heterogeneity in cell size in each fraction is taken into account. This is plotted for size classes 11-22.


11
12
13
14
15
16
100
90
80
70
60
50
12
14
16
18
20
22
median volume


17
18
19
20
21
22
100
90
80
70
60
50
12
14
16
18
20
22
median volume

Correlation between cell size and cell age.

Synchronous cultures.

a) Cell division.

In order to find the cell age of a given fraction from the zonal rotor, synchronous cultures were grown from fractions 2 and 8, median volumes 12 and 16 respectively (fig. 3-10). These cells had not been treated with antibiotics, and the zonal run was done at 15°C. Although the culture which was started from the cells in fraction 8 is less synchronous than the one from fraction 2, it clearly divides much sooner than the other. In fact, cells with a median volume of 12 units grow for 60 minutes before they divide, but cells with an average volume of 16 units only take 32 minutes before they divide. The size difference of 4 units therefore corresponds to a difference of 28 minutes.

The growth of a cell is probably very nearly exponential with time (Donachie et al., 1976). Fig. 3-11 shows exponential relationship between cell volume and age. (time), assuming a newborn cell has a volume of 11 units, and the generation time is 58 minutes. The observed age difference of 28 minutes corresponds closely to the expected 24 minutes.

b) Cell cycle markers.

Synchronous cultures were started from small cells and at intervals of several minutes the incorporation

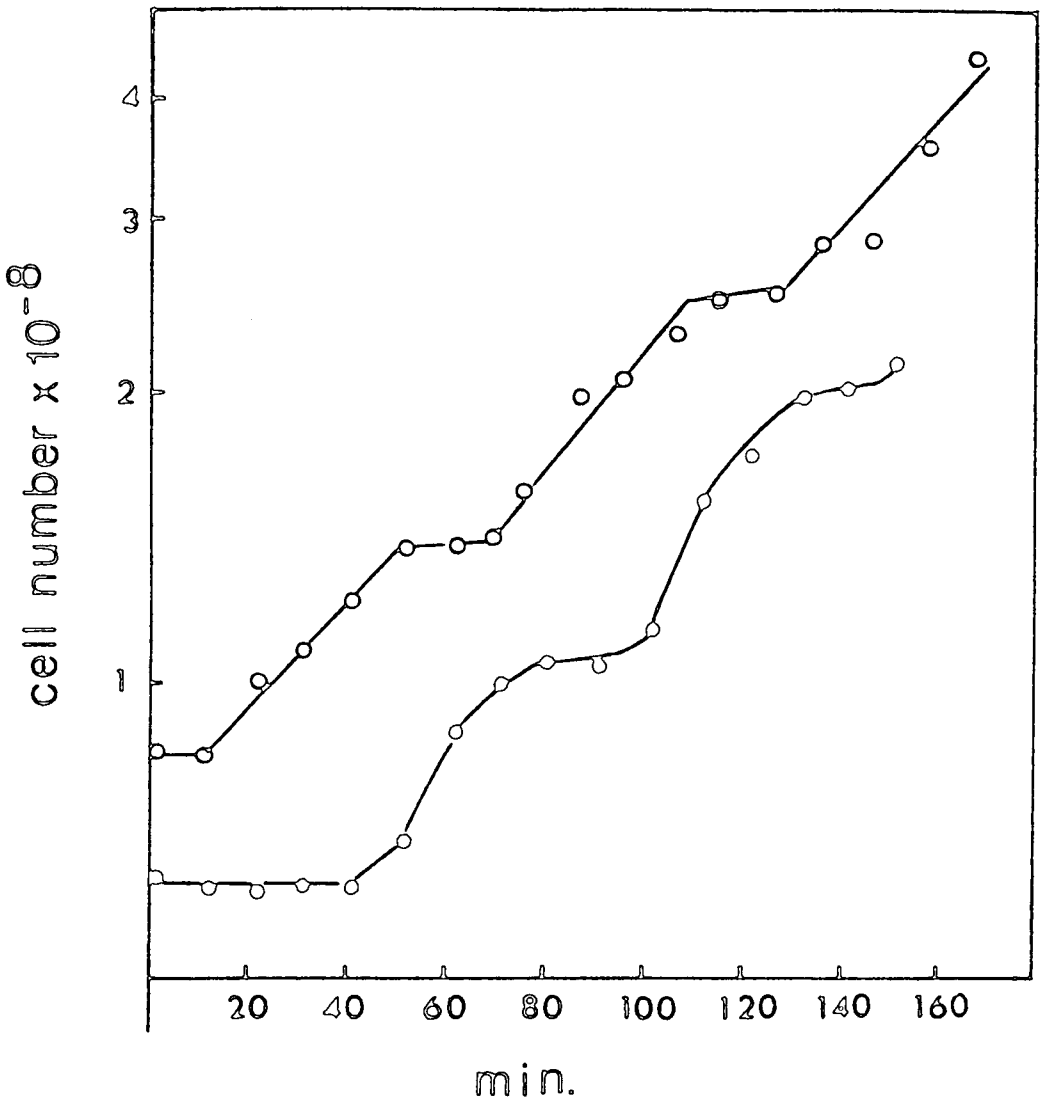


Fig. 3-10

Cell counts of synchronously growing cells from fractions 2 (o) and 8 (o) from the zonal rotor. The cells were diluted into prewarmed medium as described in Methods, and samples were withdrawn at 10 min. intervals for counting.

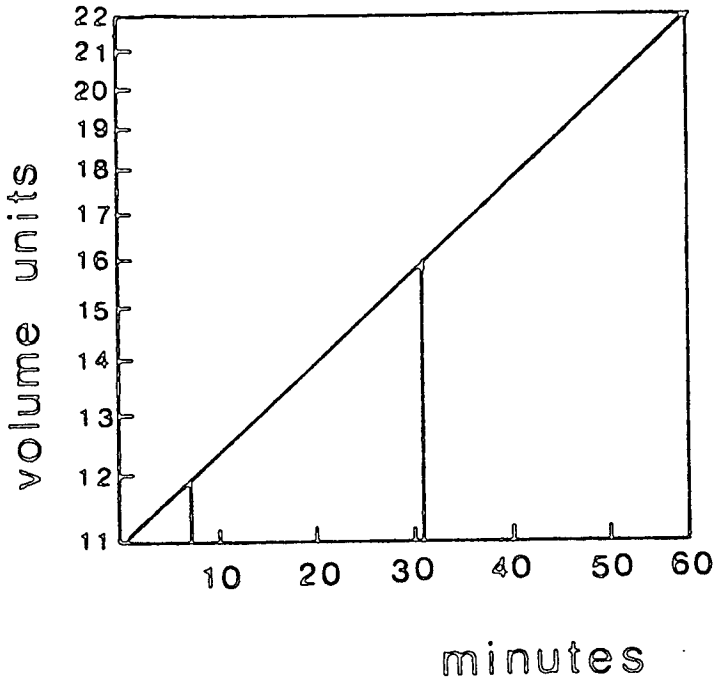


Fig. 3-11 Exponential relationship between cell volume and age (time) Size difference of 4 units corresponds to an age difference of 24 minutes.

of ^{3}H-thymidine during 1 min. pulses and the amount of β-galactosidase after 6 min. pulse inductions were measured. The results are shown in fig. 3-12. It can be seen that DNA synthesis doubles in rate once per cell cycle and shows the decrease in rate characteristic of long generation times in which synthesis is discontinuous (Cooper and Helmstetter, 1968).

The initial rate of synthesis of β-galactosidase after induction has been used to measure the number of copies of the gene specifying the enzyme in most previous studies of F'*lac* replication during the cell cycle. The presumption is that the rate at which the enzyme can be induced in a particular cell is proportional to the number of gene copies in that cell and will remain constant until the gene is duplicated. If the gene is duplicated simultaneously in all cells in a culture the rate at which the enzyme specified can be induced will double at the time of duplication. If the halfrise time is taken to represent the actual time in the cell cycle where the change in rate occurred, the first cell division takes place after 59 minutes, which is very close to a generation time, and DNA initiates at 28 minutes in the cell cycle, or at age ca 0,5. It may be seen (fig. 3-12) that the initial rate at which β-galactosidase can be induced in the synchronous culture is very low. This depressed inducibility is characteristic of cells recently centrifuged through sucrose solution (Donachie and

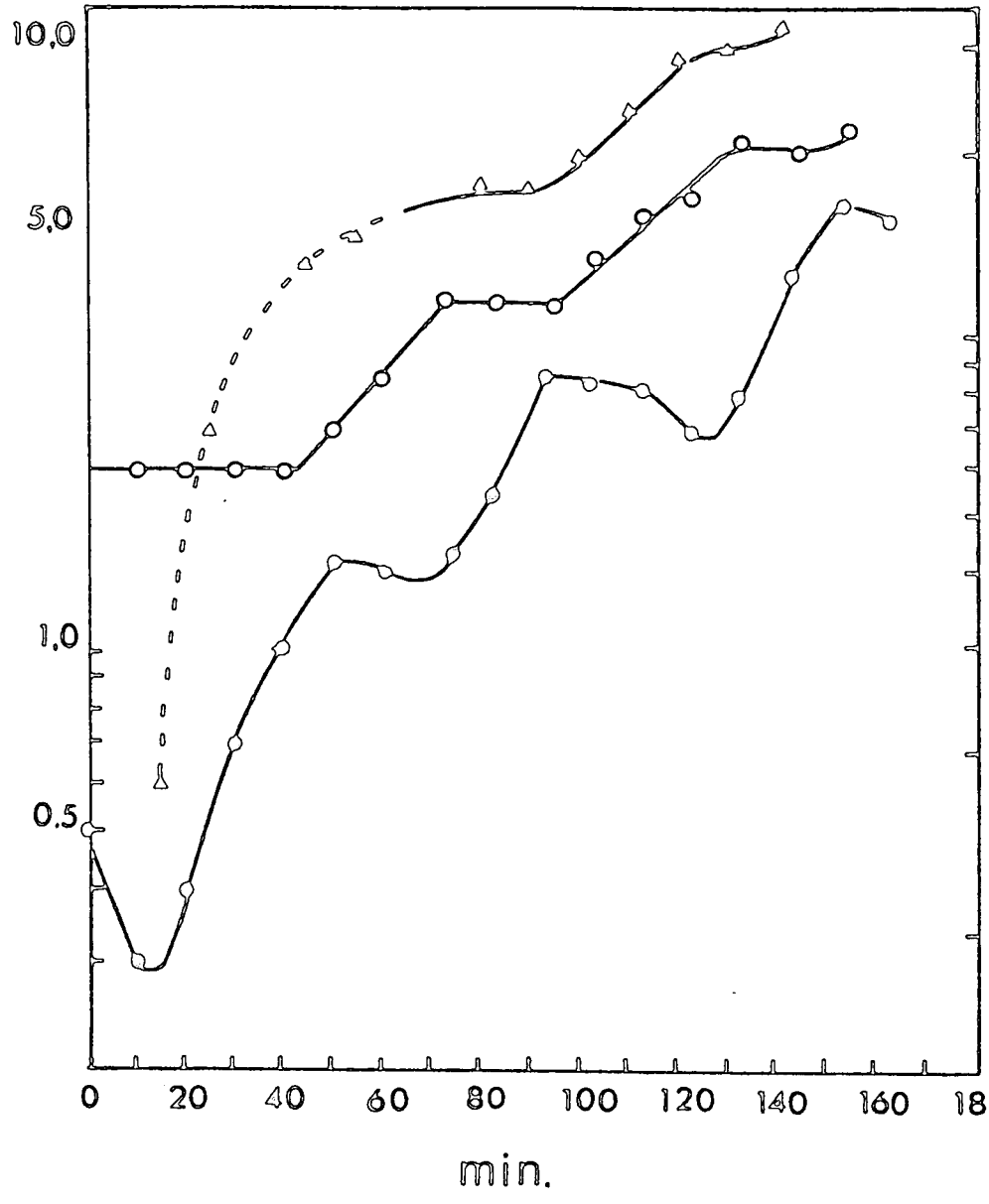


Fig. 3-12 DNA and β-galactosidase synthesis in a synchronous culture of *E. coli* B/r F *lac*$^-$/F′*lac*. Cells from fraction 2 in the zonal rotor were diluted into prewarmed medium. At 10 min intervals several 1 ml aliquots of cells were withdrawn from the culture. To measure DNA synthesis one of these aliquots was pulse labelled with ^{3}H thymidine (0,5 µCi/ml, 50 Ci/mmol) for 1 min. before adding 1 ml 10% cold TCA containing 100 µg/ml unlabelled thymidine. β-galactosidase was induced by adding 10^{-3}M IPTG to a second 1 ml aliquot. Induction was terminated after 5 min. by adding 250 µg/ml chloramphenicol. A third aliquot was preserved in 10% formaldehyde for later counting of cells. Radioactivity (○), enzyme (△) and cell numbers (○) in each sample were estimated as described in Methods.

Masters, 1966), and obscures any changes in inducibility due to gene dosage that may be expected to have occurred during the first cell cycle after synchronization. Inducibility is soon recovered, however, and a stepwise doubling in the rate at which the β-galactosidase can be induced is evident at about the time of the second cell division. This suggests that a stepwise doubling of the genes coding for β-galactosidase has occurred at this time, presumably because F′lac has replicated simultaneously in all cells.

Thus synchronous cultures of B/r E lac⁻/F′lac behave similarly to those of strains studied previously (Donachie and Masters, 1966 ; Nishi and Horiuchi, 1966) in that the rate at which β-galactosidase synthesis can be induced doubles suddenly, once per cell cycle.

Exponential cultures.

a) Rate of DNA synthesis.

The rate of DNA synthesis in cells of different sizes was measured for both B/r E lac⁺ and B/r E lac⁻/F′lac. This was done by pulsing an exponentially growing culture with ^{3}H-thymidine for 1 minute. The pulse was terminated by a chase with cold thymidine. There is no detectable thymidine pool in the strain as fig. 3-13 shows. Five minutes later a mixture of antibiotics was added to stop all macromolecular synthesis and the cells were collected and separated in the zonal rotor. It can be seen (fig.3-14)

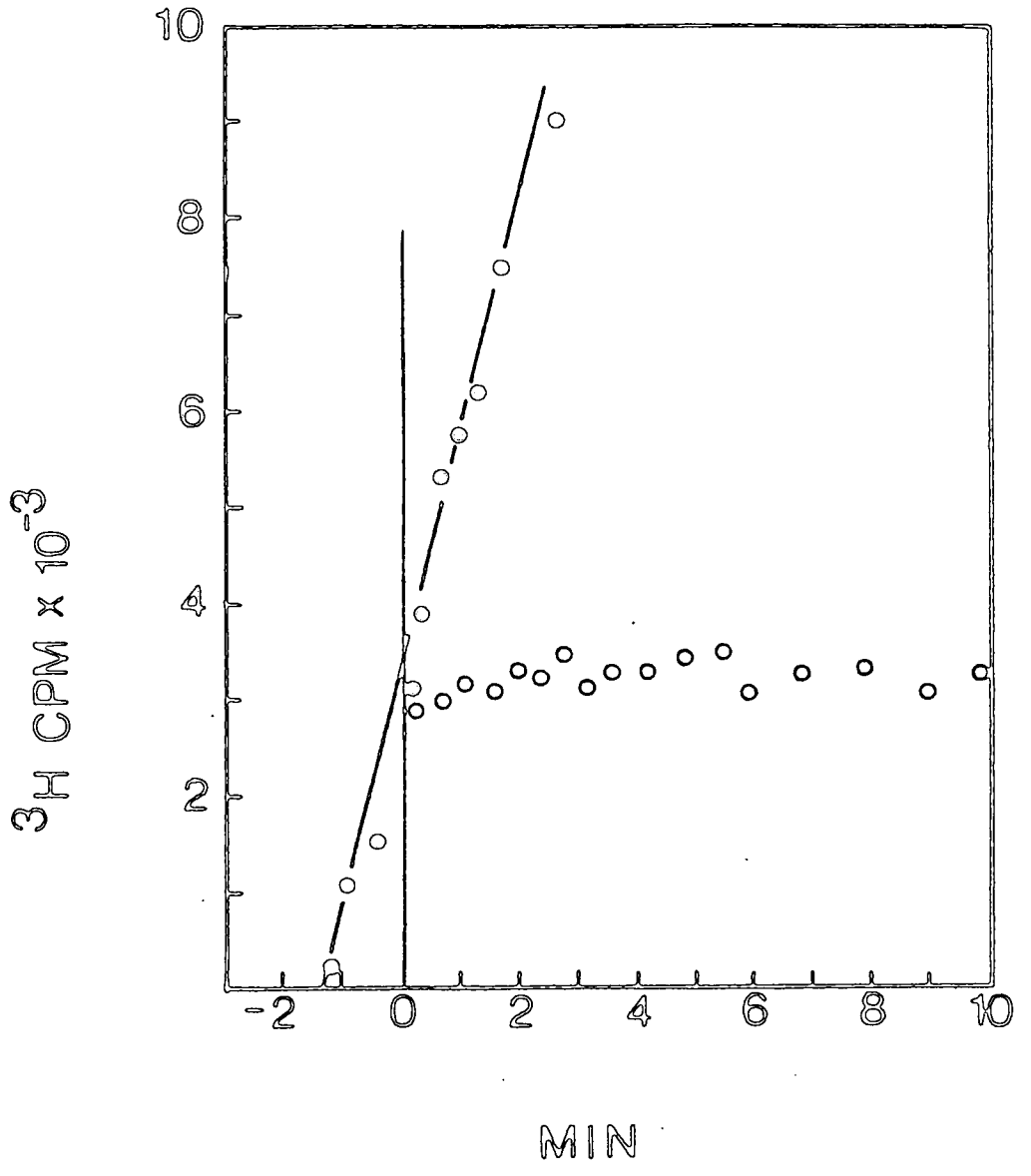


Fig. 3-13 Thymidine pool in B/r E lac$^-$/F'lac . Cells were pulse labelled with ^{3}H thymidine (0,5 µCi/ml, 50 Ci/mmol) for two minutes, then 100 µg/ml unlabelled thymidine were added to half the culture. Incorporation of ^{3}H into TCA precipitable material was followed before (o) and after (o) addition of unlabelled thymidine.

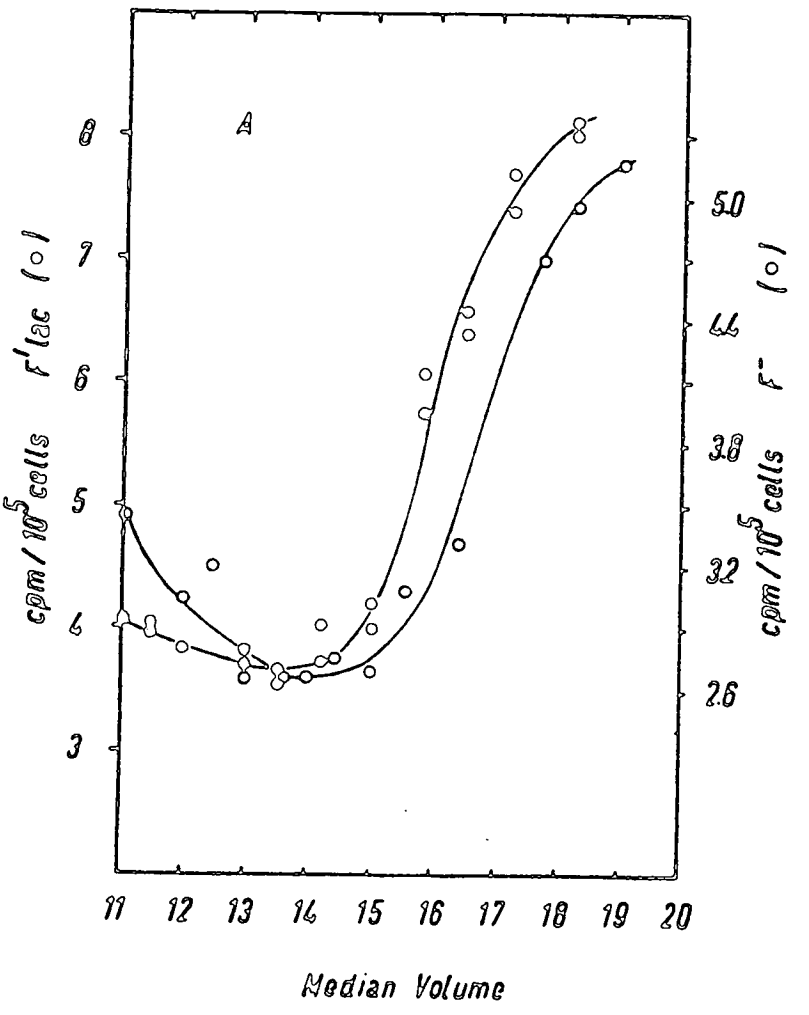


Fig. 3-14 Rate of DNA synthesis in B/r F lac$^+$ (○) and B/r F lac$^-$/F'lac (○). ^{3}H - thymidine (0,5 µCi/mmol) was added to exponentially growing cells of each of the strains. After one minute 100 µg/ml unlabelled thymidine was added. After a further five minutes a mixture of antibiotics was added and the cells harvested and fractionated as described in Methods.

that the rate of DNA synthesis approximately doubles at a median volume of 16 or 17 units. This volume corresponds to 26 and 31 minutes from cell birth when the 5 min. cold chase has been corrected for and it is assumed that the size of a newborn cell is 11 units and the cells grow exponentially (fig. 3-15). The time of initiation of the DNA synthesis did vary between 26 and 33 minutes from experiment to experiment. This time of DNA initiation agrees closely with the results from the synchronous culture and is somewhat earlier in the cell cycle than that predicted for cells with a generation time of 58 minutes (Cooper and Helmstetter, 1968), and may reflect a difference between substrain E and other substrains of B/r (Helmstetter and Pierucci, 1976). The time of termination cannot be unequivocally determined from these experiments, but if the C time is 45 minutes, as the residual DNA synthesis experiment described above suggests, then termination occurs 13 to 20 minutes after division.

b) Rate of synthesis of β-galactosidase.

The inducibility of β-galactosidase and the rate of DNA synthesis was determined for both B/r E *lac*$^+$ and B/r E *lac*$^-$/F′*lac*. IPTG, an inducer of β-galactosidase was added together with ^{3}H-thymidine to exponentially growing cultures prepared as described above. After one minute pulse labelling was terminated by the addition of excess of cold thymidine. After a further five minutes

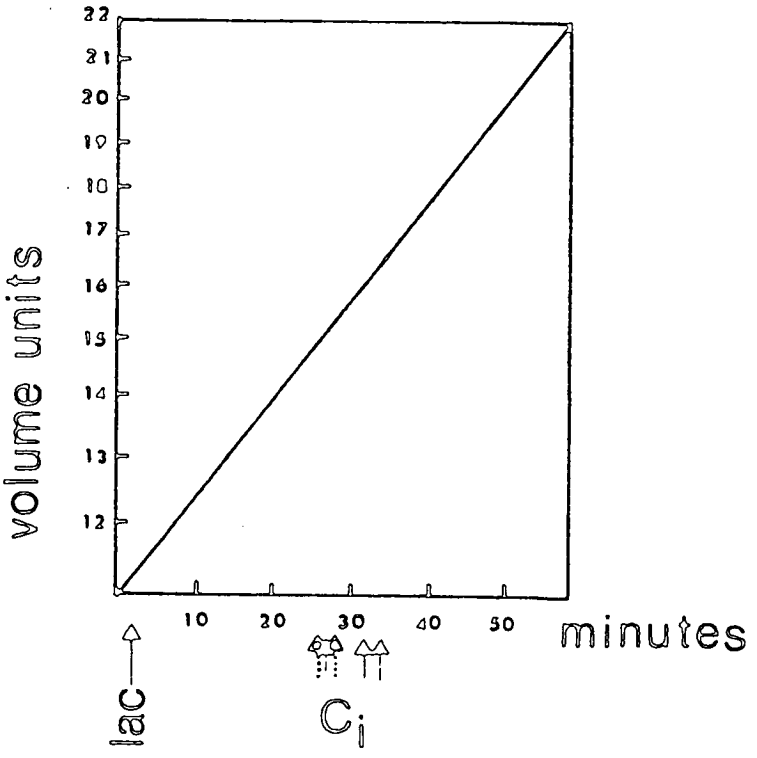


Fig. 3-15 Time of chromosome initiation and replication of the lac genes in cells that grow exponentially from 11 to 22 units. The initiation of DNA replication was variously found to be at the volume of 16, 17 and 17 1/2 units. After correction for the 5 minutes cold chase, this corresponded to 26 to 33 minutes. Open arrows represent results from synchronous cultures, while filled arrows represent results from exponential cultures fractionated in the zonal rotor. The β-galactosidase inducibility was found to double at to volume of 12 units, and after correction for the initial lag in inducibility, this was found to correspond to a time just after division.

chloramphenicol was added to terminate induction and the cells were harvested and centrifuged in the zonal rotor. Fig. 3-16 shows a β-galactosidase induction curve for B/r E *lac*$^+$, B/r E *lac*$^-$ and B/r E *lac*$^-$/F′*lac*. There is an initial lag of 3-5 minutes before the β-galactosidase starts to be produced. This is in accordance with the findings of others (Pardee and Prestidge, 1961). The mid-point of the actual time of β-galactosidase production can therefore be assumed to be at 4-5 minutes after the addition of inducer. Figure 3-17 shows the way in which enzyme inducibility varies as a function of cell size in B/r E *lac*$^+$ and B/r E *lac*$^-$/F′*lac*. The step in the induction curve for B/r E *lac*$^+$ should be displaced 4 or 5 minutes to the left, or to a time just after division. The *lac* genes are located at 8 minutes on the *E. coli* map, or 50% of the distance between origin and terminus (Bachmann et al., 1980). They will therefore be expected to replicate at or just after division in cells that have the volume of 22 units when they divide (see fig. 3-15).

In contrast, however, the amount of enzyme induced in the F′*lac* cells increases continuously with increasing cell volume, and there is not a prominent step in the induction curve.

Direct measurement of F′lac DNA.

It was decided to measure the replication of plasmid DNA directly by pulse labelling and isolating plasmid DNA.

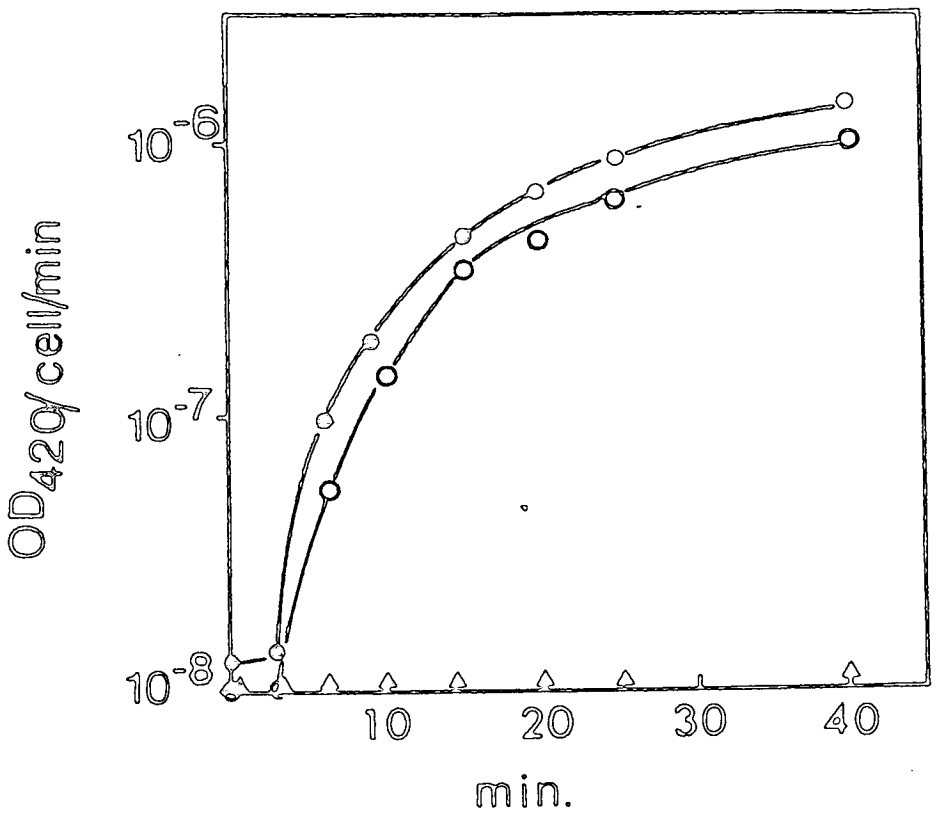


Fig. 3-16 β-galactosidase induction curve for B/r E *lac*$^+$ (○), B/r E *lac*$^-$ (△) and B/r E *lac*$^-$/F′*lac*.(○). The cells were grown to 10^8 cells/ml, IPTG was added to a final concentration of 10^{-3}M and 100 µl samples were removed from the culture at the times indicated into 10 µl 2 mg/ml chloramphenicol. The enzyme was assayed as described in Methods.

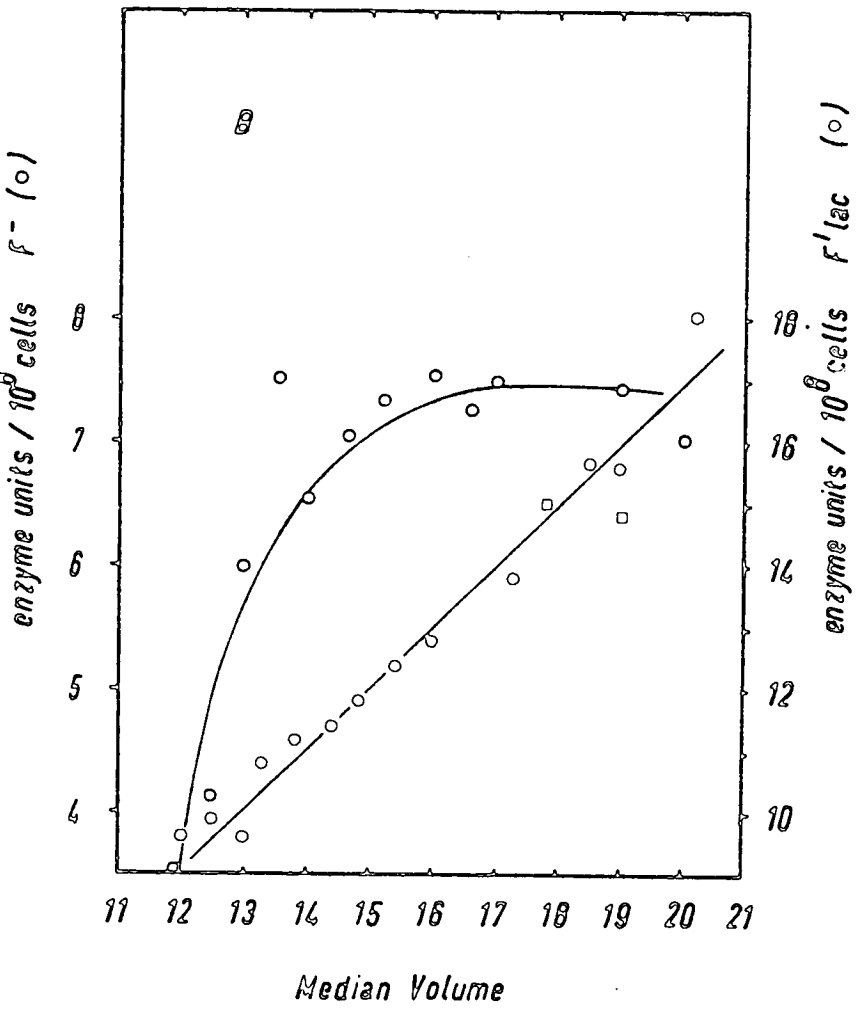


Fig. 3-17 Rate of induction of β-galactosidase in B/r E lac$^+$ (○) and B/r E lac$^-$/F'lac (○). 10^{-3} M IPTG was added to exponentially growing cells and after 6 minutes of induction a mixture of antibiotics was added to the cells and they harvested and fractionated as described in Methods.

Exponential cultures were pulse labelled for 1 minute with ^{3}H-thymidine and chased with cold thymidine for 5 minutes to ensure that replicating molecules were completed and in the closed circular form. The cells were then harvested and separated in the zonal rotor. Plasmid DNA was purified from each fraction and examined directly for evidence of incorporation of pulse label. If plasmid replication occurred mainly in cells of a particular size, one would expect to find pulse label only in the fractions containing cells of that size. This procedure should provide a more sensitive test of discontinuous synthesis than one such as enzyme induction where the maximum observable difference in the rate of enzyme synthesis could not exceed two fold (see figures 3-7 and 3-9).

a) Analysis of F'lac DNA in agarose gels.

Two different methods were employed to analyse the pulse labelled plasmid DNA in different fractions from the zonal rotor. The first was to analyse the plasmid DNA with the aid of agarose gels. Cleared lysates were made from every size fraction obtained from the pulse labelled culture, and purified as described in the Methods section. The lysates were then run on an agarose slab gel. Fig. 3-18 shows that plasmids could be isolated from all fractions and is absent from the F^{-} strain. The intensity of the plasmid bands was determined in a densitometer (fig. 3-19), and this was used as a measure of the

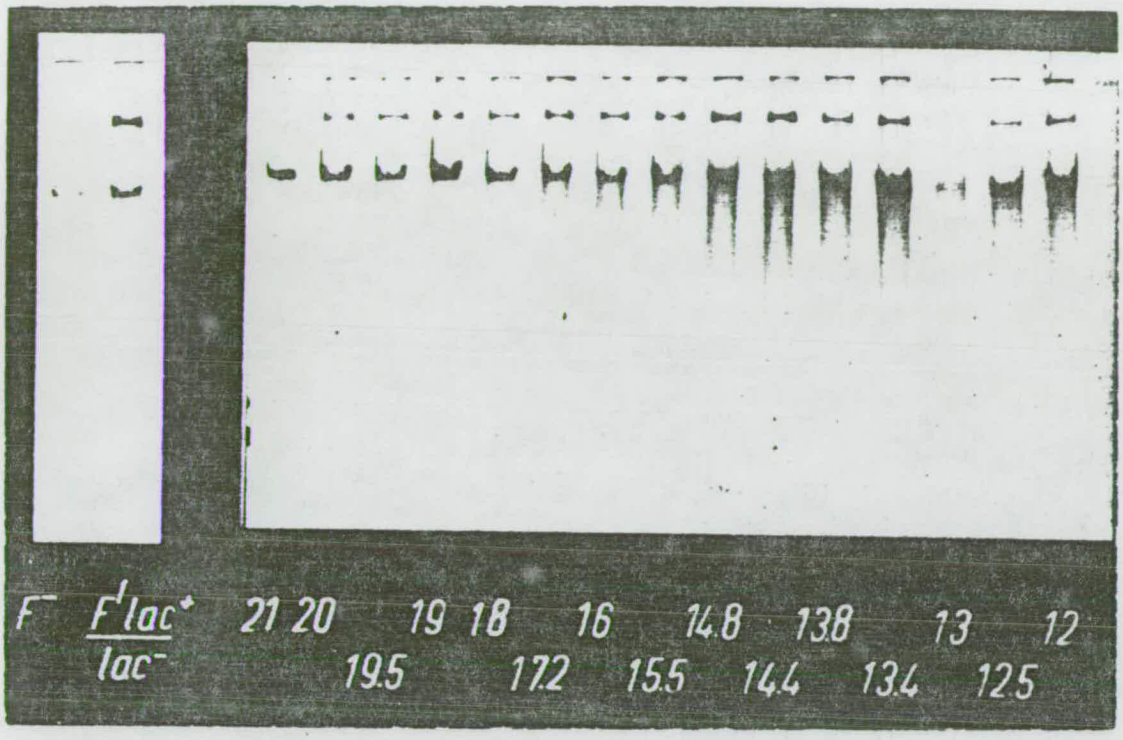


Fig. 3-18 Slab gels showing chromosomal and F'lac DNA in cells of different sizes. Cultures were pulse labelled and fractionated as described in fig. 15. Cleared lysated were prepared, partially purified, loaded on gels and run as described in the Methods section. The gels were stained with EtBr and photographed with UV light. The median cell volume for each sample is indicated below the appropriate track in the photograph.

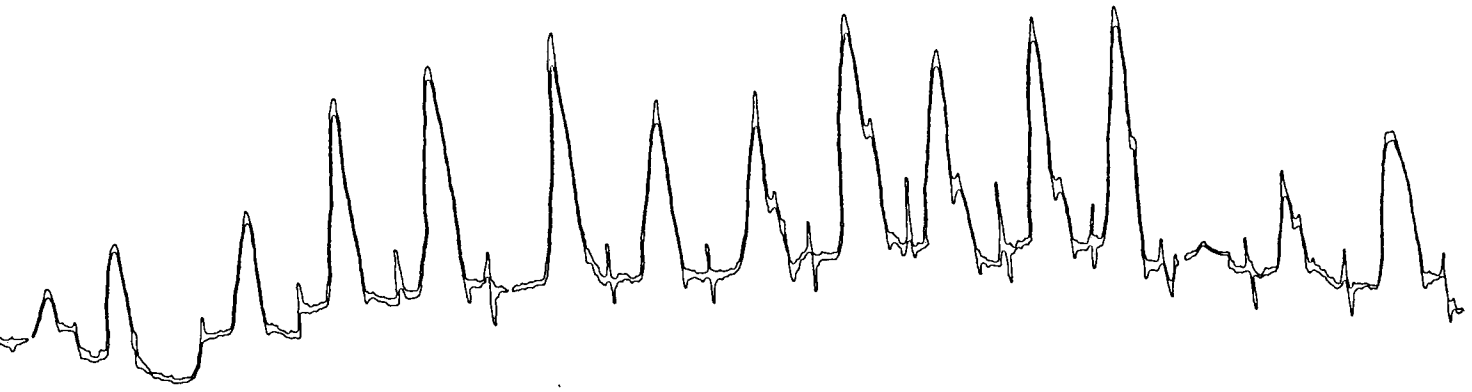


Fig. 3-19 Densitometer tracings of plasmid bands. A negative of slab gel photo was scanned by a Joyce Loebl densitometer.

amount of plasmid DNA that was there. The area under each peak was determined by tracing the densitometer scan on graphpaper with $1mm^2$ squares and counting the squares in each peak. Each figure is an average of three independent estimates. (3 scans and 3 counts).

Fig. 3-20 shows the ratio of labelled plasmid DNA to total plasmid DNA in every fraction in two separate experiments. There was labelled plasmid DNA in every fraction, and the proportion of labelled plasmid DNA does not vary over a more than twofold range.

It will be noted in fig. 3-18 that some DNA always sticks to the top of the gel. To eliminate the possibility that pulse labelled plasmid DNA preferentially stuck to the top of the gel (since open circular DNA migrates considerably more slowly than CCC DNA in the gel (Aaij and Borst, 1972), and it was conceivable that the pulse labelled molecules had not had time to form covalently closed circles), these were also cut out of the gel and counted in the same way as the plasmid bands. Fig. 3-21 shows that the ratio of ^3H to total DNA is about the same in every fraction.

b) Analysis of F'lac DNA on CsCl-EtBr gradients.

Pulse labelled plasmid DNA was also analysed using CsCl-EtBr gradients. Aliquots of cells from different size fractions were mixed with 10^9 cells of the F'lac strain which had been fully labelled with ^{14}C thymine,

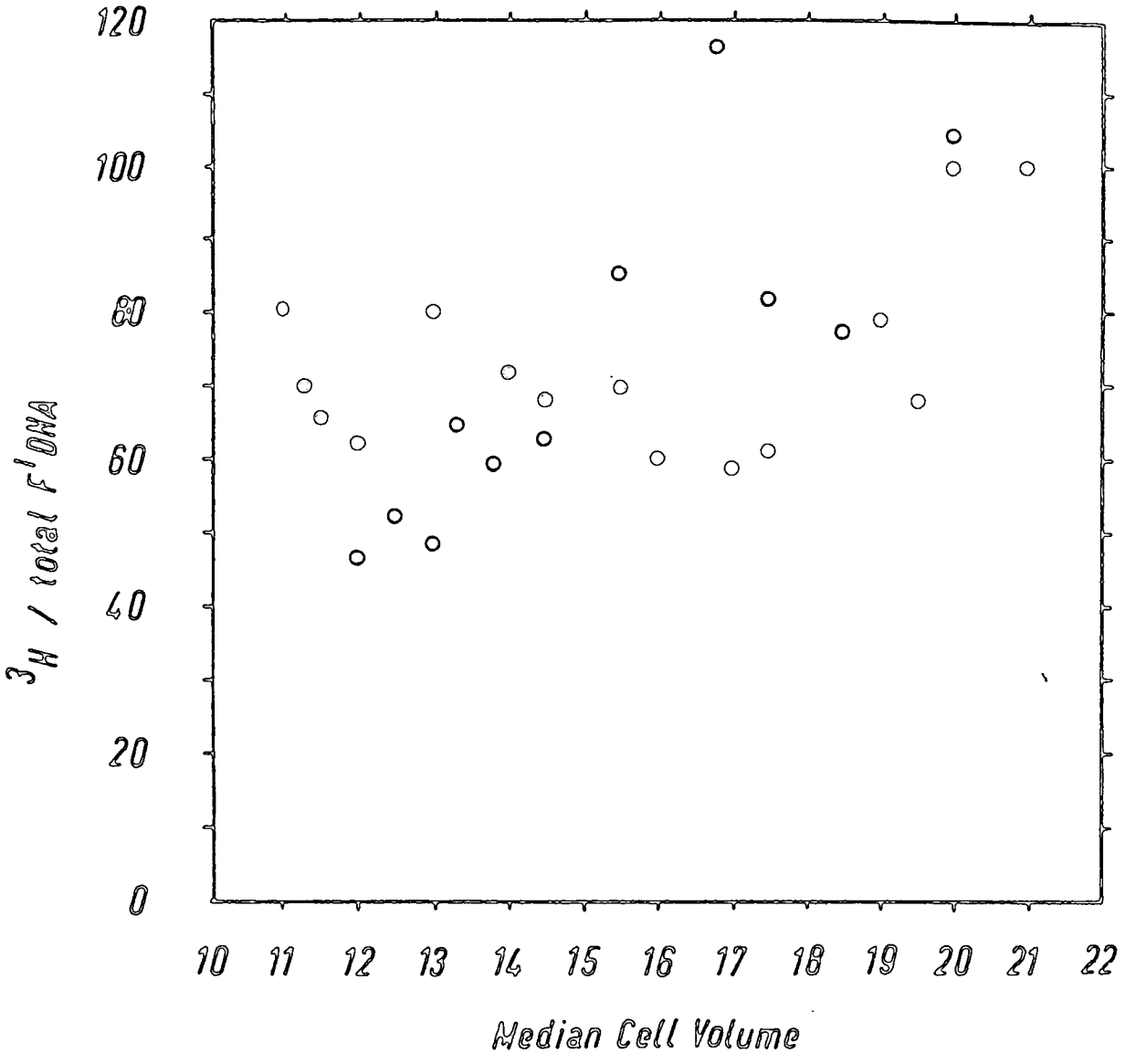


Fig. 3-20 Label incorporated into plasmid DNA in cells of B/r E lac$^-$/ F'lac of different sizes: plasmid and chromosomal DNAs separated on slab gels. Cultures were pulse labelled and fractionated as described in fig. 18. The amount of DNA in the plasmid fraction of each of the samples was determined by photographing the gles, scanning each band on the photograph in a densitometer, and measuring the area under each peak. Each band in the original gel was cut out and solubilized as described in the Methods section, and the ^3H content determined. The ^3H to DNA ratio was calculated for each fraction and plotted as a function of median cell volume. Open and closed circles represent data from two completely separate experiments.

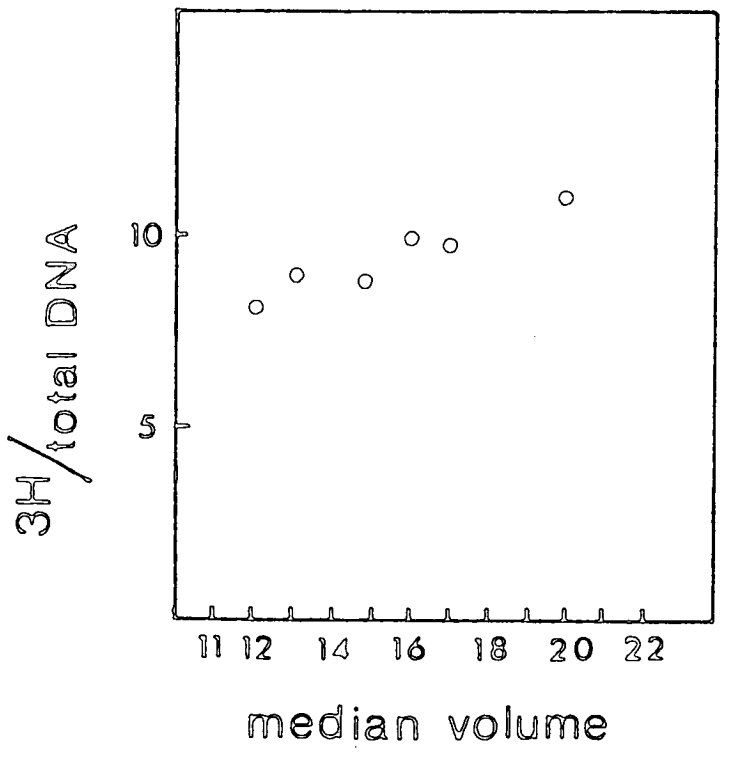
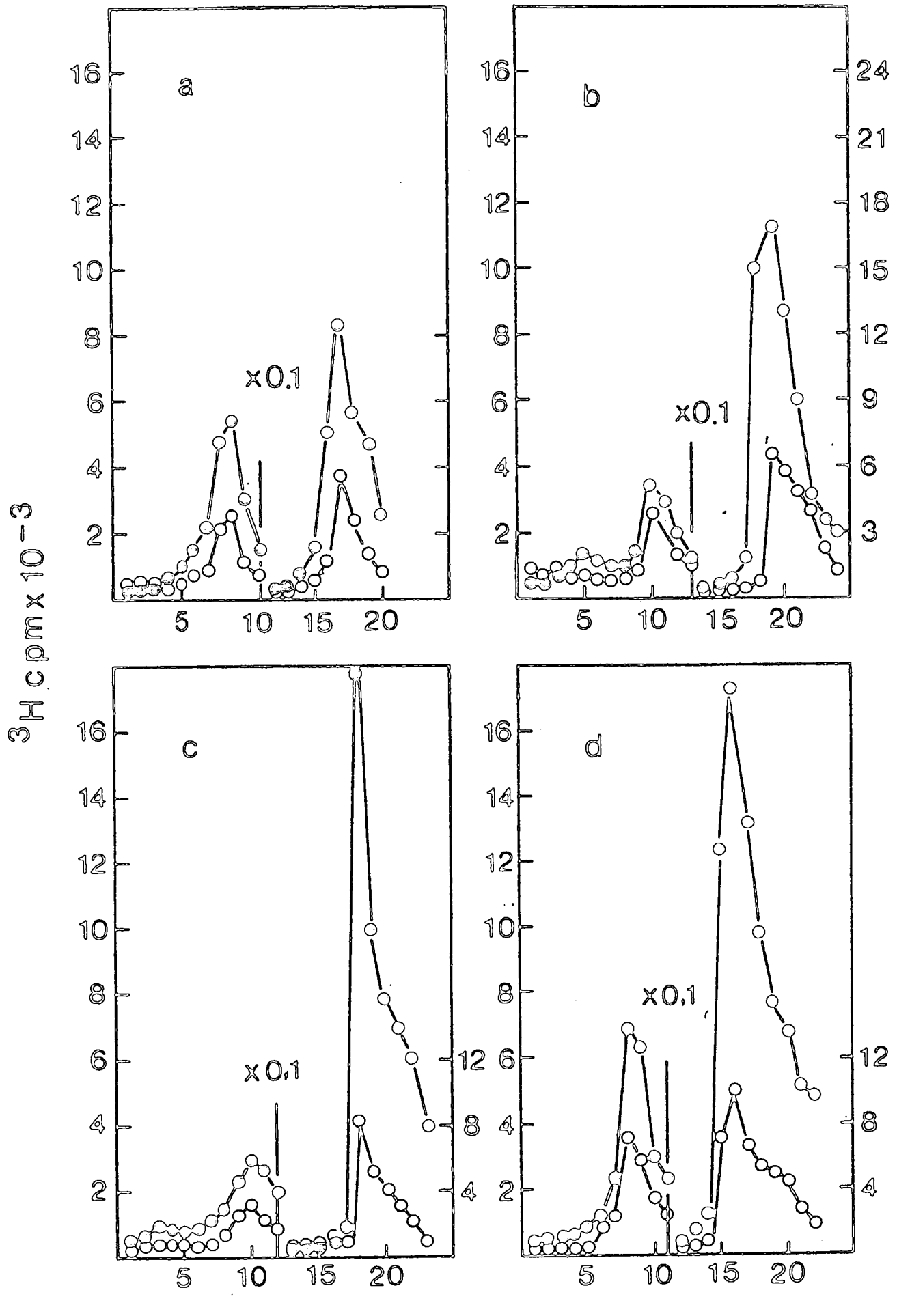


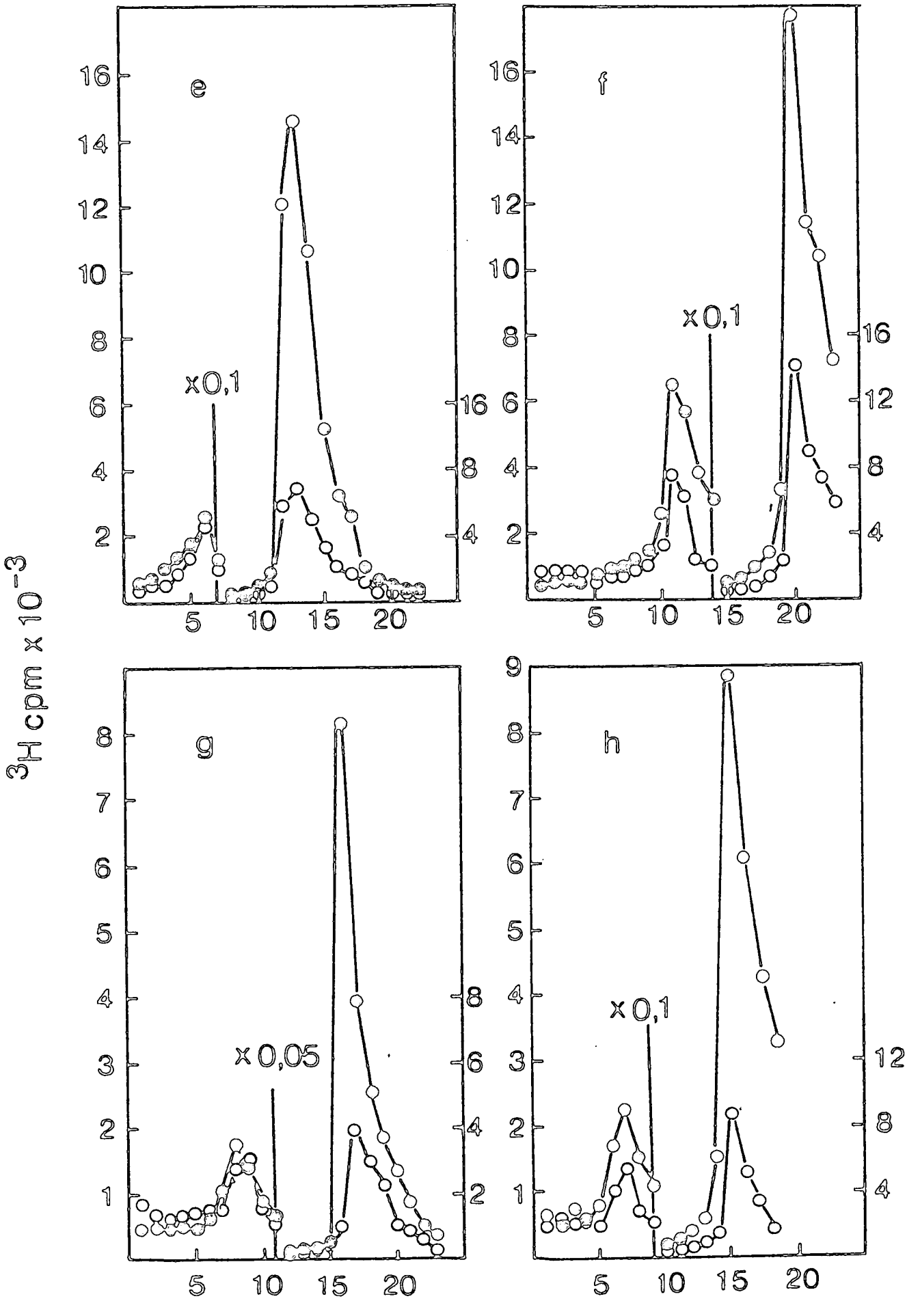
Fig. 3-21 Label incorporated into top bands of the slab gel shown in fig 18. Each point is determined as described in fig. 20.

and the mixture was lysed and centrifuged. The fixed number of ^{14}C labelled cells were added as an internal measure of the recovery of plasmid DNA. The lysates were then centrifuged in CsCl-EtBr gradients for 2-3 days (fig. 3-22). The $^{3}H/^{14}C$ ratio per cell was calculated in both the plasmid and the chromosomal peaks (fig. 3-23). It can be seen that there is good agreement between the chromosomal DNA curve obtained by spotting samples of whole cells from every fraction on filterpaper and that obtained from the chromosomal peak in the CsCl-EtBr gradient. The pulse labelled plasmid DNA can once again by found in all fractions examined.

If F'lac is replicated at a discrete cell size, the proportion of that cell size in every fraction should vary in the same way as the labelled DNA does. Fig. 3-24 shows the labelled plasmid per cell divided by the proportion of a given size class in fractions from the zonal rotor. This is plotted for every size class and should give a horizontal line for the size class where the labelled plasmids were at the time of lysis. It can be seen that this ratio is far from being constant in any size class, it can therefore be concluded that there is no one size class in which the pulse labelled plasmids were situated at the time of lysis. Neither are there any two or three adjacent size classes that added together give a constant label/size ratio.

Fig. 3-22 Pulse labelled plasmid and chromosomal DNA in cells of different sizes separated on CsCl-EtBr gradients. Cultures were labelled and fractionated as described above. The cells from each fraction were lysed with lysozyme-SDS and centrifuged in a 50Ti rotor of a Beckman 2L-50 centrifuge at 34 krpm and 18°C for 48-64 hours. a: fractions 1 and 2 from the zonal roto
b: fraction3; c: fraction 6; d: fraction 7; e: fraction 8;
f: fraction 9; g: fraction 10; h: fraction 11.


a
b
c
d
x0.1
x0.1
x0.1
x0.1
^{3}H cpm x 10^{-3}
^{14}C cpm x 10^{-2}
FRACTION NO.


e
f
g
h
x0,1
x0,1
x0,05
x0,1
^{3}H cpm x 10^{-3}
^{14}C cpm 10^{-2}
FRACTION NO.

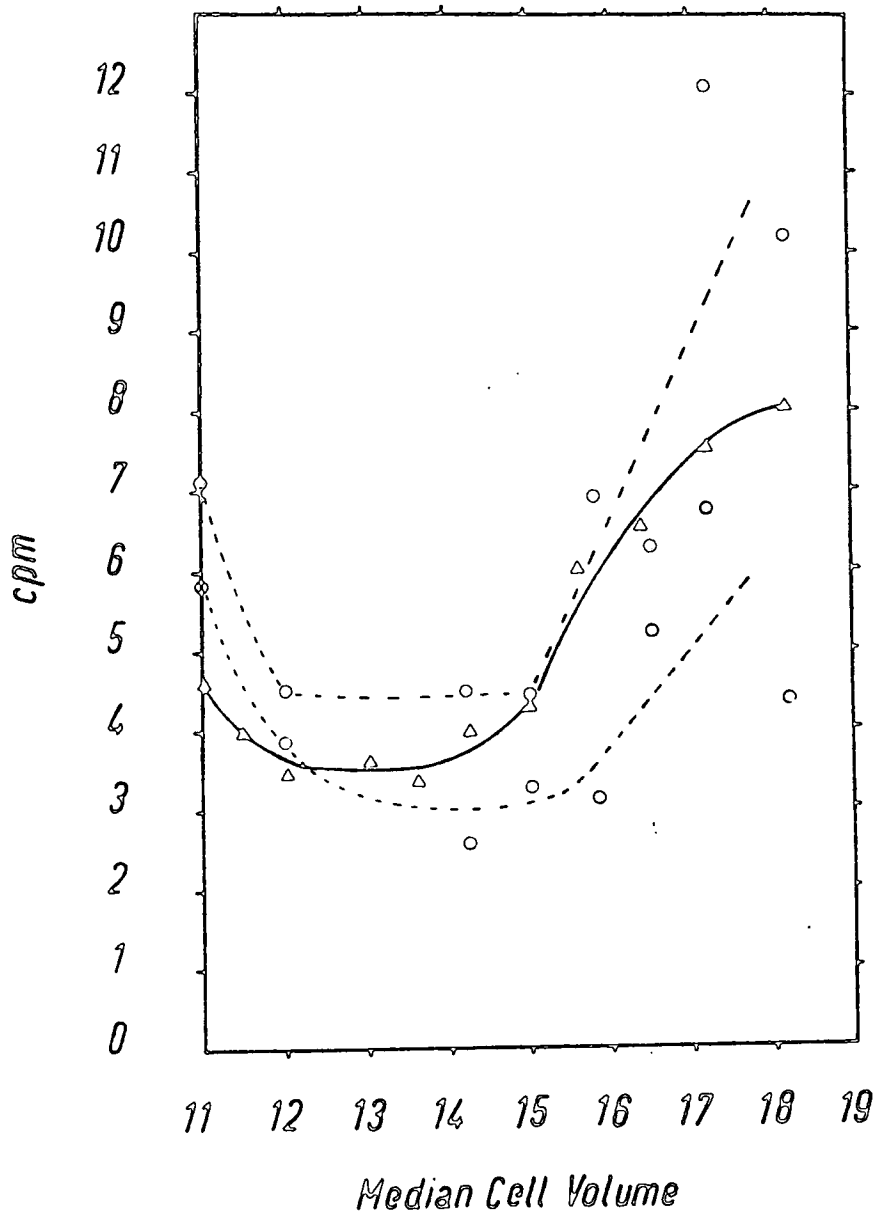
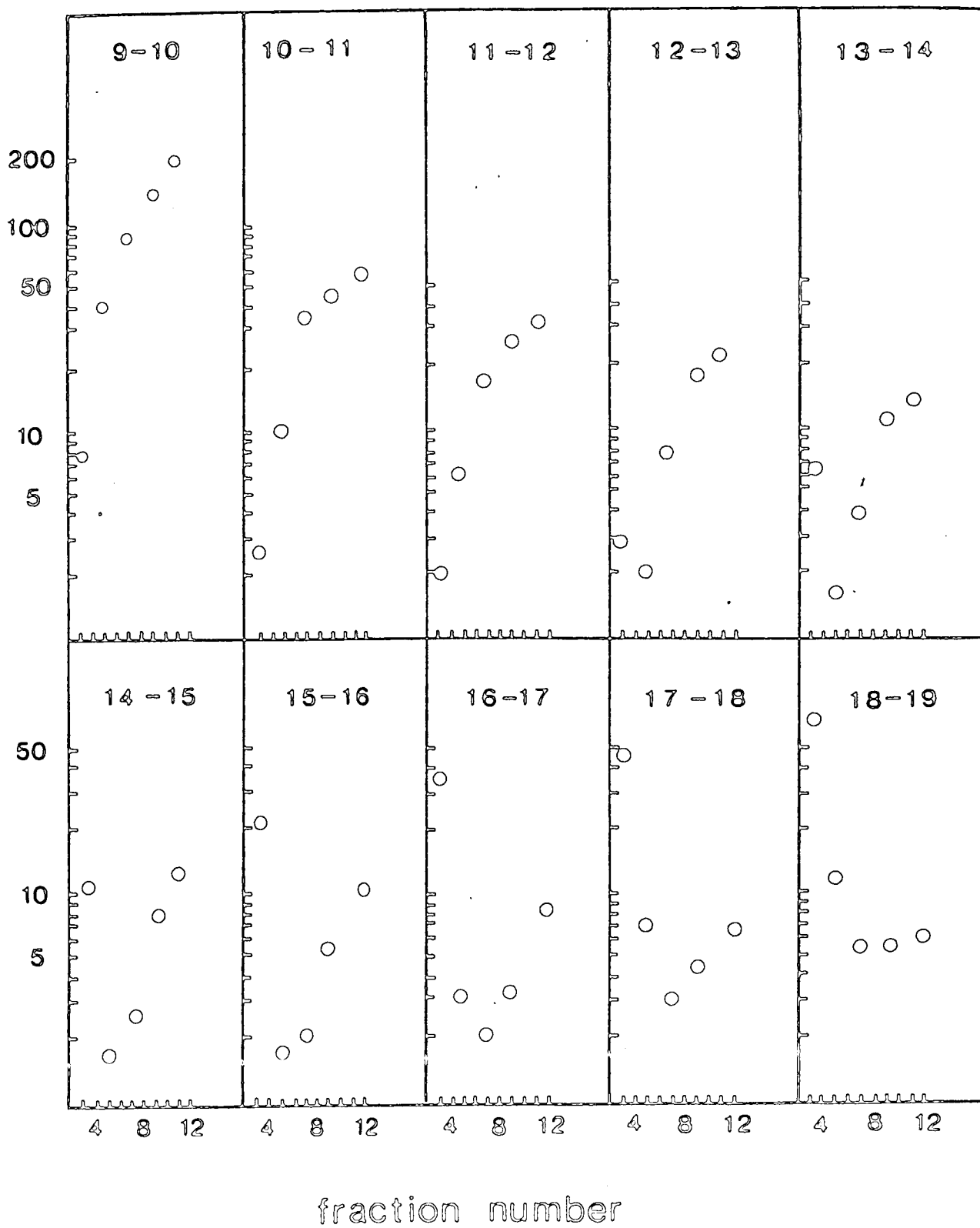


Fig. 3-23 Label incorporated into plasmid and chromosomal DNA in cells of different sizes: plasmid and chromosomal DNAs separated by centrifugation in CsCl-EtBr. Cultures were labelled and fractionated as described above. The ^{3}H/cell in each fraction was determined (△), selected fractions were mixed with 10^{9} cells of B/r E lac^{-}/F' lac fully labelled with ^{14}C thymine, lysed and centrifuged as described in Methods. Fractions were collected and the ^{3}H and ^{14}C measured in the separately pooled plasmid and chromosomal DNA containing fractions. The ratio of $^{3}H/^{14}C$ was calculated for both plasmid and chromosome and divided by the number of cells in the original fraction. The ratio is plotted as a function of the median cell volume for the sample for both plasmid (o) and chromosomal (o) DNA.


9-10
10-11
11-12
12-13
13-14
14-15
15-16
16-17
17-18
18-19
200
100
50
10
5
50
10
5
4
8
12
fraction number

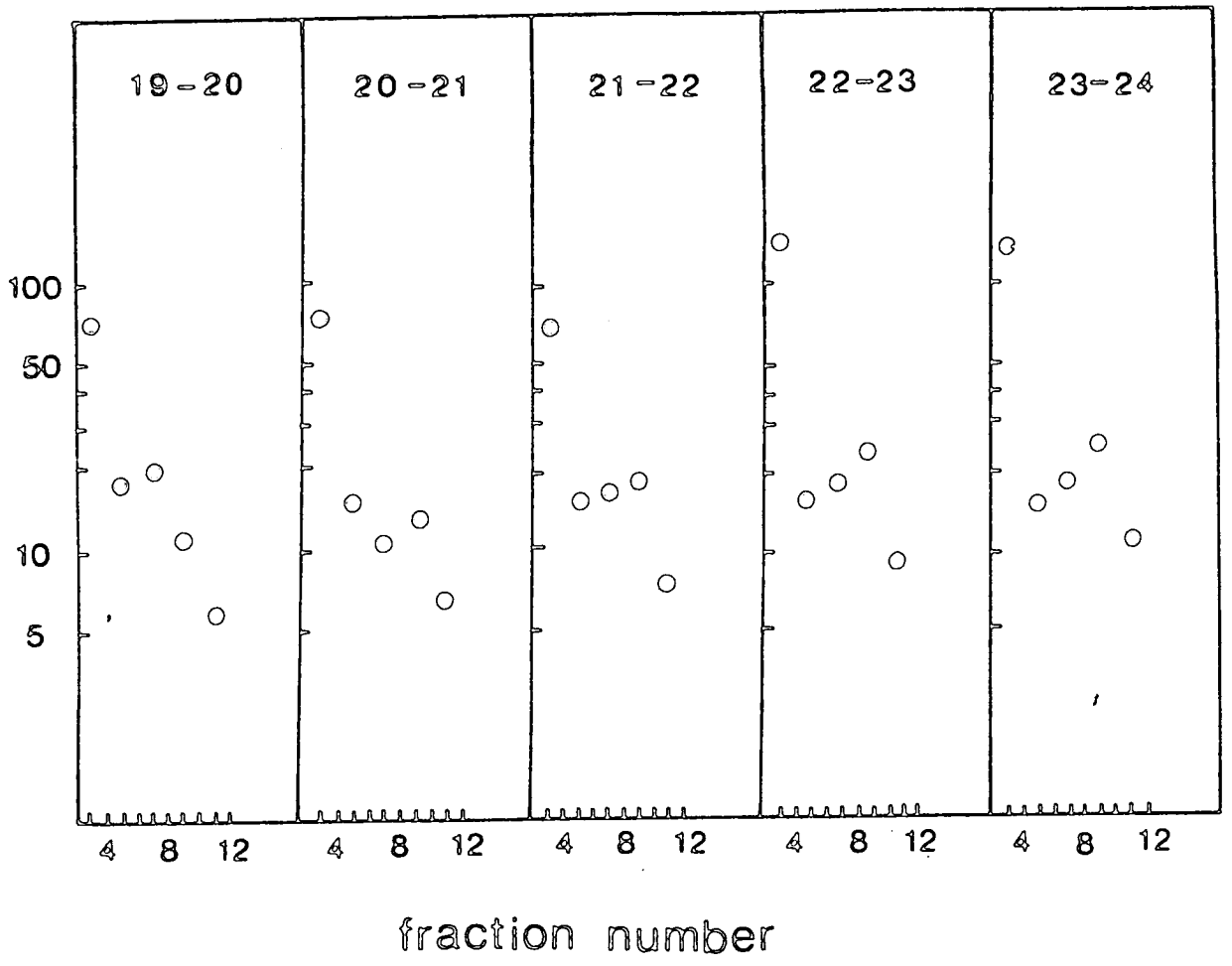


Fig. 3-24 The ratio of the proportion of radioactive plasmids in the fractions from the zonal rotor (from fig. 23) to the proportion of a given cell size class in these fractions. This is plotted for every cell size class from 9 - 24.

Copy number.

The highest recovery of fully labelled plasmid in the CsCl-EtBr gradient was 5,8% of chromosomal label. If the molecular weight of the chromosome is $2,5 \times 10^9$ daltons (Cooper and Helmstetter, 1968) and the MW of F'lac is 90×10^6 daltons, then this corresponds to 1,6 plasmid copies / chromosome equivalent. The values that have been reported for F containing cells growing at this growth rate vary from 0,7 to 1,4 copies per chromosome equvalent. (Finkelstein and Helmstetter, 1977; Collins and Pritchard, 1973; Frame and Bishop, 1971; Gustafsson et al., 1978).

The number of genome equivalents per cell can be found using the equation derived by Cooper and Helmstetter (1968):

$$G = \frac{\tau}{C \times \ln 2} \left(2^{(C+D)/\tau} - 2^{D/\tau}\right)$$

where C is the replication time for the chromosome, D is the time from termination of chromosome replication to cell division and τ is the generation time. In our case C is 45 min., D is 45 min. and τ is 58 min. Using these values, G becomes 2,27 genome equivalents per cell. There are therefore at least 1,6 x 2,27 = 3,6 plasmid copies per cell.

Stability of F'lac.

In order to see if F'lac were unstable in our strain, we measured its rate of segregation through six generations of growth on glycerol minimal medium (g=58min) and through fifteen generations of growth on broth (g=30 min) by plating cells on McConkey lactose agar once per generation. The

experiments were performed at low cell density (10^3 cells/ml) at which reinfection of segregants by mating would be unlikely to occur. Several hundred colonies were examined for each time tested, but no segregation was observed to occur during the course of the experiments. At the end of the experiments all cells were lac^+, and the culture still F′ as demonstrated by its sensitivity to acridine curing.

Discussion.

The timing of F′*lac* replication in the cell cycle was studied both in synchronous and exponential cultures. In the synchronous cultures there was a stepwise change in β-galactosidase inducibility towards the end of the division cycle. A step in the inducibility of β- galactosidase has also been found in all previous studies with synchronous cultures and exponential cultures separated into age classes using the membrane elution technique.(Helmstetter, 1967). However, the time in the cell cycle at which F′*lac* replicates has not been agreed on, and Pritchard et al. (1975), who looked at β-galactosidase inducibility in cells with different replication velocities, were not able to correlate the replication of F′*lac* with any other event in the cell cycle. Table 3-1 shows the results of three groups (Zeuthen and Pato (1971), Davis and Helmstetter (1973), and Finkelstein and Helmstetter (1977)), who looked at the inducibility of β-galactosidase in B/r A lac^-/F′*lac*, by using the membrane elution method of Helmstetter (1967). An exponential

Table 3-1

Generation time	1		2		3	
min	DNA	B-gal	DNA	B-gal	DNA	B-gal
24	0,65	0,55-0,6				
24-25					0,4-0,5	0,5-0,6
27			0,9	0,8		
26-29					0,6-0,8	0,6-0,7
30-33					0,9-1,2	0,7-0,8
35	0,37-0,5	0,35-0,4				
36			0,3	0,3		
40	0,5-0,57	0,6				
41-43					0,5-0,6	0,9-1
50	0,8-0,9	0,7-0,8				
55			0,82	0,78		
50-58					0,7-0,9	0,7-0,8
70	0,95-1,2	0,4-0,7				
85			0,0	0,82		
100	0,1	0,6-0,7				
115-125					0,0-0,1	0,6-0,7

Results from the experiments of Zeuthen and Pato (1), Davis and Helmstetter (2), and Finkelstein and Helmstetter (3), using the inducibility of B-galactosidase as a measure of F'lac replication, and the incorporation of ^{3}H thymidine during a pulse as a measure of chromosome replication. The strain was B/r A lac^{-}/F'lac, the membrane elution technique was used to separate the cells into age classes. The figures in the table represent fractions of a cell cycle after division.

culture was pulse-induced, and the cells were grown on a membrane surface, off which they fell after division. Cells which were collected in a short time interval would therefore all be at the same age with respect ot cell division when the culture was pulsed. It can be seen in the table, that there is neither agreement as to when in the cell cycle F'lac replication is, nor when chromosome replication occurs, at similar growth rates, although the same strain and the same method was used. This may be because of different experimental conditions or inaccuracy in interpreting the results. Most of the steps seem quite clear, and usually take over about 1/3 to 1/2 the cell cycle.

When we examined exponentially growing cells, after size separation on a sucrose gradient in a zonal rotor, both the inducibility of β-galactosidase and direct measurement of F'lac DNA showed that F'lac DNA which had replicated during a 1 minute pulse of radioactive thymidine or during a 6 min. period of β-galactosidase induction was situated in cells of all sizes. It therefore appears that F'lac is replicated throughout the cell cycle. It seems difficult to reconcile these results with earlier results which all showed a step in the production of β-galactosidase somewhere in the cell cycle. In the studies done on synchronously dividing cultures (Donachie and Masters, 1966; Nishi and Horiuchi, 1966) it is conceivable that the process of synchronization might have alligned otherwise asynchronous plasmid replication. In the more recent studies, the limitations of synchronous

cultures were avoided by pulse-inducing exponentially growing cells and separating their descendants by the membrane elution technique into fractions which had been at the same age at the time of induction. It is more difficult to reconcile the results of these experiments with F'lac replicating in cells of all sizes. It may be pointed out that since the cells must grow on a membrane surface after induction in order to separate them by age, it is difficult to ensure that the enzyme/cell does not alter during the period of growht subsequent to induction. However, although this limitation might conceivably obscure existing steps, it is difficult to see how they might cause steps.

When interpreting the results from the zonal rotor, it is important to analyse the cell sizes in the fractions from the zonal rotor in detail. When looking at the cell size distribution of an exponential culture (fig. 3-5), it will be noted that the cell sizes range over more that twofold. This may be a result of a variance in cell size when it divides, or asymmetric division. There is no data on asymmetric division, but this has been reported by others to be substantial (Koppes et al., 1978). In the absence of any such data it was assumed that all newborn cells were the result of symmetric divisions, and cells which had a larger volume than twice that of the smallest cell were assumed to be at the same stage regarding DNA replication as the cell they were a multiple of (but not, for instance, that a cell divided late because it initiated DNA replication late).

Using these criteria there was a good fit between the experimental curve of DNA replication and a calculated curve where it was assumed that chromosome replication started in cells of size 16 units and stopped in cells of size 13 units, and the heterogeneity of the cell sizes in the fractions from the zonal rotor was taken into account (fig.3-25). The graph for β-galactosidase inducibility in B/r F *lac*$^+$ cells is also very close to what can be expected if β-galactosidase is being produced in cells of size 12 and 24 units. (fig. 3-26).

The curves of the pulse labelled plasmids in the fractions from the zonal rotor suggest that there could be twice as many replications in the big cells as there are in the small cells. The curves of the two types of radioactive plasmid identification, i.e. the CsCl-EtBr gradients and the gel analysis are not exactly identical, although both indicate F′*lac* replication in cells of all sizes, and approximately double frequency in large cells compared to small cells. The results from the CsCl-EtBr gradients indicate that there is a relatively high level of replication in small cells which then drops and rises again. There is however a possibility that the first point in that graph is too high, possibly because of too few counts in the ^{14}C in that fraction, since the first point in the counts from the chromosome is too high compared to the estimate from the whole cells (fig 3-23).

Figures 3-27 and 3-28 show calculated points where the heterogeneity in cell size in the fractions is taken into account and it is assumed that the frequency of F′*lac* repli-

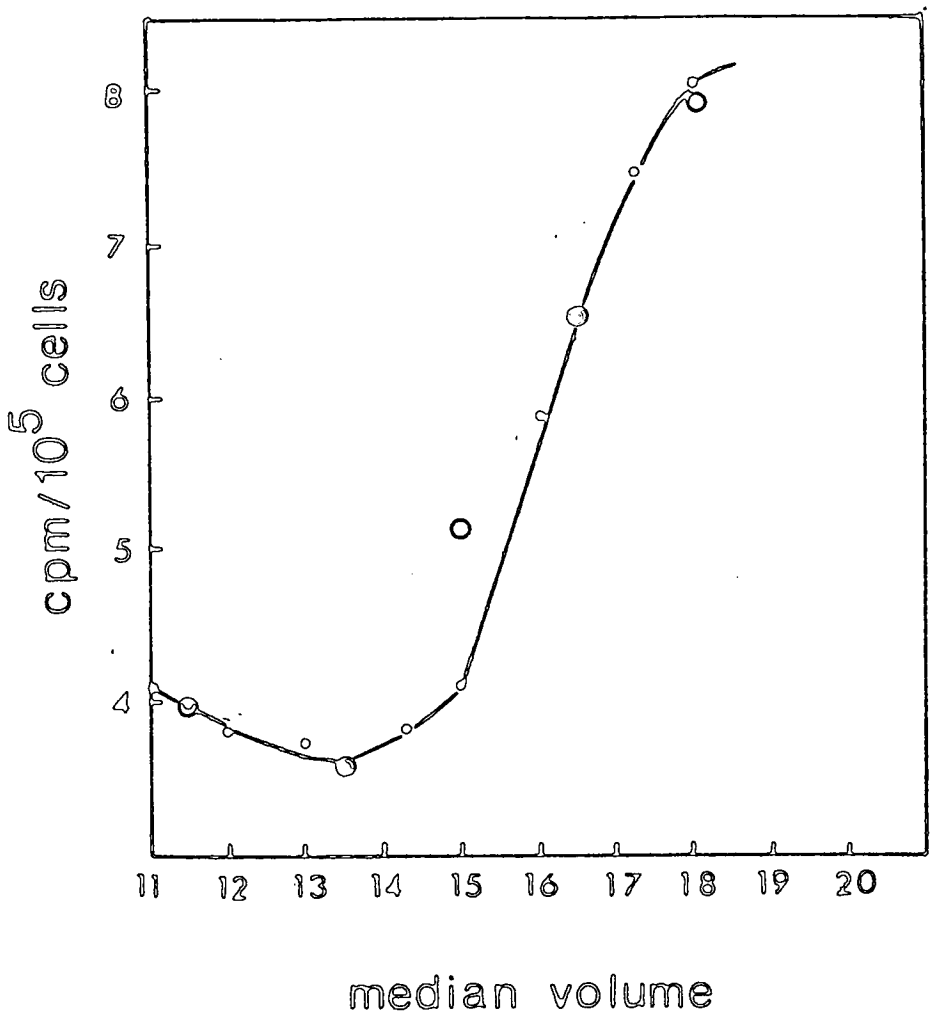


Fig. 3-25 Rate of DNA synthesis in B/r E lac⁻/F′lac (∘), and superimposed on the graph are points that would be expected if DNA replication initiated at size 16 units and terminated at size 13 units (O). The points represent fractions 2, 5, 7, 9, and 11. The corresponding median volumes are plotted.

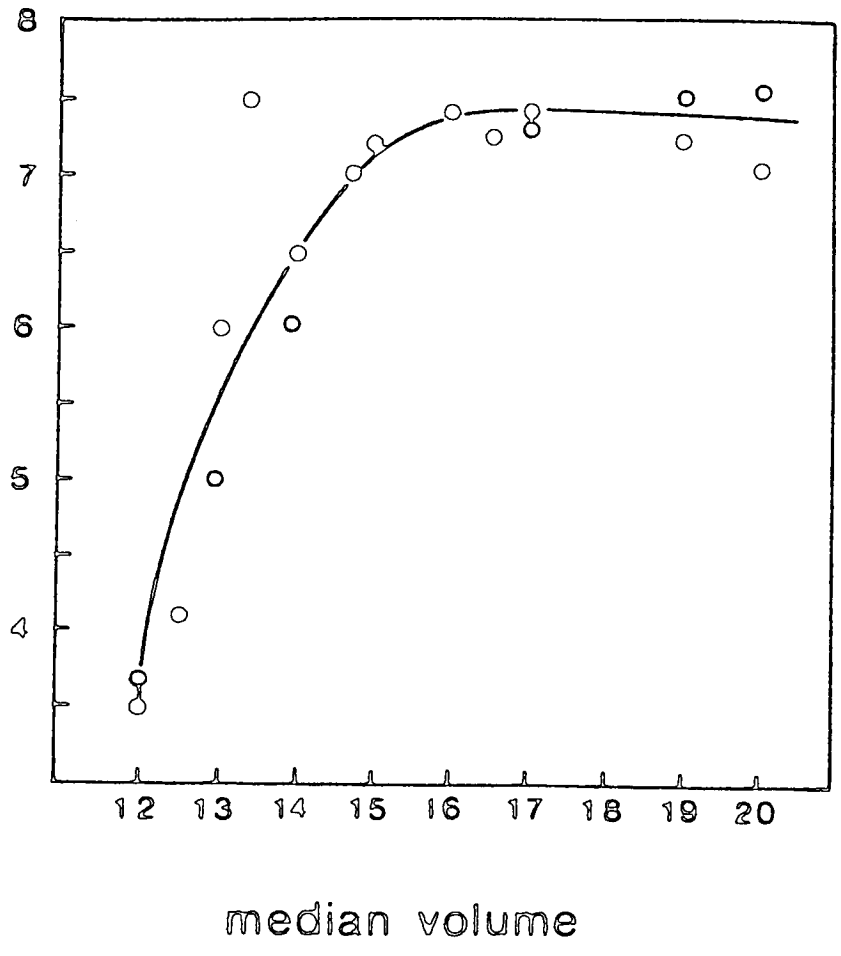


Fig. 3-26 Rate of β-galactosidase inducibility in B/r E *lac*$^{+}$F^{-} cells (○), and superimposed on the graph are points that would be expected if β-galactosidase is being produced in cells of size 12 and 24 units (○).

cation is twice as high in large cells as it is in small cells and increases linearly with cell size. It can be seen that the calculated points fit quite well the results from the agarose gel experiment, but they do not fit the CsCl-EtBr experiment as well, but as noted earlier the first point there may be too high. Figures 3-29 and 3-30show calculated points for the β-galactosidase inducibility, and it can be seen that these fit the results from the agarose gel experiment quite well, but those from the CsCl-EtBr experiment less well. It seems that the data fit the calculated points sufficiently well to conclude that the actual frequencies of F'lac in the cell sizes could be some approximation to the ones assumed here.

The cells do not all divide at the same cell size, as mentioned earlier. If F'lac replicated late in the cell cycle, as the experiments with the synchronous cultures would suggest, then some cells which had got the pulse label might divide during the 5 minutes cold chase, while others might not. This could result in label being both in large and small cells at the time of lysis. Figures 3-31 and 3-32show how label can be expected to be distributed among the fractions from the zonal rotor if it is assumed that F'lac has replicated in cells of sizes 18-20, and labelled plasmids are situated in cells of sizes 20-22 units and 10-11 units at the time of lysis. It is assumed that each cell of 10-11 units has half the amount of plasmids that the cells of 20-22 units have. Figures 3-31 and 3-32 represent the CsCl-EtBr gradient and

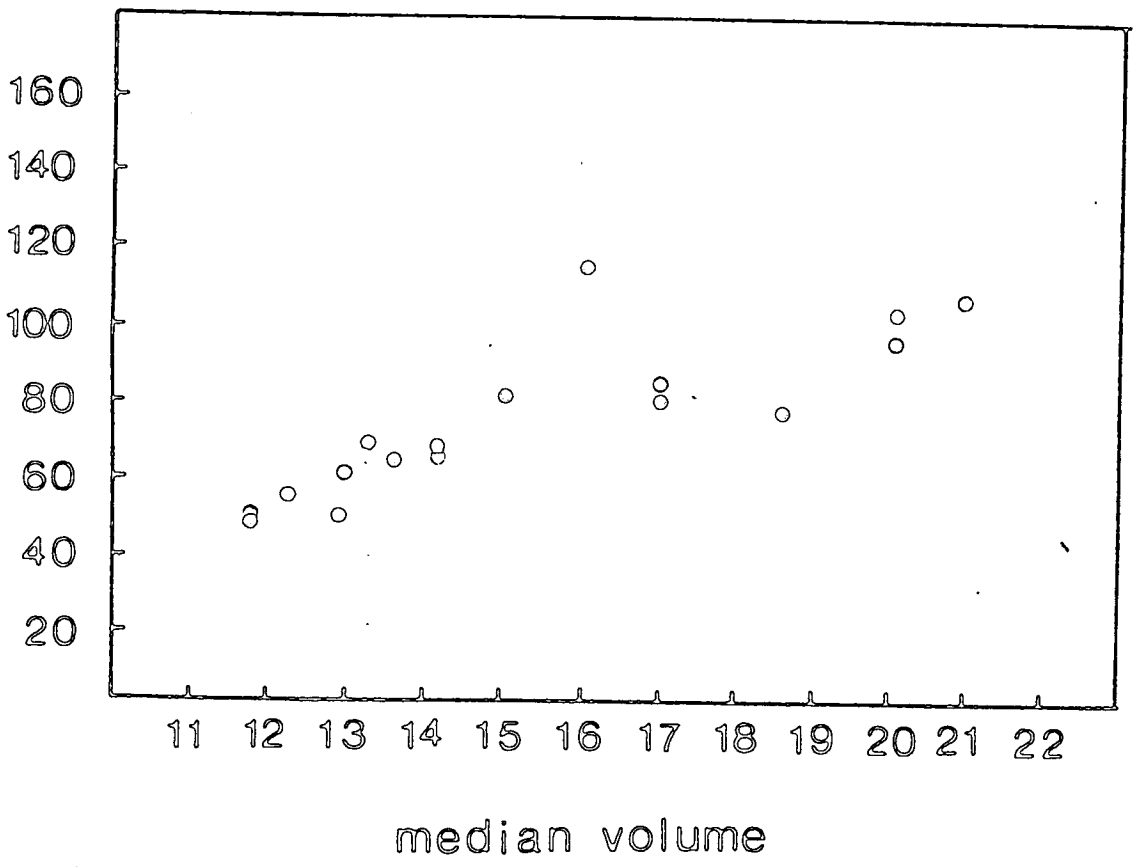


Fig. 3-27 Measured points of radioactive plasmids from the fractions of the zonal rotor, isolated by the agarose gel method (from fig. 3-20), (○), and calculated points where the heterogeneity of the cell sizes in the fractions from the zonal rotor is taken into account, and it is assumed that F′*lac* replication is twice as frequent in large cells as it is in small cells and increases proportional with cell size (○). The median volumes of the fractions are plotted.

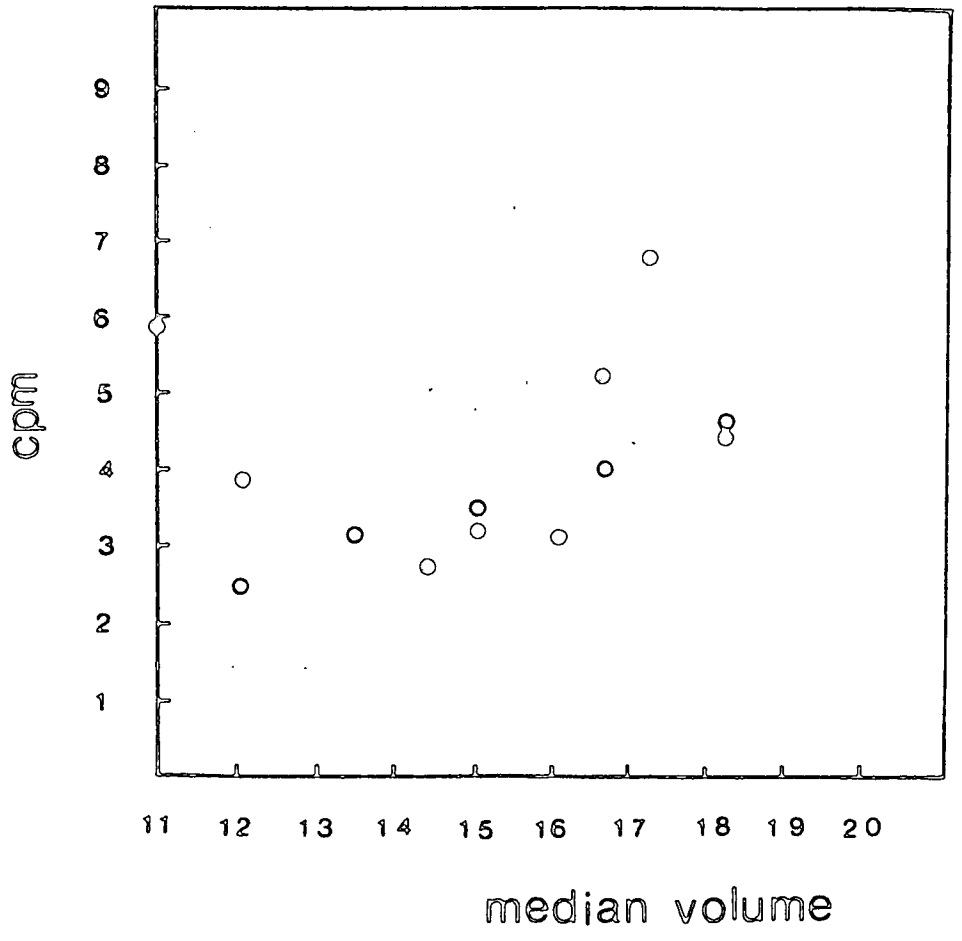


Fig. 3-28 Measured points of radioactive plasmids from the fractions of the zonal rotor, isolated by CsCl-EtBr centrifugation (from fig. 3-23), (○), and calculated points where the heterogeneity of the cell sizes in the fractions from the zonal rotor is taken into account, and it is assumed that F'lac replication is twice as frequent in large cells as it is in small cells and increases proportional with cell size (○). The median volumes of the fractions are plotted.

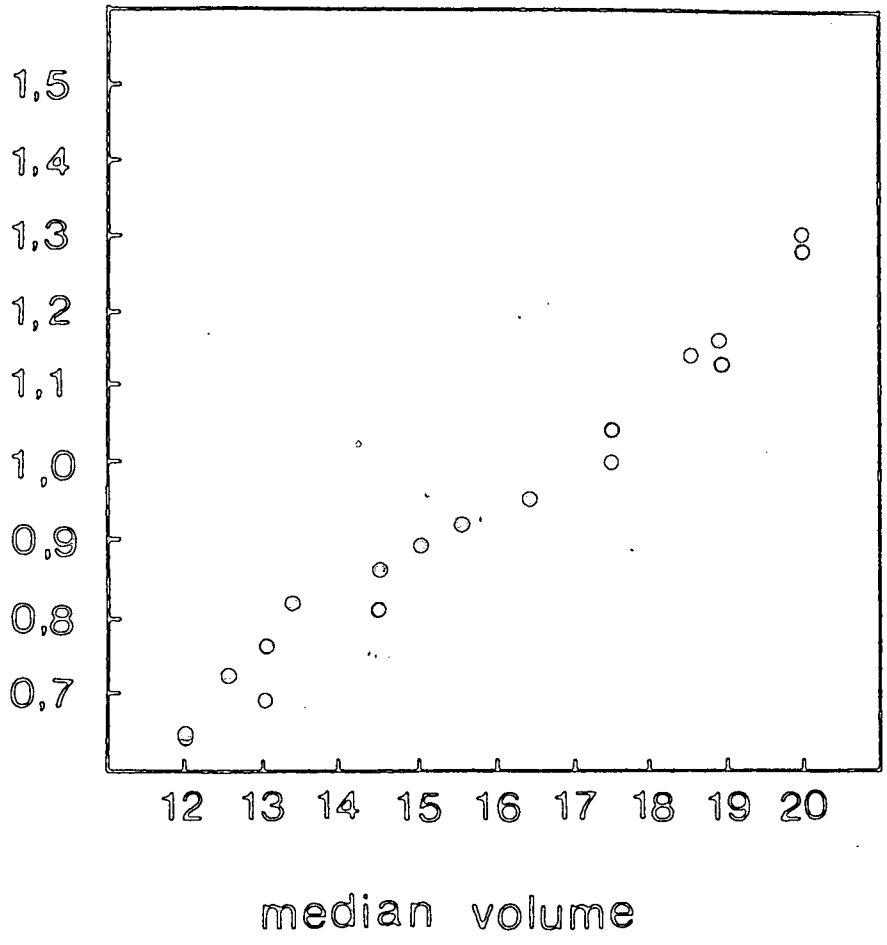


Fig. 3-29 Rate of β-galactosidase inducibility (○), and calculated points where it is assumed that F'lac replication is twice as frequent in large cells as it is in small cells and increases proportional with cell size(○). Data from the same experiment as the agarose gels.

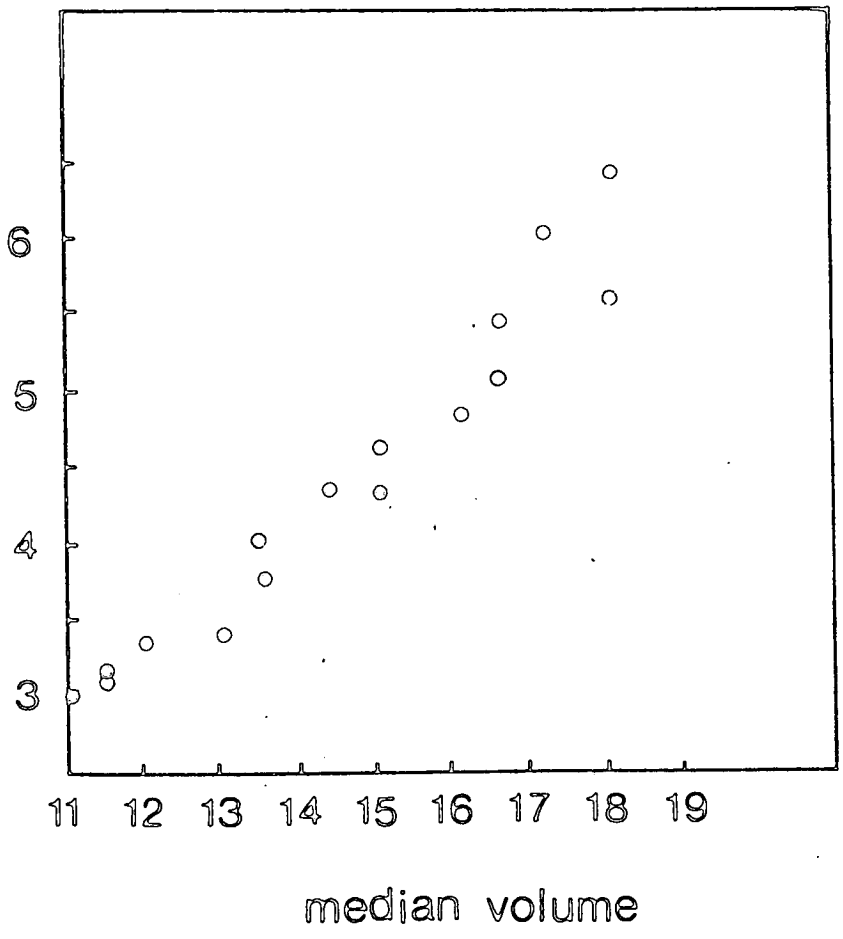


Fig. 3-30 Rate of β-galactosidase inducibility (○), and calculated points where it is assumed that F′*lac* replication is twice as frequent in large cells as it is in small cells and increases proportional with cell size (○). Data from the same experiment as the CsCl-EtBr gradients.

and agarose gel experiments respectively. While the results from the CsCl-EtBr experiment fit the calculated points quite well, there is not as good fit between the measured and calculated points derived from the agarose gel experiment. Figures 3-33 and 3-34 show the increase in β-galactosidase inducibility in the two above mentioned experiments. It can be seen that the calculated rise in enzyme between large and small cell fractions would not be more that 40-60%, whereas the observed increase in enzyme always was 100%. The β-galactosidase curves therefore do not support the assumption that F'lac is replicated late in the cell cycle. It would be preferable however, to do an experiment where penicillin was added to the culture after the 1 min. pulse, then no cells which had got the pulse label would have a chance to divide, so a clear peak of radioactive plasmids should appear at the end of the cycle if F'lac replication had indeed occurred there.

Another group, Gustafsson et al., have reported similar results as we have, and their suggestion was that if a newborn cell has 3 plasmid copies, then these plasmids are replicated one at a time at intervals 1/3 of a cell cycle apart. This idea is based on density shift experiments, where it was found that ca. 15% of the plasmids which had replicated one generation after the shift to light medium were fully light, i.e. some of the plasmids had replicated at least twice in one generation. An equally big part had not replicated at all. This was taken as a proof that the plasmids were selected randomly for replication. Further, the fact that

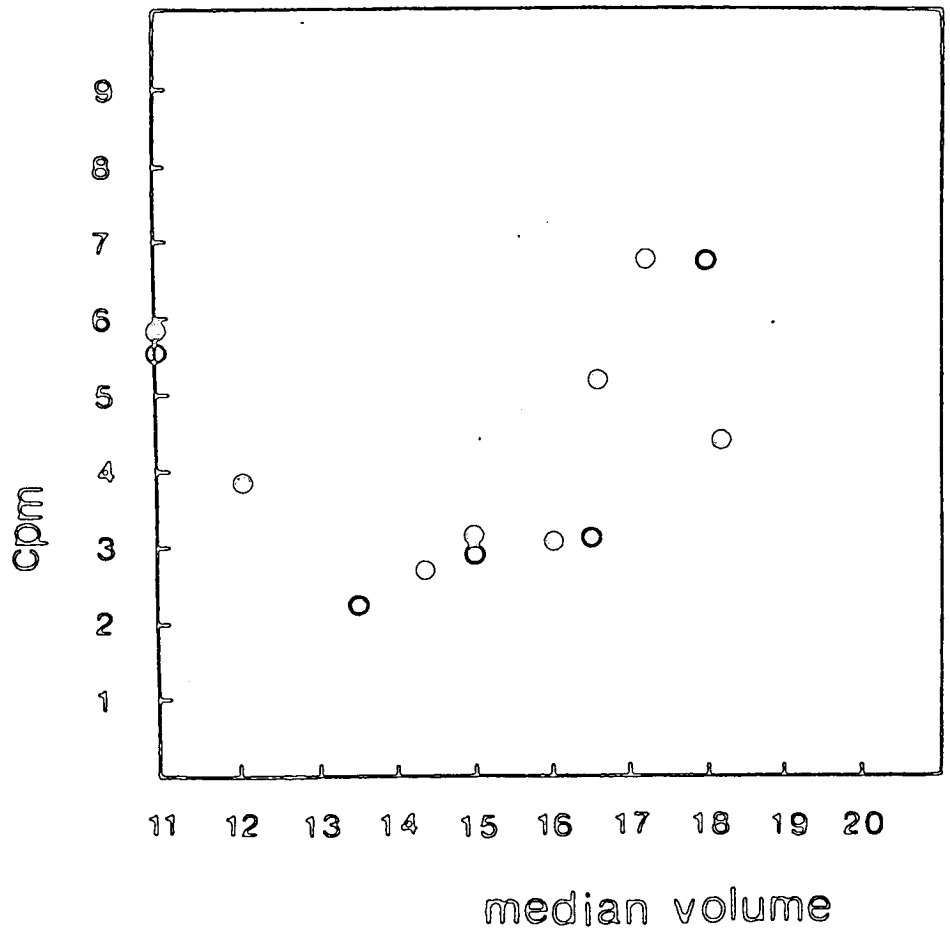


Fig. 3-31 Plasmid radioactivity in cells of different sizes (O) (from Fig. 23) and points (O) that would be expected if there was label in cells of sizes 10-11 and 20-22 units. It is assumed that cells of size 10-11 units have half the amount of radioactivity of those of 20-22 units. The measured points (O) represent fractions 1&2, 3, 6, 7, 8, 9, 10, 11 from the zonal rotor, and the calculated points represent fractions 2,5,7,9,11. The respectiv median cell volumes are plotted.

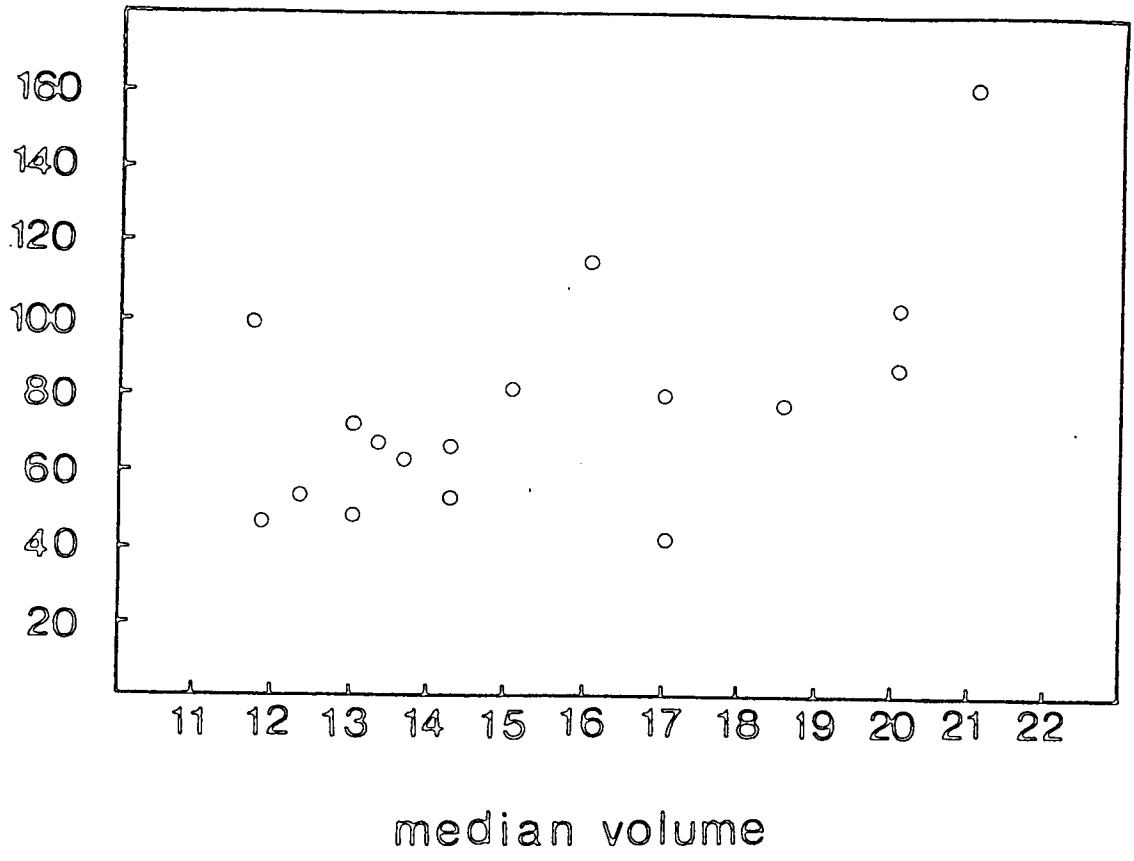


Fig. 3-32 Radioactive plasmids, isolated in agarose gels, in cells of different sizes (○) (from fig. 3-20), and calculated points where the heterogeneity of the cell sizes in the fractions from the zonal rotor is taken into account, and it is assumed that there is label in cells of sizes 10-11 units and 20-22 units(○). It is assumed that cells of size 10-11 units have half the amount of radioactivity of those of 20-22 units. The median cell volumes of the fractions are plotted.

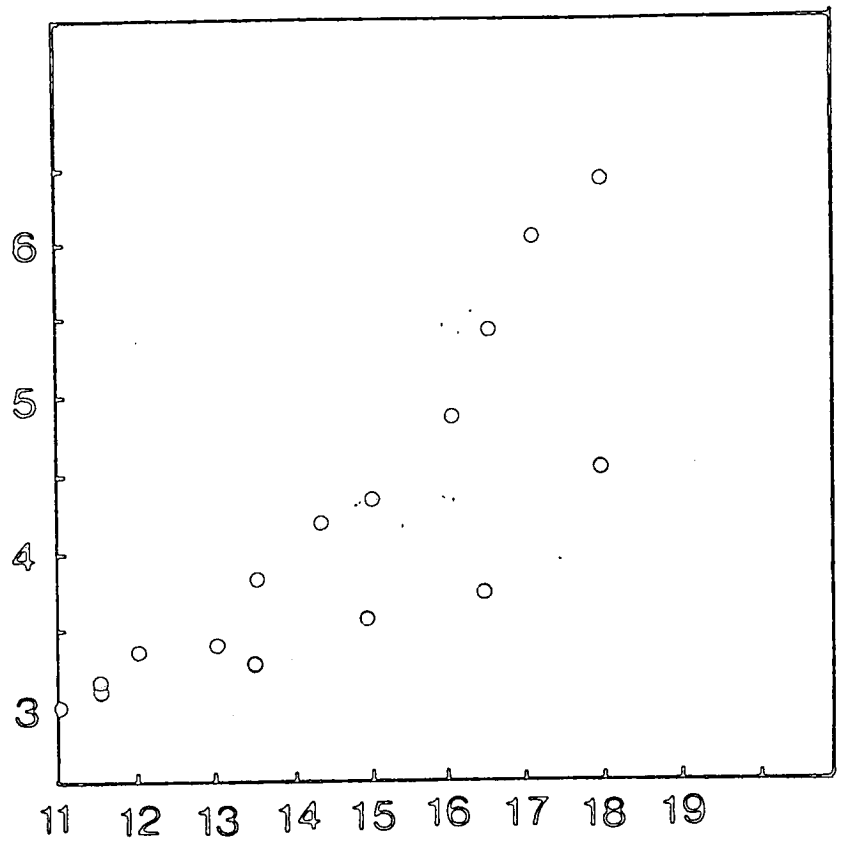


Fig. 3-33 Rate of β-galactosidase inducibility (○), and calculated points where it is assumed that F′ lac replication occurred in cells of size 18-20 units, (○). Data from the same experiment as the CsCl-EtBr gradients.

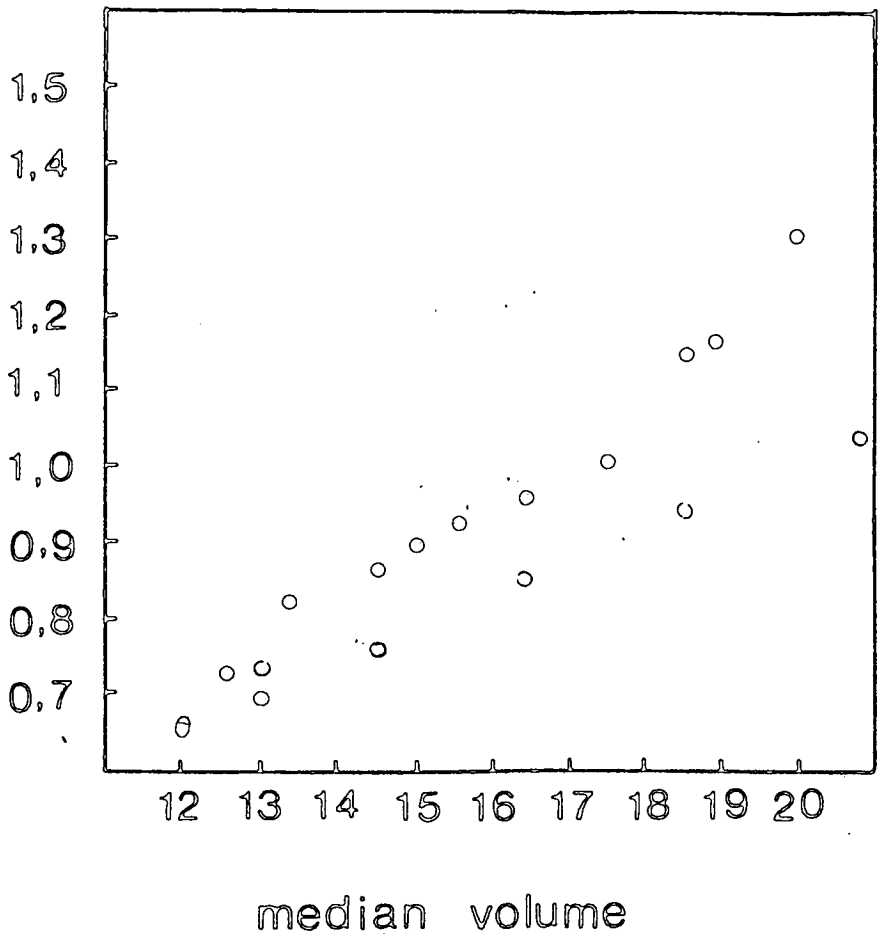


Fig. 3-34 Rate of β-galactosidase inducibility (○), and calculated points where it is assumed that F′*lac* replication occurred in cells of size 18-20 units (○). Data from the same experiment as the agarose gels.

fully light plasmids started to appear at about a third of the cell cycle and increased gradually was taken as an indication that the plasmid molecules of a cell are replicated one at a time, and that a third of a cell cycle passes between two replications. Unfortunately, however, Gustafsson et al.'s experiments don't seem to be quite unambiguous. The density shift involves cooling of the cells and repeated centrifugations. The generation time was 17% shorter in the light medium than in the heavy medium. It seems unproven that these perturbations do not affect the timing of plasmid replication. Gustafsson et al. showed that there was no lag in DNA synthesis after the shift, and the rate of DNA synthesis was approximately the same as the growth rate. Furthermore, newly synthesized plasmid approximately doubled during one generation. Howeveer, these measurements seem too crude to exclude for example multiple fork formation in the cooling period that could lead to premature replication in the light medium.

Gustafsson et al. (1978) also separated exponentially growing cells according to size in a sucrose gradient. They found plasmids which had replicated during a 6 min pulse in every fraction from the gradient. Very little attempt was however made to assure balanced growth right up to the time of killing and analysisng the cells, and little attempt was made to analyse the size and age distribution of the cells in each fraction.

We have assumed in doing these experiments, as all previous studies on the timing of F'lac replication have

done, that F'lac replicates rapidly. There is some evidence for this. The same polymerase is used for the replication of F as for the chromosome (Thompson and Broda, 1973). F replicates bidirectionally (Eichenlaub et al., 1977), and if F'lac is 1/27 the size of the chromosome and replicates at the same rate, then it should take ca. 1 1/2 minutes to replicate. Gustafsson et al. (1978) found by doing pulse-chase experiments that it took R1 less than 4 minutes to complete one replication cycle.

The highest copy number which was obtained for F'lac in the B/r E lac$^-$ strain was 3,6 copies per cell, or 1,6 plasmids per chromosome equivalent. The negative control model of Pritchard et al. (1969) was partly proposed to account for the fact that when F is integrated into the chromosome the replication is initiated at the chromosomal origin, but not at the F origin (Pritchard, 1978), the reason being that the repressor level of the replicon with the lowest copy number was maintained too high for the replication of that replicon to initiate. If F had a higher copy number than the chromosome, an Hfr would be expected to initiate replication at the site of F. However, our results do not rule out the negative control model for F'lac or the chromosome, since there is no information about the integration of this F'lac into the B/r strain.

Since the copy number of F is so low, and F is maintained stably in the cells, it is obvious that some mechanism must exist which ensures that the plasmids are replicated in each

cell cycle. If this is not assured by strict control of the timing of replication of F'lac, how is it then done? If F were able to block cell division until its replication were completed this would ensure that daughter cells free of F were not produced. Such a mechanism has been suggested as the way in which the cells assure that the chromosome has completed replication before division occurs (Clark, 1968; Helsmstetter and Pierucci, 1968). If this were the case, one might expect that F'lac containing strains would have larger cells than their F^- parents , as some of the cells would by chance have failed to replicate the plasmid and thus be prevented from dividing. We examined cultures of our strains growing on minimal medium with a generation time of 58 minutes to see if the F'lac strain had larger cells than its F^- parent. We found, as did Finkelstein and Helmstetter (1977) that both strains had very similar volume distribution when measured with the Coulter Counter and Channelizer (see fig. 3-5).

It can thus be seen that F'lac is stably maintained in the cell at low copy number without an inhibitory effect on cell division. It seems that F'lac is replicated throughout the cell cycle, but a control of the timing of the replication of F'lac in the cell cycle has not been ruled out. The method used in these experiments, i.e. the pulse labelling of an exponential culture and subsequent separation of the cells according to size in a zonal rotor has the advantage over other methods which have been used to study F'lac repli-

cation in the cell cycle, that there is no perturbation of the cells until they are killed and analysed, but a detailed analysis of the size composition of the fractions from the zonal rotor is essential. Further experiments using this method should give unequivocal answers about the timing of F'lac replication in the cell cycle.

4. F-primes carrying chromosomal DNA from the region of the origin of DNA replication.

Introduction.

The following experiments were initially carried out in order to locate the origin of chromosome replication more precisely than had been done previously. The region of initiation had been determined to be either in the lower left quarter of the *E.coli* map or near *ilv*. Various techiques had been used, these included enzyme induction studies in synchronously dividing cultures, the change in frequency of mutation induced by nitrosoguadinine at the replication fork during synchronous growth, frequency of markers transduced by P1, and determination by DNA-DNA hybridization of phage mμ inserted at various points in the chromosome relative to phage lambda inserted at a fixed point at the lambda attachment site (Donachie and Masters, 1969 ; Pato and Glaser, 1968 ; Hohlfield and Vielmetter, 1973 ; Masters and Broda, 1971; Jonasson, 1973; Bird et al., 1972; Louarn et al., 1974).

While this work was in progress and since, *oriC* has been mapped very precisely. Hiraga (1976) isolated F′s which were able to replicate in an Hfr, and these F′s all had in common the region between *dnaA* and *bglB*. These F′s were supposed to carry *oriC* because they could replicate in an Hfr and were presumably using *oriC* to overcome incompatibility with the Hfr. In a later paper, Wada et al., (1977) found that these presumtive F′*oriC*s were also

able to replicate in mafA strains, where F replication normally is inhibited. Masters (1975) isolated a series of F's, which were also concluded to carry oriC since these F's had less DNA per cell than normal and showed abnormal growth properties that were attributed to an extra oriC in the cell. These F's carried the segment between bgl and mtl. Both Masters' and Hiraga's estimates for the location of the origin proved to be incorrect, however, although their experimental rationales probably were right.

Von Meyenburg et al. (1977) isolated a series of deleted derivatives of an F-prime carrying the chromosomal region between ilv and aroE. Strains containing this F-prime had similar characteristics as those reported by Masters (1975) in that they grew more slowly than normal and had a very heterogeneous and increased cell size. The deleted derivatives were obtained by selecting for resistance to male specific phages and loss of Ilv^+. By doing EcoR1 restriction fragment analysis of these plasmids, von Meyenburg et al. were able to locate the locus giving rise to the heterogeneous cell size between the genes bglB and asn. It was suggested that this locus, called het, was identical with oriC. Later, von Meyenburg et al. (1979) located het in the unc genes at 83,4 minutes on the revised E.coli map (Bachmann and Low, 1980), separable from oriC.

Von Meyenburg (1978) and Miki et al. (1978) isolated

specialized lambda transducing phages carrying various genes in the oriC region. Some of these phages were able to establish themselves as plasmids in a lambda lysogenic recA⁻ host, presumably by using oriC for initiation of replication. It was possible by digesting these lambda transducing phages with endonuclease and ligating again to obtain small autonomously replicating plasmids, which did not contain any lambda DNA, and which must therefore have been using oriC for initiating replication (Messer et al., 1978). Similar oriC plasmids have been isolated by mixing purified Ap fragments with an EcoR1 digest of the E.coli chromosome and isolating Ap resistant transformants of a recA strain (Yasuda and Hirota, 1977). These minichromosomes use the chromosomal replication system, and need dnaA and dnaC as well as transcription and protein synthesis for replication (von Meyenburg et al., 1979). OriC has now been accurately mapped at 83,5 minutes on the E.coli genetic map (Bachmann and Low, 1980), and the region has been narrowed down to 232-245 base pairs (Oka et al., 1980).

Results.

A series of Hfrs formed by the insertion of the temperature sensitive $F_{ts}114$ at various points on the chromosome had been studied by Masters and Broda (1971). Amongst these Hfrs at least two strains, ED3029 and ED2436 had F inserted in the region near ilv, where the origin

of replication was believed to be. If F-primes made from these Hfrs did carry the origin of chromosomal replication, oriC, it could be expected that these would now be temperature resistant, because they would be able to make use of the oriC they carried to replicate at the restrictive temperature. It was hoped that by isolating a series of F-primes, some of which were temperature resistant and some of which were not, it would be possible by comparing the regions carried by these F-primes to locate the region giving rise to the temperature resistance.

Isolation of F-primes.

The strains which were used in these experiments are listed in table 4-1. The Hfr strains ED3029 and ED2436 have an $F_{ts}114$ inserted into the chromosome at two different places near the origin of DNA replication. ED3029 transfers bgl early, and to locate the origin of transfer more precisely, P1 lysates were made on the Hfr strain and the cotransduction frequencies of tna-ilv and bgl-ilv to AB2147 were measured. Reduction in linkage of the markers compared to that obtained using a lysate prepared from a female donor would indicate that the F was inserted between the respective markers (Pittard, 1965). The cotransduction frequencies are shown in table 4-2. While the cotransduction of bgl-ilv is about the same for lysates prepared on both ED3029 and JC411(F^-), the cotransduction of tna-ilv is reduced to almost zero when P1 are prepared on ED3029,

Table 4-1 Bacterial strains.

	Sex type	genotype and comments
ED3029	Hfr	thi lac-pro$_{XIII}$(F_{ts}114 lac$^+$)
ED2436	Hfr	(Masters and Broda, 1971). Origin and directions of transfer indicated in fig. 4-1
ED3029bgl$^+$	Hfr	A bglB derivative of ED3029
AB2147	F$^-$	tnaA1 hisG1 ilv-192 metB1 argH1 thi-1 lacY1 gal6 xyl7 mtl2 malA1 tonA2 tsx7 λ^- strA (P1)
AB2147bglB	F$^-$	A bglB derivative of AB2147
MM303	F$^-$	thi-1 ilv-192 argH1 metB xyl$_4$ lacY malA his pyrE tnaA uhp (P1) $\phi 80_s$ λ^r str^r T$_6^r$. A derivative of AB2147
MM318	F$^-$	A recA derivative of MM303
MM318-1	F$^-$	As MM318, but cured of P1
JC411	F$^-$	argG leu metB his malA xyl mtl lac gal str^r
MM9	F$^-$	argG leu metB his malA xyl lac gal ilv pyrE uhp recA str^r nal^r T$_6^r$. A derivative of JC411
χ478	F$^-$	leu lac proC purE try lys metE ara ton tsx str xyl mtl thi
MM202	F$^-$	B$_1$ cysE argE his proA thr leu mtl xyl ara galK lacY str^r

Table 4-1 cont.

	Sex type	genotype and comments
JC5088	Hfr	recA ilv thr
JC166	F^-	rha metE recA56
NF279	F^-	As JC411, but also ilv pyrB rbs recA
DC104	F^-	Δlac_{X74} rbs ara bglB B1 glmS nalA
AN382	F^-	$uncB_{401}$ argE3 thi-1 mtl-1 xyl-5 galK strA (λ)
ER	F^-	B_1 asnA asnB

Table 4-2. Cotransduction of tna and bgl with ilv.

		JC411	ED3029
Exp. 1	tna^+/ilv^+	8/48	0/74
	%	17	0
Exp. 2	tna^+/ilv^+		2/297
	%		0.6
	bgl^-/ilv^+	12/50	32/100
	%	24	32

P1kc lysates were prepared on ED3029 and JC411 as described in the Methods section. AB 2147bgl^+ was transduced with each of the lysates, ilv^+ progeny selected and scored for tna^+ and the ability to grow on salicin minimal plates.

suggesting that the F is inserted between the genes tna and bgl. The orientation of transfer was found to be bgl, rbs, ilv, etc. ED2436 transfers metB early (Masters, 1975). In order to isolate the new F's a recA⁻ recipient strain, which had several markers in the origin region, was made from MM303 by conjugation with an Hfr which transfers the recA⁻ gene early as described in the Methods section. This strain was called MM318. In order to be certain that later results would not be influenced by the presence of the P1 plasmid, P1 was cured from MM318 by displacing the resident P1 with P1 Cmts and selecting cured survivers after 42°C treatment. This strain called MM318-1 was used in subsequent experiments. Hfr strain ED3029 was made Bgl⁺ by selection on salicin indicator plates.

Matings were carried out between the recA⁻ female strain and the two Hfr strains, and proximal and distal markers were selected. The frequency of Ilv⁺progeny in a 30 min. mating between MM318 and ED3029 was about 1 per $5x10^4$ donor cells. About 60 of these were tested for ability to transfer plasmid markers to a recA⁻ strain, but all proved to be transfer defective. When incompatibility towards an F'his was tested by infecting with an F'his, selecting His⁺ progeny and looking for coinheritance of Ilv⁺ and His⁺, it turned out that all His⁺ had lost Ilv⁺. It was therefore concluded that the Ilv⁺ progeny were in fact transfer defective plasmid strains.

Table 4-3。 List of F-primes。

	xyl	cysE	pyrE	uhp	tna	bglB	glmS	unc	asnA	rbs	ilv	metE	metB
pED601	-	+	+	+	+	+	-	nt	nt	-	-	nt	-
pED602	-	+	+	+	+	+	+	+	+	+	+	-	-
pED603	-	+	+	+	+	+	-	nt	nt	-	-	nt	-
pED604	-	-	+	+	+	+	-	nt	nt	-	-	nt	-
pED605	-	+	+	+	+	+	-	nt	nt	-	-	nt	-
pED606	-	-	+	+	+	-	-	nt	nt	-	-	nt	-
pED607	-	+	+	+	+	+	-	nt	nt	-	-	nt	-
pED608	-	-	+	+	+	+	-	nt	nt	-	-	nt	-
pED609	+	+	+	+	+	-	-	nt	nt	-	-	nt	-
pED610	+	+	+	+	+	+	-	nt	nt	-	-	nt	-
pED611	-	+	+	+	+	-	-	nt	nt	-	-	nt	-
pED612	-	+	+	+	+	+	-	nt	nt	-	-	nt	-
pED613	-	-	+	+	+	+	-	nt	nt	-	-	nt	-
pED614	-	-	+	+	+	+	=	nt	nt	-	-	nt	-
pED615	-	+	+	+	+	+	+	+	+	+	+	+	-

Table 4-3. cont.

	xyl	cysE	pyrE	uhp	tna	bglB	glmS[1]	unc[1]	asnA[1]	rbs	ilv	metE	metB
pED617	-	+	+	+	+	+	+	+	+	+	+	-	-
pED618	+	-	+	+	+	+	+	+	+	+	+	-	-
pED619	-	-	+	+	+	+	+	+	+	+	+	+	-
pED620	+	+	+	+	+	+	+	+	+	+	+	-	-
pED621	-	-	-	-	+	+	nt	nt	+	+	+	+	-
pED622	-	-	-	-	+	+	nt	nt	+	+	+	+	-
pED403	-	-	+	+	+	+	-	nt	-	-	-	-	-
pED508	nt	nt	-	-	-	nt	nt	nt	nt	nt	-	nt	+
pED509	nt	nt	-	-	-	nt	nt	nt	nt	nt	+	nt	+

Genes carried by the plasmids. The presence of these genes on the plasmids were tested by transfer to appropriate recipient strains (nt = not tested).

[1] Tests carried out by M. Masters.

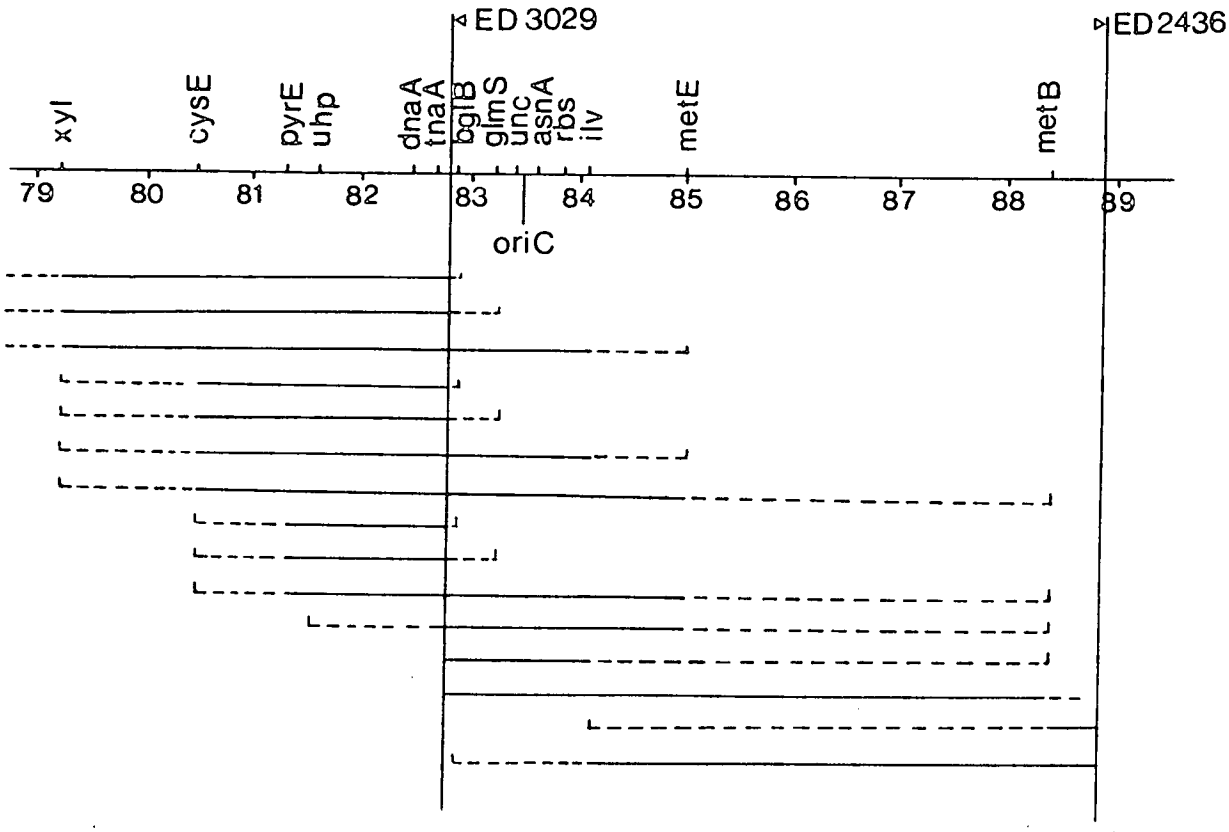


Fig. 4-1

Types of F-primes isolated in this study. The genetic markers on the E. coli chromosome are based on the map published by Bachmann and Low (1980). The origins and directions of transfer of the Hfrs ED3029 and ED2436 are indicated. Each horizontal line represents a class of F-primes. Solid lines represent regions of the genome known to be contained in the F-primes, broken lines represent regions they have not been excluded from containing. The two F-primes isolated from ED2436 are transfer deficient.

When distal markers were selected (*pyrE* or *tna* in the case of ED3029), about 1 Ura$^+$ colony was obtained for every $5x10^6$ - 10^7 donor cells. These were all able to transfer plasmid markers. The markers carried by these plasmids are shown in table 4-3, and the regions covered by the F-primes are depicted in fig. 4-1. None of these F-primes appeared to be deleted for any intermediate markers. The F-primes which were isolated from ED2436 all turned out to be transfer defective. Two of these are shown in fig. 4-1.

The plasmids pED601-pED615 in table 4-3 were all isolated as Ura$^+$ progeny in a single mating between ED3029 and MM318. It is interesting to note that the right hand side end points are mostly confined to the ca 0,3 min region between *bglB* and *glmS*; 10 of the 15 plasmids have been excised in this region, while 3 plasmids have end points between *tna* and *bgl*, 1 between *ilv* and *metE*, and one to the right of *metE*. The left hand side end points are much more dispersed; 8 of them are in the 1,2 min region between *cysE* and *xyl*, 5 are between *pyrE* and *cysE*, which is about 0,8 min, and 2 plasmids were excised to the left of *xyl*. The preferred excision between *bglB* and *glmS* may be due to IS sequences or transposons in this region, or it could be imagined that plasmid strains containing the region to the right of *glmS* on the plasmid are less viable than others.

Characteristics of F-prime strains.

i) Temperature sensitivity.

Cells were grown on minimal medium selective for plasmid markers and then plated on minimal medium with and without selection for plasmid markers at 30°C and 42°C. As expected, some of the plasmid strains showed increased temperature resistance, while others did not. Strains which carried plasmids covering the region between *bgl* and *ilv* showed temperature resistance in that between 0,09 and 100% of the cells viable at 30°C formed colonies at 42°C, but the strains bearing plasmids not covering this region only had up to 0,002% viability at 42°C. Data are given for a few representative plasmids in table 4-4. The temperature resistance of a few clones of pED620 are shown, and it can be seen that these vary a great deal. It can also be seen in table 4-4, that not only were strains carrying plasmids that did not cover the *bgl-ilv* region temperature sensitive, but that the viability of the cells was very much reduced at high temperature even when plasmid markers were not selected for. It seems as if the presence of the temperature sensitive plasmid limits viability of the cells at the restrictive temperature, even in the fully supplemented medium.

Table 4-4. Temperature resistance.

	viability at 32°C (cells/ml)		viability at 42°C (cells/ml)		% viability at 42°C	
	-ura	+ura	-ura	+ura	-ura	+ura
pED403	$1,2 \times 10^8$	2×10^8	5×10^3	4×10^5	0,004	0,2
pED604	7×10^7	5×10^7	$1,2 \times 10^3$	10^5	0,0017	0,2
pED609	$3,2 \times 10^8$	$4,3 \times 10^8$	5×10^3	$2,2 \times 10^5$	0,0015	0,05
pED615	$1,8 \times 10^7$	10^7	$5,4 \times 10^5$	$2,1 \times 10^6$	3	21
pED620-8	$3,5 \times 10^7$	4×10^7	3×10^4	$8,2 \times 10^4$	0,09	0,2
pED620-11	$6,5 \times 10^7$	10^7	$7,5 \times 10^4$	$3,5 \times 10^6$	0,115	3,5
pED620-12	$4,5 \times 10^8$	$4,9 \times 10^8$	$4,5 \times 10^8$	$3,5 \times 10^8$	100	71
pED620-24	$3,1 \times 10^8$	$2,7 \times 10^8$	$1,2 \times 10^8$	$1,5 \times 10^8$	38	55
pED620-28	$6,5 \times 10^7$	$2,6 \times 10^8$	$3,7 \times 10^5$	$6,0 \times 10^5$	0,56	0,23
MM318		$1,2 \times 10^8$		$1,0 \times 10^8$		83

Cells were grown at 32°C in casamino acids medium with trp added (ura was used as a selective plasmid marker). 20µl were spread on plates with and without uracil and incubated at 42°C and 32°C .

ii) Plasmids carrying the bgl-ilv region transfer chromosomal markers.

When transfer properties of the temperature resistant strains were examined, it turned out, unexpectedly, that these transferred chromosomal markers as well as plasmid markers at 30°C. 20 clones, which were isolated at 40°C and mated doing patch matings, no longer transferred plasmid markers with high frequency, but transferred chromosomal markers instead. So rather than using oriC to replicate at high temperature, it appeared that the plasmids had integrated into the chromosome, and this was allowing the cells to grow on the selective medium at high temperature. Data for transfer of chromosomal markers to the rec^+ strain 478 and transfer of plasmid markers to MM9, which is a $recA^-$ F^- strain are given in table 4-5. Transfer properties of a clone of pED620 which was isolated at 42°C, grown at 42°C and then reinoculated at 30°C and 42°C are also shown. Plasmid integration appears to be stable in that even after regrowth at 30°C, the colonies which survived at 42°C continue to transfer predominantly chromosomal markers. Another possible reason for this apparent lack of excision of the F' could be the poor growth properties of the F' bgl-ilv strains, as will be seen later. The integration of the plasmids into the chomosome happens quite quickly, since on second restreaking all clones tested transferred both plasmid and chromo-

Table 4-5. a) Transfer properties of clones isolated at 30°C.

	Number of progeny per donor cell			
	plasmid markers (ura^+)		chromosomal markers (leu^+)	
	32°C	42°C	32°C	42°C
pED609	$1,3 \times 10^{-3}$	$1,5 \times 10^{-6}$	$1,9 \times 10^{-7}$	$1,9 \times 10^{-7}$
pED620	$9,5 \times 10^{-4}$	$1,1 \times 10^{-6}$	$1,7 \times 10^{-4}$	$3,0 \times 10^{-4}$

b) Transfer properties of a clone isolated at 42°C.

pED620 isolated at 42°C	Number of progeny per donor cell	
	plasmid markers (ura^+)	chromosomal markers (leu^+)
grown at 30°C	$1,1 \times 10^{-6}$	$1,8 \times 10^{-3}$
grown at 42°C	$2,0 \times 10^{-6}$	$3,0 \times 10^{-3}$

a) Donor and recipient strains were grown at 32°C and mixed in a ratio of 1 donor : 10 recipient cells. After 60 min. mating, the cells were plated on selective plates. The recipient strain for measuring transfer of plasmid markers was MM9, and χ478 for chromosomal markers.

b) A clone of pED620 was isolated at 42°C and inoculated and grown overnight at 42°C. From this culture overnight cultures were set up at 30°C and 42°C. Matings were performed as above.

somal markers at 30°C.

The site of integration was determined by doing interrupted matings at 42°C to λ 478 and examining the time of entry of metE, leu, pro, trp and lys. MetE was in all instances transferred early and lys late (fig. 4-3).

As noted earlier, oriC has now been located very precisely between the markers unc and asn. By doing further mating experiments, M. Masters and H. Wolf-Watz showed (Masters et al.,1979) that the integrated strains transferred bgl$^+$ and unc$^+$ early, but ilv$^+$ and ura$^+$ late, suggesting that integration occurs between unc and asn on the chromosomal DNA carried by the plasmid, and is not mediated by the IS sequences of F, which is generally thought to be the basis of normal Hfr fromation (Davidson et al., 1975). M.Masters also showed that miniplasmids, which contained a small chromosomal fragment including oriC, would make cointegrates with the F-primes carrying the bgl-ilv region, but not with others. This was further evidence that the site of integration was very close to oriC, and the locus at which it occurs has been termed osr, for "origin specific recombination".

iii) Ability of plasmids carrying the bgl-ilv region to replicate in an Hfr recA$^-$ strain.

It was suggested by Hiraga (1976) that the ability of F-primes to replicate in an Hfr strain could be used as a criterion forpossession of oriC ; plasmids carrying

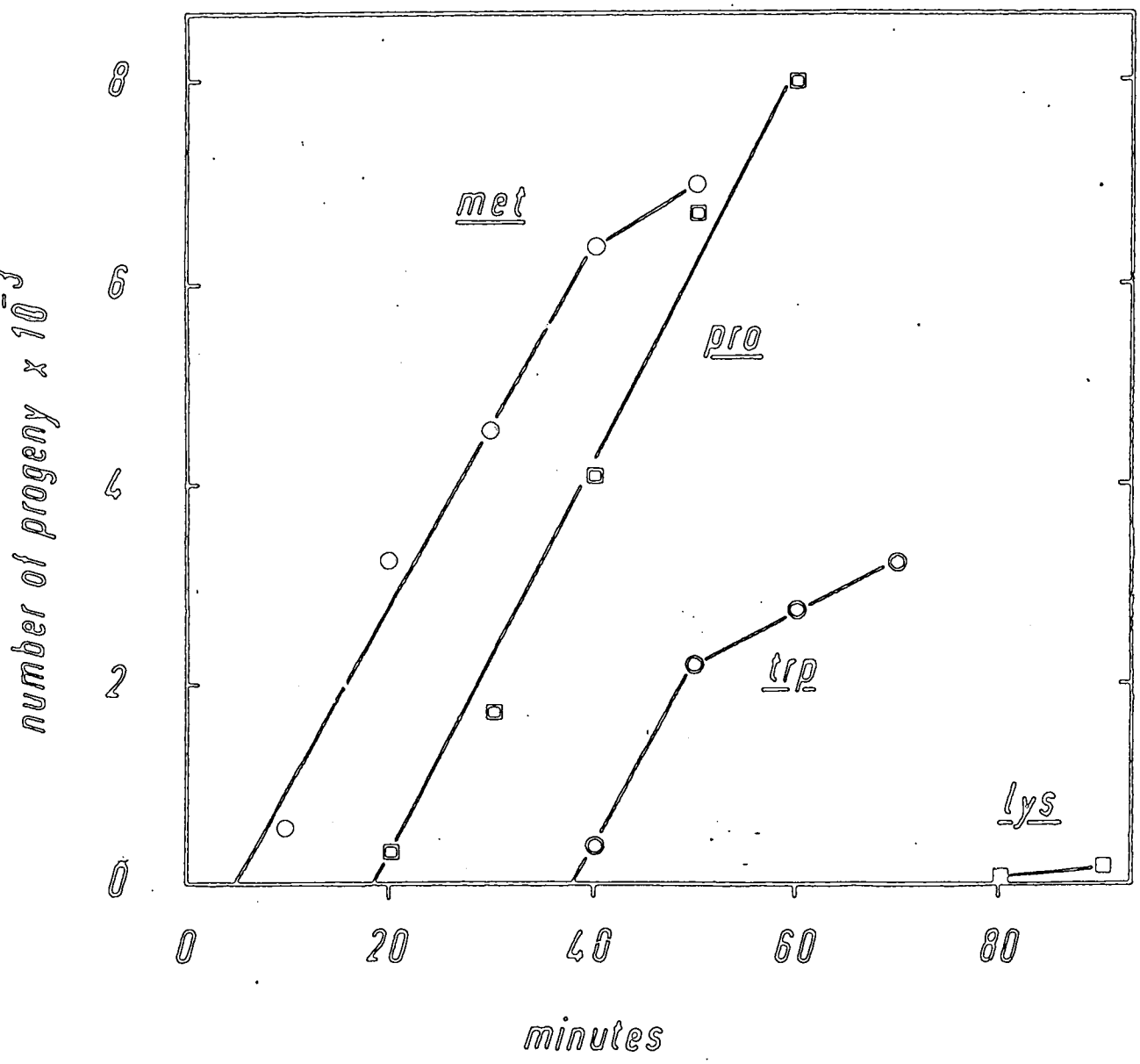


Fig. 4-2

An interrupted mating at 42°C with pED620/MM318-1 as donor. The donor was grown overnight at 42°C on medium selective for plasmid markers from an inoculum grown at 30°C. It was reinoculated into selective medium at 42°C and allowed to grow for four mass doublings. It was then mixed with χ478 (metE proC try purE lys leu), and samples were taken as indicated and plated to measure Met^+, Pro^+, Try^+, and Lys^+. The experiment was done by H. Wolf-Watz.

oriC would be able to overcome incompatibility by using oriC for initiation of replication. A number of these plasmids were isolated by Hiraga, and the locus was termed poh for "permissive on Hfr". The plasmids carrying the bgl-ilv region were in fact able to replicate in the Hfr JC5088 $recA^-$, while the others were not. JC5088 was made Bgl^+ and bgl used as a selective marker. Results of cross-streak matings are shown in table 4-6. It seems, that while the temperature sensitive plasmids were not able to overcome the obstacle in replication at 42°C by using the oriC, they are in fact able to make use of oriC to overcome incompatibility with an Hfr.

iv) Growth characteristics of plasmid strains.

When cell size and growth rate were measured, it turned out that these varied with different plasmids. Plasmid strains that extended past bgl to the right had larger cells and grew more slowly than strains harboring plasmids which did not extend past bgl. Plasmid strains which extended towards bgl from ED2436 and went only as far as rbs did not have large cell size. Strains in which the plasmid had integrated into the chromosome did on the other hand produce cells that were only slightly larger than the parent strains. Data for pED609, which does not cover the bgl-ilv region and for several clones of pED620, which does cover the bgl-ilv region are shown in fig. 4-3 (growth rates) and figs. 4-4 and 4-5 (size distribution).

Table 4-6. Transfer of F-primes to Hfr JC5088 (cross-streaks).

	bgl^+
pED601	-
pED602	+
pED603	-
pED604	-
pED605	-
pED606	nt
pED607	-
pED608	-
pED609	nt
pED610	-
pED611	nt
pED612	-
pED613	-
pED614	-
pED615	+
pED617	+
pED618	+
pED619	+
pED620	+
pED621	+
pED622	+
pED403	-

Cross-streak matings were done between JC5088*bgl*$^+$ and the F′strains. F′strains where the plasmid did not cover *bgl* were not tested (nt).

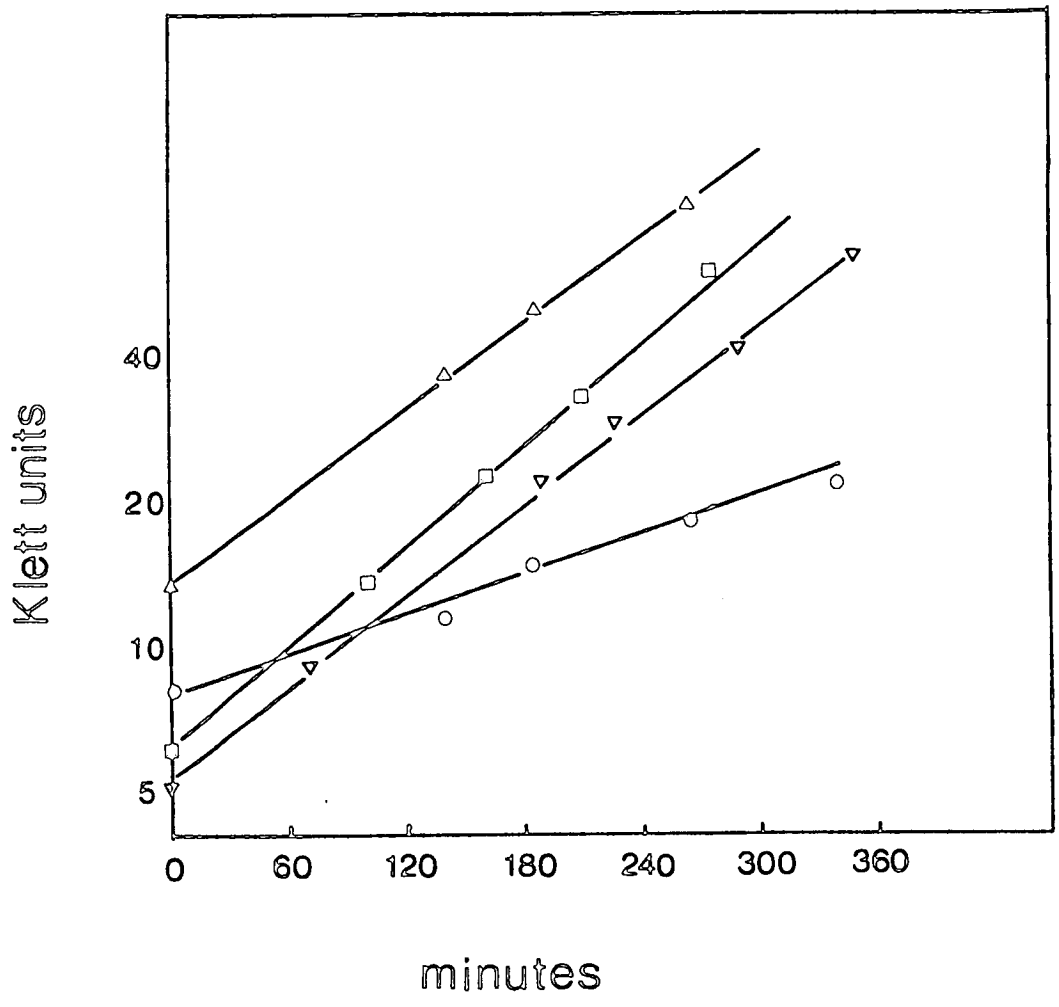


Fig. 4-3

Growth rates of MM318-1 (□), pED609/MM318-1 (▽), pED620-8/MM318-1 (
and pED620-12/MM318-1 (△). The cells were grown in medium
containing casamino acids and try at 32°C.

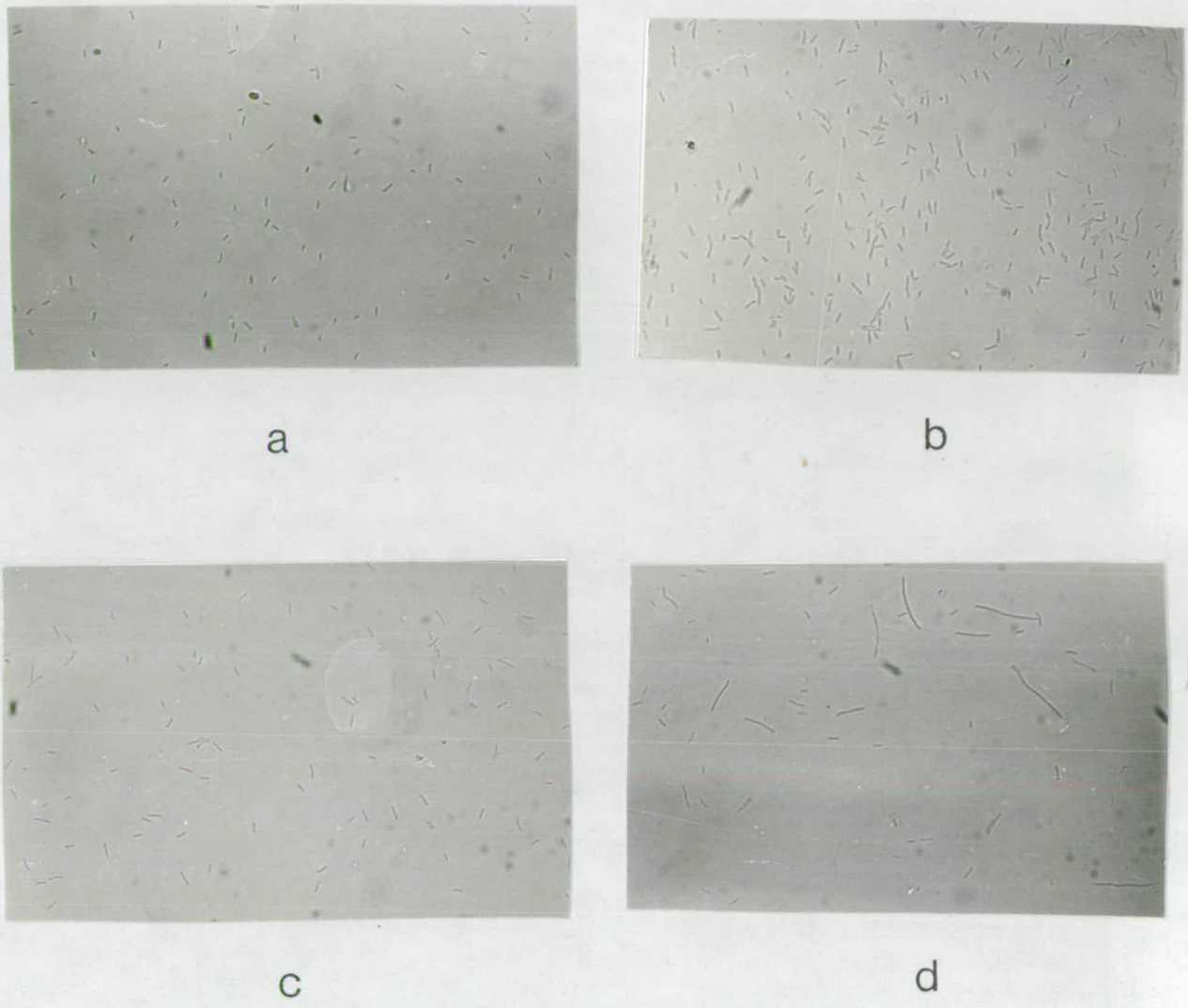


Fig. 4-4 Photographs of a) MM318-1 b) pED609/MM318-1 c) pED620-12/MM318-1 d) pED620-8/MM318-1 . The cultures were spread on agar-covered slides (to immobilize the cells), and photographed using a Leitz Orthomat Photomicroscope. The overall magnification is x375. The cell lengths were measured from the photographs by using a magnifying glass with a built-in scale.

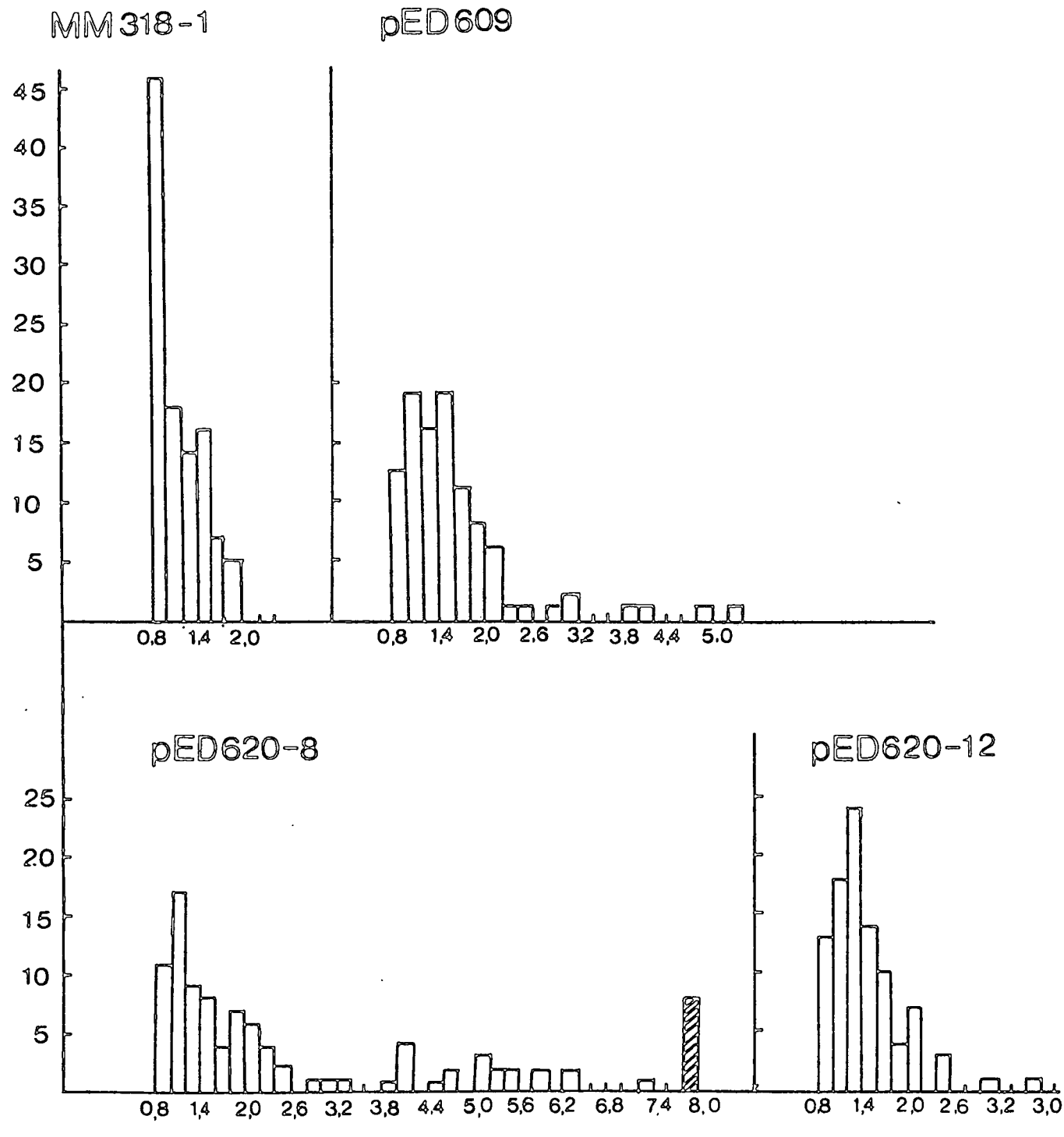


Fig. 4-5

Size distributions of MM318-1, pED609/MM318-1, pED620-8/MM318-1 and pED620-12/MM318-1. Each distribution contains a hundred cell length measurements. The lengths of all cells in randomly selected fields were determined. Each scale unit represents 2,6 μ. The shaded area contains both cells of the indicated size and all longer ones observed.

Table 4-7 lists the temperature sensitivities and transfer properties of these strains as well as average cell sizes and generation times. The clone of pED620, containing the fewest Hfr cells has a growth rate that is twice as long as that of the parent F^- strain, and the average cell size is more than twice that of the F^- strain. The clone, which seems to be fully Hfr, pED620-12, is only slightly bigger than the F^- and grows at a similar rate.

Table 4-7

	% viability at 42°C		F′ transfer	chromosome mobil.		poh	gen. time	average cell size
F-prime	+ura	-ura	ura to MM9	leu to λ478	ilv to JC5088		min	μ
pED609	0,05	0,0015	+	-		n.t.	100	4,5
pED620-8	0,1	0,08	+	+		+	200	7,6
pED620-11	3,5	0,12	+	+		+	172	
pED620-12	71	100	-	+		-	100	3,8
pED620-24	55	35	+	+		+	160	
pED620-28	0,21	0,56	+	+		+	190	
MM318-1	85						90	3,3

Transfer properties were tested by doing cross-streak matings.

Discussion.

F-primes were isolated from matings between Hfrs and a recA$^-$ recipient. The formation of F-primes carrying proximal markers was much more frequent than F-primes carrying distal markers. The F-primes carrying only proximal markers turned out to be transfer defective. This is in accordance with the findings of Guyer and Clark (1976), who studied the progeny of matings between an Hfr donor and a recA$^-$ recipient. Guyer and Clark suggested that the transfer defective F-primes in fact were formed in the recipient cell after conjugal transfer rather than in the donor cell prior to transfer as suggested by the Campbell model (Campbell, 1962). Guyer et al. (1977) did heteroduplex studies with these F-primes and found that they were in fact lacking the region on F that is transferred late by an Hfr donor. The F-primes carrying proximal markers which were transfer proficient were on the other hand probably formed in the donor as suggested by the Campbell model.

The plasmid strains did not survive well at 42°C even in the fully supplemented medium. Survival of F'oriC$^-$ at 42°C was no more than 0,2% of survival at 30°C, whereas survival of the F$^-$ host was 100%. Survival of F'oriC$^+$ was 0,2-100% due to integration of the plasmid into the chromosome. It seems that the presence of the plasmid inhibits colony formation even in fully supplemented medium at the restrictive temperature. This implies a

connection between plasmid replication and cell division, possibly through attachment of the plasmid to the membrane. This would suggest that the mutation in the plasmid is either in DNA elongation or segregation rather than initiation, since the extra origin, oriC, present on some of these plasmids, does not rescue F replication at the high temperature as it does in an Hfr strain.

There have been reports about temperature sensitive plasmids that interfere with cell division at the restrictive temperature. These include ColVBtrp (Koyama and Yura, 1975), Rts1 (Yamamoto and Kaji, 1977), P1 (Scott, 1970) and F'lac (Miki and Horiuchi, cited in Koyama and Yura, 1974). It has also been reported that the introduction into a host cell of certain plasmids that have been impaired in replication by UV irradiation prevents the cell from dividing (Donachie, 1974; MacQueen and Donachie, 1977). The introduction of UV irradiated plasmids into a cell induces lambda as well as inhibiting cell division. These phenomena have been integrated into the so-called SOS repair hypothesis that states that the recA gene product is induced by DNA degradation products to inactivate repressors of various functions (Hanawalt et al., 1979). Donachie (1974) and MacQueen and Donachie (1977) proposed on the other hand that cell division is inhibited by these irradiated plasmids because a division block is set up at initiation of these replicons and not released until termination. If there is indeed a division block set up

when these plasmids initiate replication at the F origin that is not released at 42°C because replication cannot terminate, and if this block is not relieved even by a subsequent initiation at oriC, if present, then this could be the reason for the cells not dividing at the high temperature. There is some evidence, however, that the inhibition by irradiated plasmids of division is suppressed by the recA mutation (Teather, 1974). Since the strain used in these experiments is recA$^-$, the temperature sensitivity in fully supplemented medium observed in our strain may not reflect the same phenomenon as the division block set up by the irradiated replicons.

We have found that F-primes which carry the origin region of the chromosome can be divided into two distinct classes; those which include the region between bgl and ilv, in which oriC is, and those which don't. The plasmid strains which do carry this region show abnormal cell division, are able to replicate in Hfr strains, and integrate into the chromosome of a recA$^-$ host at a very high rate. Plasmids strains which do not contain this region of the chromosome do not have these characteristics.

Why should this be the case ? Let us first consider Hfr formation.

The F'bgl-ilv formed Hfrs at a high frequency, which does not normally happen in a recA$^-$ strain (Wilkins, 1969; Cullum and Broda, 1979). The site of recombination with the chromosome was determined by doing mating experiments,

and it was concluded that the site of integration was between unc and asn, indistinguishable by this type of experiment from oriC. It was shown that the site of recombination was not at the IS sequences of F, which probably normally mediate integration of F into the chromosome. Our suggestion is that this site-specific recombination is due to the unique structure of, and/or enzymatic events near the origin. This would suggest that some event occurs near the origin which obviates the need for and may thus be analogous to the action of the recA protein.

The recA protein seems to be involved in early events in recombination (Radding, 1978). These include the formation of so-called D-loops, which are formed when single strands interact with superhelical DNA, which is believed to be one of the initial stages in recombination (Holloman and Radding, 1976), and so-called cutting-in-trans, which is cutting of circular DNA, induced by the presence of repairable lesions in homologous molecules, or by the presence of homologous single strands. This may represent the cleavage of a D-loop (Radding, 1978).

The DNA sequence at the origin allows for extensive single-stranded looping out. It is possible that the single strands somehow promote recombination without the aid of recA. Another possibility is that the DNA is nicked at initiation and that this allows recombination to bypass recA. It has been shown that an increased frequency

of single-stranded breaks or gaps in the DNA results in a hyper-rec phenotype (Konrad, 1977). It is uncertain whether this integration is reversible, it looks as if the integrated plasmids do not excise as easily as they were integrated, but the fact that the plasmid strains would grow with only half the rate of the integrated strains may have resulted in selection against them, making them difficult to detect.

I would next like to consider the abnormalities in cell division which seem to occur in the F'*oriC* strains.

The cell size of the *oriC*-plasmid strains was considerably larger than that of the plasmid strains in which the plasmids did not extend to *oriC*, or the strains where the plasmid was integrated into the chromosome. Heterogeneity in the size of cells diploid for the *oriC* region has been reported previously (Masters, 1975; von Meyenburg et al., 1977; Wada et al., 1977), and the locus which gives rise to the heterogenous cell size has been mapped within the *unc* genes at 83,4 minutes on the *E. coli* map (Bachmann & Low, 1980) which code for the 8 subunits of ATP synthetase (von Meyenburg et al., 1978). The integration into the chromosome of the plasmids containing this *het* region results in almost normal cell size. It appears therefore not to be a double dose of this site *per se* that inhibits cell division, but an extra copy *in trans*.

It is tempting to speculate whether this behaviour

could be related to the control of DNA replication, and whether it can reveal anything about its control. Termination of chromosome replication is needed for cell division, and another set of F'oriCs have been reported to contain less DNA per mass than normal (Masters, 1975). The positive control theory assumes that each initiation event requires a substance or structure produced in limited quantity and made available for use only when the cell reaches initiation mass (Helmstetter et al., 1968; Donachie and Masters, 1969). If het were a receptor site for such initiation substance, there might be competition between the het on the chromosome and that on the plasmid for the limiting material, and the chromosome might sometimes miss it, which would result in fewer initiations.

On the negative control model, one would expect that het coded for a repressor for initiation, and an elevated dose of repressor resulted in fewer initiations. This is however unlikely, since it has been demonstrated that het is in the ATP synthetase genes. ATP synthetase is needed for ion transport over the membrane, but these ions play a part in cell division. An elevated copy number of this region could therefore affect cell division.

The trans-dependence of the het effect makes it unlikely that it is due to a diffusable gene product, unless the copy number of the plasmids is higher in the autonomous state than when integrated.

The copy number of the minichromosomes containing

the *het* region has been reported to be low, Hirota et al. (1979) reported 1-3 copies per chromosome, and Messer et al. (1979) 2-4 copies per chromosome, while von Meyenburg et al. (1979) estimated that there were 6-12 copies per chromosome, taking into account the amount of plasmidless cells in the population. The copy number of the F'*oriC*s is not known, but all estimates of the copy number of F per cell agree that it is very close to one per chromosome. This therefore rather speaks for the *het* effect being due to a site on the chromosome rather than a diffusable gene product.

Various groups have claimed to find attachment between the origin region and the membrane (Hirota et al. 1979; Wolf-Watz and Masters, 1979), and *het* seems to be a likely candidate for such an attachment site. However, *het* is not required for the replication of minichromosomes, since a number of these exist which lack this region (Messer et al., 1979; von Meyenburg et al., 1979). *Het* is therefore not required for replication from *oriC*, it may however be needed for chromosome replication, or it may have a regulatory role in DNA replication although it is dispensable. The *het* effect thus provides interesting prospects for further studies on DNA replication.

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Evidence that F′ *lac* Replicates Asynchronously During the Cell Cycle of *Escherichia coli* B/r

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Summary. The replication of F′ *lac* was studied in exponentially growing cultures of *E. coli* B/r. The cells were pulse induced for the synthesis of β-galactosidase and their DNA pulse labelled with ^{3}H thymidine. The cells were then separated into age classes by centrifugation through a sucrose gradient in a zonal rotor. Plasmid replication was measured in each age fraction by three methods: the rate at which β-galactosidase could be induced, the amount of label incorporated into CCC plasmid DNA which had been separated from chromosomal DNA on agarose gels, and the amount of label incorporated into plasmid DNA which had been separated from chromosomal DNA by ultracentrifugation through CsCl-EtBr gradients. All these methods gave the same result, that replication of F′ *lac* occurs in cells of all ages and is not confined to a part of the cell cycle.

Introduction

Certain plasmids of *Escherichia coli*, of which the plasmid F is representative, are maintained in the cell at approximately one plasmid copy per chromosome (Frame and Bishop, 1971). The number of chromosomes per cell is controlled by the fact that initiation of chromosome replication occurs only once per cell cycle at the time that a fixed mass, the initiation mass, is reached (Donachie, 1968). This mass is attained at different times relative to cell division in cells growing at different rates. A similar control system could assure that F too replicates once and only once in each cell cycle. If such a control system exists, F replication, like chromosome replication, might be expected to be initiated at a fixed time relative to division in a cell cycle of a given length.

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Several groups have studied the timing of F replication in order to see whether it could be correlated with other events of the cell cycle. This was done by measuring the rate at which a plasmid-coded enzyme could be induced and assuming that this rate was proportional to the number of copies of the plasmid. Both synchronously dividing (Donachie and Masters, 1966; Nishi and Horiuchi, 1966) and asynchronous cultures (Zeuthen and Pato, 1971; Cooper, 1972; Davis and Helmstetter, 1973; Finkelstein and Helmstetter, 1977) have been used. The exponentially growing asynchronous cultures were separated after induction by the membrane elution technique (Helmstetter, 1967) into fractions containing cells which had been of the same age at the time of induction. Although each group concluded that F is replicated at a fixed time relative to division in all cells growing at a particular rate, they disagreed as to when that time was; thus the attempts of each group to relate the time of F replication to other events in the cell cycle could not be reconciled with one another. Pritchard, Chandler and Collins (1975) attempted to see if F replication were coupled to any stage of the replication cycle of the host chromosome or to cell division, without measuring replication in cells of different ages. They were unable to demonstrate a correlation between F replication and any other event.

However, even the conclusion that F replicates at a fixed time in the cell cycle has recently been called into question. Gustafsson and Nordström (1978), using asynchronous exponentially growing cultures and a density transfer technique to measure F replication, concluded that the replication of F is not confined to cells of any particular age. They found, rather, that F was replicated in cells of all ages.

We report here results obtained from pulse induced and pulse labelled exponential populations, separated for analysis into age classes by centrifuga-

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tion through a sucrose gradient in a zonal rotor (Beck and Park, 1976; Moore, 1976). This technique allows one to examine cells which can be induced or pulsed during exponential growth but need not be allowed to grow further during subsequent manipulations. The limitations of working with synchronized cultures or with cultures which require a period of growth on the surface of a membrane after pulsing can thus be avoided. Using this technique we measured not only the rate of synthesis of plasmid coded β-galactosidase but also measured directly the amount of pulse label incorporated into covalently closed circular plasmid DNA. We conclude, as did Gustafsson and Nordström (1978) that F is replicated in cells of all ages and not at any particular time relative to cell division.

Methods and Materials

Bacterial Strains and Growth Conditions

The parent of the B/r strain used in these experiments was originally obtained from S. Glover and has had *leu*$^-$, *cysB*$^-$, *thy*$^-$ and *his*$^-$ mutations introduced by UV mutagenesis (Masters 1970). Strains from this line of descent will be referred to as B/r E. This strain was made *lac*$^-$ by using o-nitrophenyl-β-D-thiogalactoside as a selective agent as described by Smith and Sadler (1971). The *lac*$^-$ derivative obtained could not be induced by isopropylthiogalactoside (IPTG) to form β-galactosidase and the mutation was therefore judged to be in the *z* gene. No band of covalently closed circular molecules was observed when DNA from a lysate of this strain was centrifuged in a CsCl-Ethidium (Et) Br gradient and it was therefore judged to be free of plasmids. The strain was made resistant to T6 and an F′ *lac* transferred into it from a K12 strain using T6 as counterselection. This Lac$^+$ strain was shown to contain covalently closed circular DNA.

Bacterial cultures were grown at 37° in a shaking water bath in a medium containing M9 salts 0.2% glycerol and required supplements at 20 μg/ml.

Separation of Cells by Centrifugation in the Zonal Rotor

A liter of cells was grown in M9 minimal medium supplemented as described until a cell density of 10^8 cells/ml was reached. The culture was then labelled and/or induced as described for the individual experiments. DNA and protein synthesis were terminated by the addition of 20 μg/ml of nalidixic acid and 200 μg/ml of chloramphenicol respectively. Penicillin at 60 U/ml was also added to prevent large cells from dividing after they had been applied to the rotor. The cells were then rapidly chilled by pouring the culture over ice, centrifuged in the cold and resuspended in 10 ml of 3% sucrose solution in the minimal salts medium containing the antibiotics.

The cells were then applied to a preformed sucrose gradient (see below) in an MSE type A low speed zonal rotor. This rotor, which is made of perspex, has a radius of about 18 cm and a capacity of 1300 ml. The rotor was kept spinning in an MSE Mistral 6L centrifuge at 600 rpm and the cells were introduced through the central feed and sealed with a 50 ml overlay of distilled water. The gradient was centrifuged at 4° C at 2500 rpm for 20–40 min. It was then allowed to slowly decelerate to 600 rpm. Fractions of 20 ml were collected from the center by pumping 30% sucrose solution into the edge of the rotor. This was done slowly to avoid turbulence and viscous drag. The cells were generally found distributed amongst 15–20 fractions.

In order to prepare synchronous cultures, cells which had not been treated with antibiotics were fractionated at 15° C. A fraction containing small cells was suspended in prewarmed medium at a final concentration of 3×10^7 cells/ml.

Preparation of the Sucrose Gradient

A 5–20% sucrose gradient was made in the minimal salts medium, with antibiotics added if required. The sucrose was pumped into the edge of the zonal rotor which was spinning at 600 rpm. Solutions of 5% and 20% sucrose were used. The light sucrose solution was pumped in first and gradually displaced towards the center by heavier sucrose solution. As 5% sucrose solution was pumped into the rotor, 20% sucrose solution was drawn into the flask containing the 5% sucrose solution, thus creating a gradient that was linear with respect to the radius of the rotor.

Induction and Measurement of β-Galactosidase

The enzyme β-galactosidase was induced by adding 10^{-3} M IPTG either to an aliquot of a synchronous culture or to the whole of an exponential culture which was to be fractionated on a gradient. Induction was terminated after six minutes by adding chloramphenicol (200 μg/ml). The enzyme was assayed according to the procedure of Abbo and Pardee (1960).

Radioactive Labelling and Counting

DNA was labelled by adding ^{3}H thymine, ^{3}H thymidine or ^{14}C thymine to the growth medium, as described for the individual experiments. Samples were taken onto 3MM Whatman filter paper discs, washed twice with 10% TCA (trichloracetic acid), containing 100 μg/ml thymine or thymidine, once with 80% ethanol and then dried. The discs were counted in 5 ml of a 0.5% solution of Butyl-PBD (2-(4′-tert-butylphenyl)-5-(4″-biphenyl)-1,3,4, oxadiazole) (Ciba, Cambridge, England) in a Packard Tri-Carb liquid scintillation counter. When both ^{3}H and ^{14}C were present in a single sample the scintillation counter was adjusted such that less than 0.1% of the tritium counts appeared in the ^{14}C channel. The spillover of ^{14}C into the ^{3}H channel was 20% of the ^{3}H counts.

When bands cut out of a gel were to be counted, they were dissolved by shaking overnight in scintillation fluid made up as follows: 720 ml toluene, 80 ml Soluene (Packard), 2,88 g PPO (2,5 Diphenyloxazole (Packard)), 80.8 mg dimethyl POPOP (1,4-bis-(2-(4-Methyl-5-Phenyloxazolyl))-Benzene (Packard)) and 0.72 ml 10% SDS (sodium dodecyl sulphate (BDH)). The vials were then cooled and counted in the scintillation counter.

All radioactive chemicals were obtained from the Radiochemical Centre, Amersham, England.

Centrifugation of Crude Lysates in CsCl-EtBr Gradients

Exponentially growing bacterial cultures were pulse-labelled with ^{3}H thymidine and fractionated in the zonal rotor. 10 ml fractions of cells were mixed with 10^9 bacteria fully labelled with ^{14}C thymine. The cells were centrifuged and resuspended in 2 ml of TES (0.05 M tris, 0.05 M NaCl 0.005 M EDTA, pH 8.0) buffer contain-

ing 4 mg/ml lysozyme. After 15–30 min incubation at 37° C, 10% SDS was added dropwise to complete lysis. To 1.5 ml of this lysate 3.3 ml H_2O, 2.0 ml EtBr (700 μg/ml in 0.1 M Na phosphate pH 7.0) and 6.55 g CsCl were added. Samples of this mixture were centrifuged for 48 hours in a 50Ti Beckman centrifuge rotor at 36,000 rpm at 18° C. Ten-drop fractions were collected from the bottom of the tube, either by puncturing the tube or by pumping with a peristaltic pump.

DNA Electrophoresis on Slab Gels

Cleared lysates were prepared from zonal rotor fractions of 10–20 ml by the procedure of Clewell and Helinski (1969). The cleared lysates were further purified and the DNA precipitated as described by Meyers et al. (1976). Slab gels were prepared and the DNA samples run using the procedure of Sharp, Sugden and Sambrook (1973) as modified by Thompson, Hughes and Broda (1974) except that 0.7% agarose was used to make the gels. The EtBr-stained gels were examined using UV light. The intensity of the bands was determined, when necessary, by scanning photographs of the gels in a Joyce-Loebl densitometer.

Results

Separation of Cells on the Gradient

The procedure used to measure enzyme inducibility and the rate of DNA synthesis in cells of different ages growing in exponential culture was to pulse an exponential culture containing 10^8 cells/ml with an inducer for β-galactosidase and/or with radioactive thymidine to label the DNA. The cells of the exponential culture were prevented from growing further by addition of a mixture of antibiotics, collected and then separated into fractions containing cells of particular sizes by centrifugation through a sucrose gradient in a zonal rotor. To assure that the cells to be separated were in balanced growth, they were grown in minimal glycerol medium for approximately fifteen generations before treatment. The generation time in this medium was 58 min. The cells generally appeared in fifteen to twenty of the 65 × 20 ml fractions collected. Figure 1 shows how the median volume of the cells varies across the gradient. The median volume does not quite double because the largest fractions are not completely homogeneous and contain some smaller cells. The smaller fractions, on the other hand are very homogeneous. The fraction of cells with septa was estimated microscopically for each fraction and it can be seen that the septa appear with quite good synchrony in fractions 10–13.

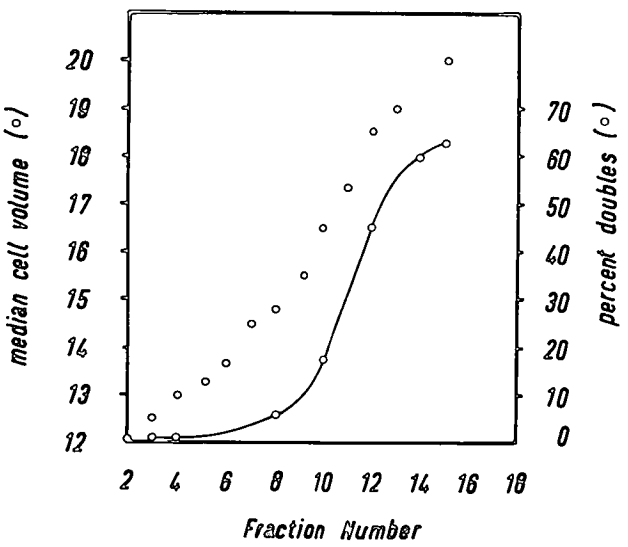

Fig. 1. Separation of cells of *E. coli* B/r E into size classes by centrifugation in the zonal rotor. A liter of cells of *E. coli* B/r E was prepared and centrifuged through a sucrose gradient in the zonal rotor as described in the *Methods* section. The median cell volume of the cells in each sample was determined using a Coulter Counter (Model Z_B) and Channelyzer (Coulter Electronics Ltd.). The volumes are expressed in relative units. The fraction of cells in each sample exhibiting a septum was determined microscopically using a Petroff-Hauser Cell Counting Chamber

Synchronous Cultures

The earliest fractions (i.e. those containing the smallest cells) prepared from a culture not treated with antibiotics could be inoculated to start a culture which divides synchronously after an appropriate interval. A synchronous culture was made by reinoculating cells from an early fraction into fresh medium. At frequent intervals thereafter the rate of DNA synthesis, the inducibility of β-galactosidase and cell number were measured (Fig. 2). It can be seen that DNA synthesis doubles in rate once per cell cycle and shows the decrease in rate characteristic of long generation times in which synthesis is discontinuous (Cooper and Helmstetter, 1968).

The initial rate of synthesis of β-galactosidase after induction has been used to measure the number of copies of the gene specifying the enzyme in most previous studies of F′ replication during the cell cycle. The presumption is that the rate at which the enzyme can be induced in a particular cell is proportional to the number of gene copies in that cell and will remain constant until the gene is duplicated. If the gene is duplicated simultaneously in all cells in a culture the rate at which the enzyme specified can be induced will double at the time of duplication.

It may be seen (Fig. 2), that the initial rate at which β-galactosidase can be induced in the synchronous culture is very low. This depressed inducibility is characteristic of cells recently centrifuged through sucrose solution (Donachie and Masters, 1966), and obscures any changes in inducibility due to gene dos-

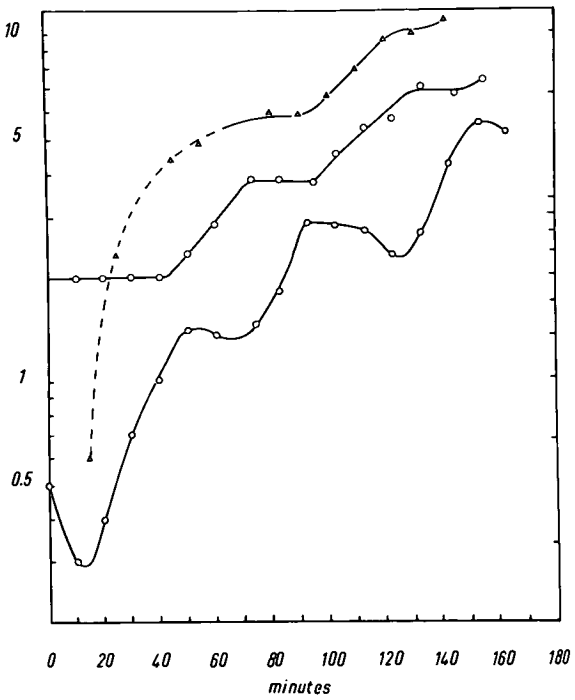

Fig. 2. DNA and β-galactosidase synthesis in a synchronous culture of *E. coli* B/r E/F′ *lac*. An exponential culture was fractionated in the zonal rotor. Reinoculating cells from Fraction 2 yielded a synchronously dividing culture. At 10 min intervals several 1 ml aliquots of cells were withdrawn from the culture. To measure DNA synthesis one of these aliquots was pulse labelled with ^{3}H thymidine (0.5 μCi/ml, 50 Ci/mmol) for 1 min before adding 1 ml 10% cold TCA containing 100 μg/ml thymidine. β-galactosidase was induced by adding 10^{-3} M IPTG to a second 1 ml aliquot. Induction was terminated after 5 min by adding 250 μg/ml chloramphenicol. A third aliquot was preserved in 10% formaldehyde for later counting of cells. Radioactivity (o) enzyme (Δ) and cell numbers (o) in each sample were estimated as described in Methods.

age that may be expected to have occurred during the first cell cycle after synchronization. Inducibility is soon recovered however and a stepwise doubling in the rate at which β-galactosidase can be induced is evident at about the time of the second cell division. This suggests that a stepwise doubling of the genes coding for β-galactosidase has occurred at this time, presumably because F has replicated simultaneously in all cells.

Thus synchronous cultures of B/r E/F′ *lac* behave similarly to those of strains studied previously (Donachie and Masters, 1966; Nishi and Horiuchi, 1966) in that the rate at which β-galactosidase synthesis can be induced doubles suddenly, once per cell cycle.

Having confirmed that F′ *lac* appears to replicate at a particular time in the cell cycle of synchronously dividing bacteria we proceeded to our analysis of its replication in exponential cultures fractionated in the zonal rotor.

Exponential Cultures Separated in the Zonal Rotor

Rate of DNA Synthesis. The rate of DNA synthesis in cells of different sizes was measured for both B/r *lac*$^+$ and B/r *lac*$^-$/F′ *lac*$^+$. This was done by pulsing an exponential culture with H^3 thymidine for one minute. The pulse was terminated by a chase of cold thymidine. Five minutes later a mixture of antibiotics was added to stop all macromolecular synthesis (see Methods) and the cells collected and separated in the zonal rotor. It can be seen (Fig. 3, Panel A) that the rate of DNA synthesis approximately doubles at a point about half way through the cell cycle in

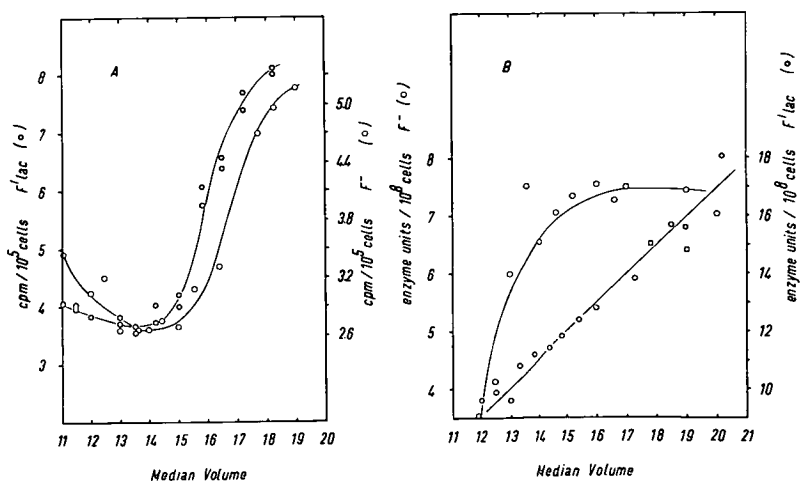

Fig. 3A and B. Rate of DNA synthesis and rate of induction of β-galactosidase in B/r E *lac*$^+$ and B/r E *lac*$^-$/F′ *lac*. ^{3}H-thymidine (0.5 μCi/ml, 50 Ci/mmol) and 10^{-3} M IPTG were added to exponentially growing cultures of each of the strains. After one minute 100 μg/ml unlabelled thymidine was added. After a further five minutes a mixture of antibiotics was added and the cells harvested and fractionated as described in Methods. Panel A shows the label incorporated and Panel B the enzyme induced in different sized cells of B/r *lac*$^+$ (o) and B/r *lac*$^-$/F′ *lac* (o)

both the F⁻ and the F′ strains. If the cell cycle in the synchronous culture (Fig. 2) is regarded as starting at the time at which half the cells have divided, the rate of DNA synthesis doubles about half way through the cell cycle under these conditions as well. This, it may be noted, is somewhat later than the time predicted (Cooper and Helmstetter, 1968) for cells with a generation time of 58 min and may reflect a difference between substrain E and other substrains of B/r.

Rate of Synthesis of β-galactosidase. The inducibility of β-galactosidase and the rate of DNA synthesis was determined for both F′ *lac* and F⁻ cells. An inducer of β-galactosidase was added together with ^{3}H-thymidine to exponentially growing cultures prepared as described above. After one minute pulse labelling was terminated by the addition of excess unlabelled thymidine. After a further five minutes chloramphenicol was added to terminate induction and the cells were harvested and centrifuged in the zonal rotor. Since measurable enzyme does not appear until three minutes after the addition of inducer this procedure permits three minutes of enzyme synthesis. Figure 3, panel B, shows the way in which enzyme inducibility varies as a function of cell size in F⁻ and F′ *lac* cells. In the F⁻ culture nearly four units of enzyme are induced in the smallest cells. The enzyme per cell increases rapidly with median volume such that cells only 20% larger than the minimum size contain twice as much enzyme. The amount of enzyme induced per cell remains constant in all larger classes of cells. Therefore, the inducibility of the β-galactosidase specified by the chromosomal *lac* gene doubles early in the cell cycle. Since the enzyme produced by each cell presumably is proportional to the number of genes present at the time inducer was added, in these experiments the enzyme/cell reflects the state of the genome in a cell six minutes (or, since g = 58 min, 1/10 of a generation) younger. The *lac* gene therefore seems to be doubling in cells just completing division. We can see from Panel A that such cells (i.e. those with a notional median volume of 22 units) will have replicated about half/two thirds of the chromosome and might be expected to be replicating the chromosomal *lac* gene, located at 8 min on the *E. coli* map, or 50% of the distance between origin and terminus (Bachmann et al, 1976).

In marked contrast, however, the amount of enzyme induced in the F′ *lac* cells increases continuously with increasing cell volume, as if the number of β-galactosidase genes were increasing continuously. This is not what would be expected if F′ *lac* were replicating at a particular time in the cell cycle as was suggested by previous studies. The fact that the inducibility of β-galactosidase increases not only continuously but approximately linearly suggests, rather, that F′ *lac* is replicated with similar frequencies in cells of all sizes.

Direct Measurement of F′ lac DNA

Since this result is so strikingly different from that obtained by all previous workers who measured the inducibility of β-galactosidase in F′ *lac* strains, we decided to confirm it by directly measuring the replication of plasmid DNA. Exponential cultures were pulse-labelled with radioactive thymidine, which was chased with cold thymidine to ensure that replicating molecules were completed and in the closed circular form. They were then harvested and separated in the zonal rotor. Plasmid DNA was purified from each fraction and examined directly for evidence of incorporation of pulse label. If plasmid replication occurred mainly in cells of a particular size one would expect to find pulse label *only* in the fractions containing cells of that size. This procedure provides a much more sensitive test of discontinuous synthesis than one such as enzyme induction where the maximum observable difference in rate of enzyme synthesis could not exceed two fold.

Two different methods were employed to analyze the pulse labelled plasmid DNA in different fractions. The first was to analyze the plasmid DNA with the aid of agarose gels. To do this cleared lysates were made from each of the size-fractions obtained from the pulse labelled culture and partially purified as described in the Methods section. The lysates were then run on agarose slab gels. Figure 4 shows that plasmid DNA could be isolated from all size fractions and is absent in the F⁻ strain. The intensity of the bands was determined using a densitometer and was used as a measure of the amount of plasmid DNA in each fraction. The plasmid bands were cut out of the gel and the H^3 DNA in each band measured as described in Methods. Figure 5 shows the proportion of pulse labelled DNA in the plasmid DNA isolated from cells of each size in two separate experiments. It is clear that there are labelled plasmids in cells of every size. Furthermore, the proportion of plasmid DNA labelled does not vary over a greater than two fold range. This experiment thus shows that although plasmids are replicating perhaps twice as frequently in the largest cells as in the smallest cells, plasmid replication occurs in cells of all sizes. These results support those we obtained by measurement of β-galactosidase induction.

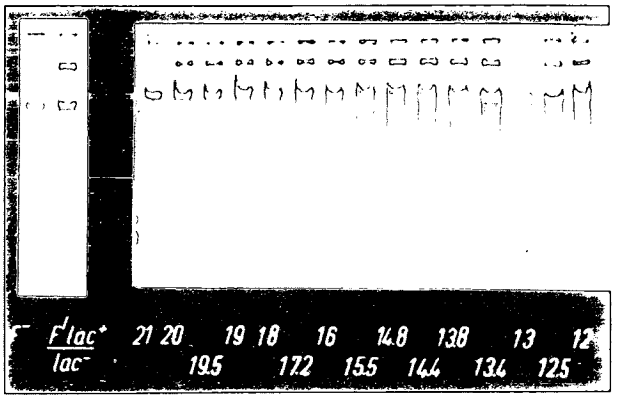

Fig. 4. Slab gels showing chromosomal and F′ DNA in cells of different sizes. Cultures were pulse labelled and fractionated as described in Figure 3. Cleared lysates were prepared, partially purified, loaded on the gels and run as described in the Methods section. The gels were stained with EtBr and photographed by UV light. The median cell volume for each sample is indicated below the appropriate track in the photograph

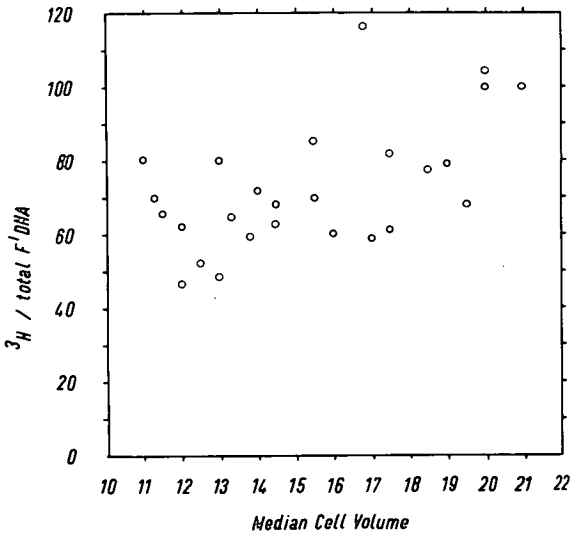

Fig. 5. Label incorporated into plasmid DNA in cells of B/r *lac*$^-$/F′ *lac*$^+$ of different sizes: plasmid and chromosomal DNAs separated on slab gels. Cultures were pulse labelled and fractionated as described in Figure 4. The amount of DNA in the plasmid fraction of each of the samples was determined by photographing the gels, scanning each band on the photograph in a densitometer, and measuring the area under each peak. Each band in the original gel was cut out and solubilized as described in Methods and the ^3H content determined. The ^3H to DNA ratio was calculated for each fraction and plotted as a function of median cell volume. Open and closed circles represent data from two completely separate experiments

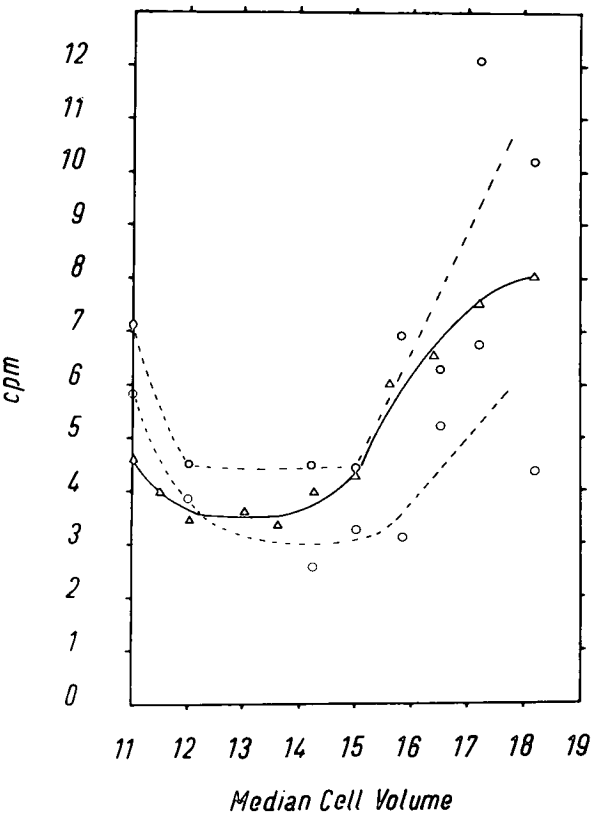

Fig. 6. Label incorporated into plasmid and chromosomal DNA in cells of different sizes: plasmid and chromosomal DNAs separated by centrifugation in EtBr–CsCl. Cultures were labelled and fractionated as described above. The ^3H/cell in each fraction was determined (△) selected fractions were mixed with 10^9 cells of B/r *lac*$^-$/F′ *lac*$^+$ fully labelled with ^{14}C thymine, lysed and centrifuged as described in Methods. Fractions were collected and the ^3H and ^{14}C measured in the separately pooled plasmid and chromosomal DNA containing fractions. The ratio of $^3H/^{14}C$ was calculated for both plasmid and chromosome and divided by the number of cells in the original fraction. This ratio is plotted as a function of the median cell volume for the sample for both plasmid (○) and chromosomal (○) DNA

Pulse labelled plasmid DNA was also analyzed using CsCl-EtBr gradients. Aliquots of cells from different size fractions were mixed with 10^9 cells of the F′ *lac* strain which had been fully labelled with C^{14} thymine and the mixture lysed and centrifuged. The C^{14} labelled cells were added as an internal standard of recovery of plasmid and chromosomal DNA. The concentration of each isotope in the separately recovered plasmid and chromosomal DNAs of each sample was measured and the H^3/C^{14} counts per cell calculated for both chromosomal and F′ DNA. The H^3/cell in an aliquot of the uncentrifuged mixture was taken as an alternative measure of chromosomal DNA synthesis. Figure 6 shows that the pattern of chromosomal DNA synthesis as measured from the chromosomal band of the lysed, centrifuged cells (corrected according to recovery of the internal standard) is similar to that derived from the uptake of label into the fractionated uncentrifuged cells. It is also similar to that shown above (Fig. 3) with a reiniti-

ation near the middle of the cycle following a termination early in the cycle. The plasmid DNA can once again clearly be seen to be replicating in all fractions examined. As with the cultures analyzed on gels, an increased rate of plasmid synthesis is apparent in the larger cells.

We have thus far assumed that there is about one F/chromosome in B/r E/F′ *lac*. If there were several copies of the plasmid to be replicated it might not be possible to observe any synchrony in their replication. The C^{14} labelled plasmid/C^{14} labelled chromosome recovered in each of the samples centrifuged in the CsCl-EtBr gradient served as a measure of the ratio of plasmid DNA to total DNA in this strain. An average value of 3.3% was obtained which is similar to that reported previously (Finkelstein and Helmstetter, 1977; Frame and Bishop, 1977) and equivalent to about 0.8 plasmids/chromosome equivalent of DNA.

Stability of F′ lac

If the copy number of F is low and its replication is not assured through strict regulation of its timing, it might be expected that Lac^- segregants would arise with high frequency. In order to see if F were unstable in our strain we measured its rate of segregation through six generations of growth on glycerol minimal medium ($g=58$) and through fifteen generations of growth on broth ($g=30$) by plating cells on McConkey-lactose agar once per generation. The experiments were performed at a low cell density (10^3 cells/ml) at which reinfection of segregants by mating would be unlikely to occur. Several hundred colonies were examined for each time tested but no segregation was observed to occur during the course of the experiments. At the end of the experiments all cells were Lac^+ and the culture still F′ as demonstrated by its sensitivity to acridine curing.

Discussion

The results reported here demonstrate by several different criteria that the plasmid F′ *lac* replicates in cells of all sizes in an exponentially growing population of *E. coli* B/r E. This result differs from that obtained in all previous studies which used the change in the rate at which β-galactosidase could be induced as a criterion of plasmid replication. All these studies report a stepwise change in β-galactosidase inducibility once per cell cycle and on that basis conclude that F replicates at a particular time in the cell cycle. In the studies done on synchronously dividing cultures (Donachie and Masters, 1966; Nishi and Horiuchi, 1966) it is conceivable that the process of synchronization might have aligned otherwise asynchronous plasmid replication (if, for instance, a repressor of plasmid replication were unstable in sucrose solution) and/or caused fluctuations in the level of production of *lac* repressor. That the latter is likely can be deduced from the fact that cultures freshly reinoculated after centrifugation through sucrose solution are initially poorly inducible by IPTG. A synchronous culture of our F′ *lac* strain also showed periodic doublings (Fig. 2) in the rate of synthesis of β-galactosidase although cells of different ages separated from the exponential culture did not.

In the more recent studies the limitations of synchronous cultures were avoided by pulse inducing exponentially growing cells and separating their descendants by the membrane elution technique (Helmstetter, 1967) into fractions which had been of the same age at the time of induction (Zeuthen and Pato, 1971; Cooper, 1972; Davis and Helmstetter, 1973; Finkelstein and Helmstetter, 1977). The results of these experiments are more difficult to reconcile with our own. It may be pointed out that since the cells must grow on a membrane surface after induction in order to separate them by age it is difficult to ensure that the enzyme/cell does not alter during the period of growth subsequent to induction. Furthermore, the enzyme assay is always performed on newly eluted cells of a single age class, a fact which could affect their behavior in the assay. However, although either of these limitations might conceivably obscure existing steps it is difficult to see how they might cause steps.

However, the most serious limitation of the above experiments is that they rely on a relatively remote event, enzyme inducibility, for measuring the time of plasmid replication. Our experiments and those of Gustafsson and Nordström (1978) have avoided this problem by measuring the label incorporated into molecules of plasmid DNA replicating during growth in exponential culture. Both of these molecular analyses agree in concluding that F′ *lac* does not replicate at a fixed time in the cell cycle. Since Gustafsson and Nordström worked with the same substrains of B/r (A and F) used by other workers it seems improbable that our observations can be attributed to a difference between substrain E and substrains A and F.

F is a plasmid with a low copy number. It has been variously estimated to be maintained at between 0.5 and 2.0 copies per chromosome (Frame and Bishop, 1971; Pritchard, Chandler and Collins, 1975; Finkelstein and Helmstetter, 1977) although Gustafsson and Nordström (1978) suggest that there may be as many as five copies per cell. How can a plasmid

be stably maintained in the cell at a low copy number? The suggestion of earlier workers (Zeuthen and Pato, 1971; Cooper, 1972; Davis and Helmstetter, 1973; Finkelstein and Helmstetter, 1977) that the replication of F is assured by strict control of the timing of its replication is not supported by our results or by those of Gustafsson and Nordström (1978). That F is stably maintained under the conditions of our experiments can be seen from the results of the segregation experiments we performed.

If F were able to block cell division until its replication were completed this would ensure that daughter cells free of F were not produced. Such a mechanism has been suggested as the way in which the cells assure that the chromosome has completed replication before division occurs (Clark, 1968; Helmstetter and Pierucci, 1968). If this were the case, one might expect that F′ *lac* containing strains would have larger cells than their F^- parents as some of the cells would by chance have failed to replicate the plasmid and thus be prevented from dividing. We examined cultures of our strains growing on minimal medium with a generation time of 58 min to see if the F′ *lac* strain had larger cells than its F^- parent. We found, as did Finkelstein and Helmstetter (1977) that the cell size, as measured by optical density per cell was the same for both the F^- and the F′ strains. We also found that the two strains had the same volume distribution when measured with the Coulter Counter and Channelyzer.

Thus we see that F is stably maintained in the cell at a low copy number without an inhibitory effect on cell division, despite the fact that the timing of its replication does not seem to be strictly controlled. F seems to be able to replicate in cells of all sizes, large ones as well as small ones, with only a two-fold range in frequency of replication. If each replication is triggered by dilution of a repressor produced in a burst at the time of the previous replication, as suggested by Pritchard, Barth and Collins (1969), then the reaction between plasmid and repressor is unlikely to be of the high kinetic order necessary to assure synchrony of plasmid replication in all cells of a particular size.

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Plasmids Carrying *oriC* Can Integrate at or near the Chromosome Origin of *Escherichia coli* in the Absence of a Functional *recA* Product

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The bidirectional replication of the chromosome of *Escherichia coli* is initiated at a specific site (Masters and Broda 1971; Bird et al. 1972; Hohlfeld and Vielmetter 1973). This site is located between the *unc* and *asn* genes at 82.5 minutes on the linkage map of *E. coli* (von Meyenburg et al., this volume). Although the base sequence of the DNA containing the replication origin has now been determined (Messer et al.; Hirota et al.; both this volume), the mechanism by which replication is initiated still remains obscure.

We shall describe a striking phenomenon which may help to elucidate the mechanism of initiation. We have observed that recombination occurs with high frequency between pairs of DNA molecules when each contains the origin of chromosome replication. This recombination occurs at a specific site at or very close to the replication origin and is independent of the bacterial *recA*-gene product. We therefore suggest that this recombination may be mediated by an enzyme normally involved in the initiation of replication.

F's Carrying DNA from the Region of the Chromosomal Replication Origin, *oriC*

We isolated a number of new F's carrying chromosomal DNA from the region of *oriC* by mating Hfr 3029 *bgl*$^+$ with MM318, a RecA$^-$ derivative of MM303 (Masters 1977). This strain contains the negative alleles of several markers in the region of the chromosomal replication origin (*pyrE*, *uhp*, *tna*, *bgl*, *ilv*), some of which are to the left and some to the right of the insertion point of the F in ED3029. Thus, we were able to isolate transfer-effective F' progeny by mating for a short time and then selecting for the simultaneous inheritance of markers normally transferred late and those normally transferred early (Low 1968).

By this procedure, we obtained 20 transfer-effective *tna*$^+$ *bgl*$^+$ F's extending various distances to the left of *tna* and/or to the right of *bglB*. None appeared to be deleted for any intermediate markers. These F's, shown in Figure 1, could be divided into two distinct classes correlated with the presence on, or absence from, the plasmid of the DNA between *bglB* and *ilv*.

Strains harboring F's which contained the DNA between *bglB* and *ilv* (of which pED620 is typical) grew slowly, had poor viability when plated on broth, and exhibited an abnormal cell-size distribution with a high proportion of filaments. These characteristics have previously been reported for other sets of F's containing this region and have been attributed to the possession of an extra copy of the replication origin (Masters 1975; von Meyenburg et al. 1977). Our F's were also transmissible to, and maintainable in, JC5088, a RecA$^-$ Hfr strain of *E. coli*, as has been reported for another set of F's which contain this region (Hiraga 1976). Strains harboring F's which did not extend past *bglB* to *ilv*, such as pED604, did not have these characteristics.

While studying the mating behavior of these RecA$^-$ strains, we noted that those harboring plasmids such as pED620, which carry *bglB-ilv*, transferred chromosomal markers to RecA$^+$ recipients and also transferred plasmid markers to both RecA$^+$ and RecA$^-$ recipients. Since all clones tested were able to transfer both plasmid and chromosome, we took this to indicate that these plasmids were integrating unstably into the chromosome to form transient Hfrs. This behavior is highly unusual, since F's do not normally mobilize or recombine with the chromosomes of RecA$^-$ strains (Wilkins 1969). We therefore decided to see whether the chromosomal transfer exhibited a marker-frequency gradient, as would be expected if mobilization were occurring from a specific site.

We mated cultures of several of the F'-containing strains with suitable recipients and measured the transfer of chromosomal and plasmid markers (Table 1). It can be seen that pED615 and pED620 transfer chromosomal markers with a much higher frequency than does pED604. *MetE*, which is near the region of homology between the plasmid and the chromosome, is transferred with nearly the same frequency as the plasmid in this cross. There is a gradient of transfer of the other markers tested consistent with the order of transfer: *metE leu proA lys*. Dr. S. Hiraga (Institute for Virus Research, Kyoto University) kindly provided us

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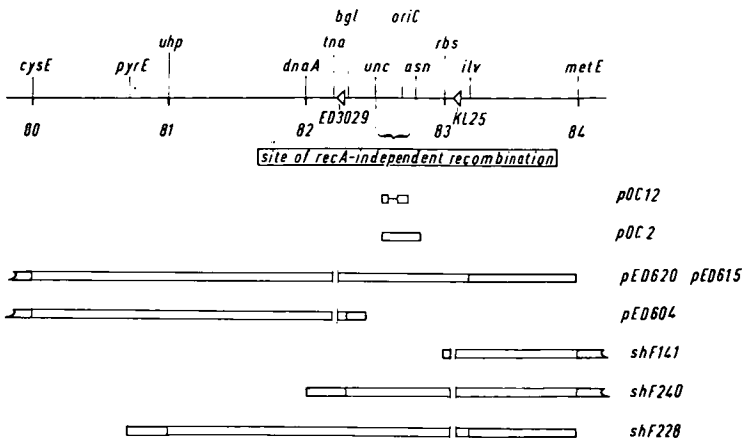

Figure 1. Part of the chromosome of *E. coli* and the plasmids used in this study. The sites of insertion and directions of transfer of F′ *ts114lac* in ED3029 and F in KL25 are indicated by arrows. The gaps in pED620, pED615, and pED604 indicate the position of F′ *ts114lac*. The gaps in shF141, shF240, and shF228 indicate the position of F. Only the chromosomal DNAs of pOC2 and pOC12 are shown. (▭) Chromosomal DNA present on the plasmid; (▭) DNA whose presence has not been excluded.

with some of the plasmids he had isolated from Hfr KL25: shF141, shF228, and shF240. These behaved as described above; shF228 and shF240, which contain the DNA between *bglB* and *ilv*, mobilized the RecA⁻ chromosome; shF141 did not. Thus, we may conclude that F′ plasmids which carry the DNA between *bglB* and *ilv*, but not those which carry only the DNA between *bglB* and *cysE* or between *ilv* and *metE*, are able to recombine with the chromosome of RecA⁻ cells.

Behavior of pED620 and pED604 under Conditions Restrictive for F Replication

Hfr 3029 was formed by the insertion into the chromosome of F′ *ts114lac*, which fails to replicate at 42°C. This Hfr was selected as the parent for the F's we planned to isolate, with the expectation that an F′ *ts114* carrying *oriC* might replicate at the restrictive temperature by making use of the chromosomal replication system.

We therefore investigated the survival of pED620 and pED604 at 42°C to see whether pED620 did indeed survive better than pED604. We grew pED620/318 and pED604/318 exponentially on minimal medium selective for plasmid markers at 30°C and then plated on selective medium at 30°C and 42°C. We found that between 0.2% and 30% of pED620/318 cells viable at 30°C formed colonies at 42°C, but that only 0.002% of pED604/318 cells did so. We then mated the 42°C survivors to see whether they were still F's. To our surprise, the surviving colonies were not F′; instead, they were all Hfr. Fifteen clones of pED620/318 isolated at 42°C and a liquid culture grown overnight at 42°C from an inoculum grown at 30°C all transferred the chromosome with the same orientation noted for the transfer at 30°C described above. However, the plasmid itself was transferred with a greatly reduced frequency (Table 1). This indicates not only that the 42°C survivors of pED620/318 are all Hfr, but also that in all cases the F′ is integrated into the chromosome to the right of *pyrE* in the region of homology between the chromosome and the plasmid, despite the absence of a functional *recA* allele to promote such integration. To further establish the order of marker transfer, we performed an interrupted mating between a culture of pED620/318 grown overnight at 42°C from an inoculum grown at 30°C and measured the time of transfer of markers to a RecA⁺ recipient. Figure

Table 1. Transfer of Chromosomal and Plasmid Markers by RecA⁻ Strains Containing F′ Plasmids

Plasmid in donor strain	Temperature (°C)	No. of progeny/donor cell					
		chromosomal markers				plasmid markers	
		MetE⁺	Leu⁺	Pro⁺	Lys⁺	PyrE⁺	Ilv⁺
pED604	30	2×10^{-6}	2×10^{-6}	2×10^{-6}	3×10^{-6}	1×10^{-2}	—
pED615	30	2×10^{-2}	9×10^{-3}	4×10^{-3}	1×10^{-4}	3×10^{-2}	n.t.
pED620	30	2×10^{-2}	1×10^{-2}	7×10^{-3}	1×10^{-4}	2×10^{-2}	n.t.
shF141	37	n.t.	$<3\times10^{-6}$	n.t.	n.t.	—	2×10^{-2}
shF228	37	2×10^{-3}	n.t.	1×10^{-3}	n.t.	—	2×10^{-2}
shF240	37	n.t.	1×10^{-3}	n.t.	n.t.	—	4×10^{-2}
pED604	42	2×10^{-4}	1×10^{-4}	2×10^{-4}	5×10^{-5}	$<10^{-6}$	—
pED620	42	3×10^{-2}	3×10^{-2}	2×10^{-3}	3×10^{-4}	10^{-5}–10^{-6}	n.t.

Donor strains were grown at the appropriate temperatures in selective medium and mixed with the recipient strain in a ratio of 1:10. After 3 hr of incubation, the mixtures were plated on selective plates. To measure transfer of chromosomal markers, the recipient strain was χ478 (*metE leu proC lys trp*). Plasmid transfer was measured by mating with the *recA* strain MM318. Classes of progeny which were not or could not be scored are indicated by n.t. (not tested).

2 shows that transfer is oriented in the order *metE pro try lys* even though individual Hfr clones had not been isolated. This shows that the integration event leading to the formation of these Hfrs must nearly always occur in the same region of the chromosome, between *pyrE* and *metE*.

The survivors at 42°C of pED604/318 also proved to be Hfr. A culture grown at 42°C from a 30°C inoculum transferred all chromosomal markers with a fairly low but equal frequency, as might be expected if integration had occurred at many different sites on the chromosome. Plasmid transfer was not detectable. Eight individual clones isolated and mated at 42°C transferred the chromosome from several different points (data not shown), indicating that the Hfrs were not formed through recombination between the homologous DNA on plasmid and chromosome. This is the behavior that might be expected from an F′ in a RecA⁻ host.

The Site at Which pED620 Integrates into the Chromosome Cannot Be Distinguished from *oriC*

The most recent evidence indicates that the origin of replication of the *E. coli* chromosome is between *unc* and *asn* (Yasuda and Hirota 1977; von Meyenburg et al., this volume). Since this stretch of DNA is carried by pED620 but not by pED604, we attempted to find out whether the site at which *recA*-independent Hfr formation occurs was distinguishable from *oriC*. We attempted to do this by determining in what order the plasmid markers of pED620 were transferred. Those proximal to the site of integration should be transferred early in an interrupted mating; those distal to the site of integration should be transferred late, after the chromosome has been transferred. We introduced pED620 into RecA⁻ hosts carrying the negative alleles of markers near *oriC*. These strains were then grown at 42°C and mated with RecA⁺, Nal^r, or Rif^r derivatives of themselves. The results were unequivocal—the plasmid markers *bgl*⁺ and *unc*⁺ were transferred early with high frequency (1·0); *rbs*⁺, *ilv*⁺, and *pyrE*⁺ were transferred late with low frequency (1×10^{-2}). This method was not used to analyze the transfer of *asn*, since the Asn⁻ strain harboring pED620 would not grow well at 42°C. Instead, individual clones of pED620/CM987 which were able to grow at 42°C were isolated and tested. Of these, 75% (12) transferred the plasmid *asn* allele late, as would be expected if pED620 had integrated between *unc* and *asn*. The remaining 25% transferred early not only *asn* but also plasmid markers which were transferred late by pED620/318 (e.g., *ilv*). We therefore infer that these latter clones have integrated pED620 in an unstable fashion peculiar to strain CM987. The results of these mating experiments are summarized in Figure 3, where the heavy line indicates the order of transfer of markers from strains containing pED620. It is clear that the site at which pED620 is integrated into the RecA⁻ chromosome is between *unc* and *asn*, i.e., the same stretch of DNA as that in which *oriC* is located.

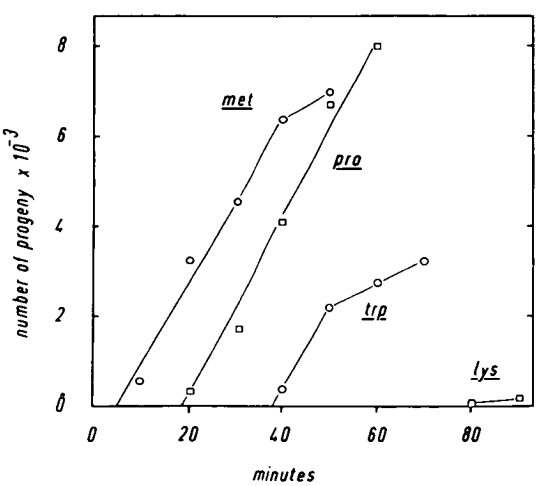

Figure 2. An interrupted mating at 42°C with pED620/318 as donor. The donor was grown overnight at 42°C on medium selective for plasmid markers from an inoculum grown at 30°C. It was reinoculated into selective medium at 42°C and allowed to grow for four mass doublings. It was then mixed with χ478 (*metE proC try purE lys leu*), and samples were taken as indicated and plated to measure Met⁺, Pro⁺, Trp⁺, and Lys⁺.

pED620 Will Form Cointegrates with Other Plasmids Containing *oriC*

Dr. W. Messer (Max Planck Institute for Molecular Genetics, Berlin) kindly provided us with pOC2 and pOC12, nontransmissible plasmids which he had constructed in vitro by ligating a gene specifying penicillin resistance (Pen^r) to *E. coli* DNA fragments containing *oriC* (Messer et al., this volume). When RecA⁻ strains harboring both pED620 and either pOC2 or pOC12 were used as donors in a mating, it was found that Pen^r could be transferred as well as the F′ markers. Pen^r and plasmid markers always segregated together from the progeny of the cross, indicating that they were probably located on a single structure, presumably a cointegrate

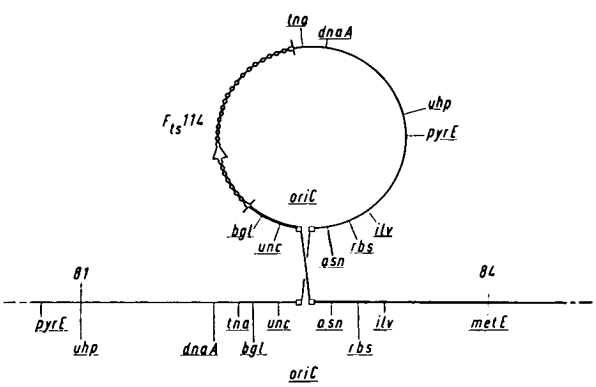

Figure 3. Postulated crossover near *oriC* between pED620 and the chromosome in *recA* strains. The arrow and heavy line indicate the markers transferred early by pED620/318 to RecA⁺ recipients at 42°C.

of pED620 and pOC2 or pOC12. The transfer of pOC2 was not promoted by pED604.

It is clear from the results reported above that pairs of replicons carrying the DNA at or near the replication origin of the *E. coli* chromosome can recombine at a site within that DNA in the absence of a functional *recA* protein. Whether this site-specific recombination can occur between nonreplicating molecules or whether it is a concomitant of replication remains to be discovered. Since the site at which the recombination must occur is within 2000 bp of *oriC* (pOC12 contains about 2000 bp of chromosomal DNA [W. Messer, pers. comm.]), it is not unreasonable to suppose that it may be mediated by an enzyme system normally involved in the initiation of replication at the chromosomal origin.

SUMMARY

F′ plasmids containing the chromosomal origin of replication recombine with the chromosome at a site very close to or identical with the chromosomal replication origin. This site-specific recombination is independent of the host *recA* function. Such F's also form cointegrates with other plasmids carrying this DNA. This was demonstrated by analyzing the consequent ability of F's containing *oriC* to transfer the chromosome or pOC plasmids from RecA$^-$ donors.

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