STUDIES ON THE CONTROL OF DNA REPLICATION IN ESCHERICHIA COIL

by

H. G. NANDADASA

Submitted in partial fulfilment of the requirements for the degree of Ph. D. of the University of Leicester.

Leicester
July, 1971
ACKNOWLEDGEMENT

I am sincerely indebted to Professor R. H. Pritchard for accepting me as a research student in the Department of Genetics and for his kind and patient supervision of my research work.

I wish to thank Dr. B. M. Wilkins for his valuable suggestions and criticisms, made during the course of my studies and in preparation of this thesis.

My thanks are also to Mrs. Joan Skinner who typed the thesis and to all those in the Department who helped me in various ways and made my stay in England a happy one.

This work was supported by Vidyodaya University of Ceylon.
## CONTENTS

### CHAPTER 1
**Introduction**

I. The chromosome of *Escherichia coli* and its replication 1
II. Sex factor F and its replication 5
III. Regulation of DNA synthesis 10
IV. Mutants defective in DNA replication 15
V. Present work 20

### CHAPTER 2
**Materials and Methods**

Results

- Isolation of T516/Flac^+ 28
- Growth properties 30
- Episome replication 34
- Mapping of the T5^- mutation 37
- Revertants of T516 39

Discussion 41

### CHAPTER 3
**Materials and Methods**

Results 45
(Chapter 3)

Reversion frequencies 45
Properties of revertants 48
Integration of the F factor into the chromosome 48
Revertants carry the Ts\textsuperscript{-} mutation 51
The site of integration 55
Effects of acridine orange on revertant strains 59
DNA synthesis in temperature-sensitive strains 68
Discussion 102
Integrative suppression 102
Complementation between the F particle and the chromosome 106
The results in relation to the Pritchard, Barth and Collins' model of control of DNA synthesis 110

CHAPTER 4

Materials and Methods 115
Results 116
Abnormal gradient of recombination 116
Recombinant frequencies 120
Sensitivity of the recombination-deficient strains to uv-irradiation and treatment with MMS 126
Location of the \textit{rec} gene 129
CHAPTER I

INTRODUCTION

1. The Chromosome of *Escherichia coli* and its replication

Using density labelling techniques Meselson and Stahl (1958) have shown that the DNA of *E. coli* replicates semiconservatively. Their experiments indicated that when *E. coli* cells grown in a "heavy" medium are transferred to a "light" medium, heavy DNA is progressively replaced by half heavy hybrid DNA during the first generation of growth. Light DNA does not appear until almost all the original heavy DNA has been transferred into hybrid DNA. These observations imply that a segment of DNA which is replicated later in one generation is not replicated early in the next cycle, and that the DNA replication is an orderly sequential process. Such a replication pattern should involve regular initiation at one or more points in the chromosome and replication in one or both directions.

The distinction between initiation and replication itself was first made by Maaløe and Hanawalt (1961). They postulated that protein or RNA synthesis is necessary for initiation of DNA synthesis but not for the replication itself. Thus, once initiated, chromosome replication was assumed to run to completion even in the absence of protein synthesis. This view was later confirmed by Lark, Repko and Hoffman (1963).

Cairns' (1963) autoradiographic data threw new light on
the structure and replication of *E. coli* chromosome. He found that if a bacterium is lysed by very gentle procedures the whole chromosome can be isolated as a single circular structure. The DNA from cultures labelled with \(^3\)H-thymidine for 2 or more generations produced rare circular tracks of 1000\(\mu\) - 2000\(\mu\) in photographic emulsions. Such tracks sometimes had two circles joined by two Y-shaped forks and the labelling pattern indicated that one of the forks represents a growing point at which replication occurs and the other fork represents the point of origin from which replication must have commenced. These observations led Cairns to the idea that the bacterial chromosome replicates as one unit with a single growing point.

Genetical evidence for a unique origin and direction of replication came from Nagata (1963). Yoshikawa and Sueoka, working on *Bacillus subtilis*, also found clear evidence for a unique origin and direction of chromosome replication. Their approach was based on the premise that, in cells which synthesise DNA continuously from a fixed origin, there will be 2 or more copies of a marker which is replicated early for each copy of a marker which is replicated later. Marker frequencies were determined by relative transforming frequencies of different markers in DNA isolated from growing cultures. Their results indicated that in rapidly growing *B. subtilis* cells the ratios between early and late replicating markers reach a value close to
showing that rapidly growing cells usually have 3 growing points per nucleus, two of which are created simultaneously before the preceding one has completed the replication (Yoshikawa, O'Sullivan and Sueoka, 1964).

Using a completely different technique, Pritchard and Lark (1964) also found that in *E. coli* cells a new round of replication is initiated before the completion of the previous cycle. They demonstrated that, after a period of thymine starvation, cells replicate DNA by newly initiated forks as well as from the completion of the previously initiated forks.

The pattern of DNA synthesis appears to depend on the growth rate of the cells. Fast growing *E. coli* cells synthesise DNA throughout the division cycle but slow growing cells synthesise DNA only during a fraction of each generation. This has been determined by counting the proportion of cells which can incorporate labelled thymine into DNA in pulse labelling experiments (see Maaløe and Kjeldgaard, 1966). Such observations suggested that the duration of the replication cycle is similar in cells growing with generation times between 40-100 minutes; Maaløe and Kjeldgaard (1966) suggested that at a given temperature, the rate of DNA synthesis per replication point is constant and independent of the growth rate of the cell. This view has been elegantly confirmed by Helmstetter and Cooper (1968) in a series of experiments using
synchronized cultures of *E. coli* B/r. They demonstrated that, between growth rates of 1 to 3 generations per hour, the time (termed C) taken by a growing point to traverse the chromosome is a constant of about 41 minutes. For cells growing with a generation time longer than C, a chromosome replication cycle is completed in a fraction of a generation. Therefore, in these cells there is a period devoid of DNA synthesis after the end of one round of replication and before the beginning of the next. In cells growing with a generation time equal to C, a new round of replication begins coincidentally with the completion of the previous round. When the generation time is shorter than C, successive cycles of replications will be initiated at intervals shorter than C. Therefore, a new round of replication begins before the completion of the previous round. In such cells DNA synthesis is continuous and chromosomes contain multiple forks at least during part of each cell cycle.
II. Sex factor F and its replication

Some strains of \textit{E. coli} carry extra-chromosomal genetic elements, or plasmids, referred to as sex factors. A cell harbouring such an element is called a \textit{male} because it is capable of transferring genetic material to a second cell, a \textit{female}, which lacks a sex factor. The best known example of a sex factor is the F factor (Jacob and Wollman, 1958).

The F factor, when present in the cell, may be found in two distinct, usually mutually exclusive, states. The autonomous, independently replicating or extra-chromosomal state, and the integrated, or chromosomally attached, state. A particle with these properties was defined as an episome by Jacob and Wollman (1958). Cells harbouring an F factor in the autonomous state are designated $F^+$ cells and those without F factors, are designated $F^-$. Cells carrying an F factor integrated within the chromosome are termed Hfr cells (because of their high frequency of marker transfer into $F^-$ cells).

On theoretical grounds (Campbell, 1962; Adelberg and Pittard, 1965) and based on genetical studies (Broda, Beckwith and Scaife, 1964; Pittard, 1965) the F factor was also regarded as a circular structure like the chromosome. Its circular nature has been subsequently demonstrated by physico-chemical studies (Hickson, Roth and Helinski, 1967; Freifelder, 1968). It is now known to
be a circular DNA ring about 1/50th of the size of the chromosome, with an approximate molecular weight of $4.5 \times 10^7$ (Freifelder, 1968).

Although in $F^+$ cells the number of copies of the $F$ factor in each cell is small, the association of these factors with the cells is very stable in growing cultures. This indicates that there is some co-ordination between the replication of the $F$ factor and the growth rate and division of the cell. However, the $F$ factor in the autonomous state can replicate at a rate different from that of the chromosome since, when $F^+$ and $F^-$ cell populations are mixed, infection of the sex factor into $F^-$ cells may occur with a frequency approximately one hundred per cent and, in newly infected cells, it replicates faster than the bacterial chromosome (De Haan and Stouthamer, 1963).

Hfr strains arise in $F^+$ cell populations as a result of genetic recombination between the chromosome and the $F$ factor (Campbell, 1962; Broda, Beckwith and Scaife, 1964). Integration of the $F$ factor into the chromosome occurs at one of many different sites on the chromosome (Falkow and Citarella, 1965; Boyer, 1966). The pattern of replication of a chromosome containing an integrated $F$ factor is not clear. Nagata (1963) claimed that in Hfr strains chromosome replication starts at the site of integration of the $F$ factor. This view has received support more recently by Vielmetter et al. (1968). On the other hand, Berg and Caro (1967), and Caro
and Berg (1968) studied by P1 transduction relative gene frequencies in exponentially growing cultures of isogenic Hfr strains with the F particle integrated at different sites and with different orientations and came to the conclusion that integration of F does not affect the origin or direction of replication. Abe and Tomizawa (1967) and Wolf et al. (1968) came to the same conclusion using methods similar to those described above. Cerda-Olmedo and Hanawalt (1968), studying sequential mutagenesis of replicating chromosomes with MTG (N-Methyl-N'-nitro-N-nitrosoguanidine), also confirmed this view. However, it is possible that the role of the F factor as an origin for chromosome replication is strain specific or dependent on cultural conditions.

F factors of many Hfr strains have the ability to return to the autonomous state. Such strains sometimes produce cell populations with an autonomous F factor carrying one or several chromosomal markers. Such F factors are termed F prime factors (F'). The chromosomal DNA of F prime factors is replicated under the total control of the F factor replication system.

An important feature of cells carrying F factors, in either the autonomous or the integrated state, is their immunity towards superinfecting F factors. The presence of one F factor prevents the autonomous replication of another in the same cell. Scaife and Gross (1962)* studied the nature of superinfection immunity of

* The nomenclature used in this thesis is based on the suggestions made by Demerec et al. (1966). The symbols are generally those used by Taylor (1970).
Hfr and $F^+$ cells by infecting them with $\text{Flac}^+$. Although both Hfr and $F^+$ cells are more resistant to infection by $\text{Flac}^+$ than are $F^-$ cells, infection does occur. In $F^+ \times \text{Flac}^+$ crosses the $\text{Lac}^+$ conjugants obtained formed varigated colonies on E.M.B. lactose plates. Pure $\text{Lac}^+$ colonies were infrequent. This shows that the superinfecting $\text{Flac}^+$ particle cannot replicate together with the resident $F^+$ particle in the recipient cell, although the $\text{Flac}^+$ particle can replicate if it replaces the resident $F$ particle. In Hfr $\times \text{Flac}^+$ crossings, $\text{Lac}^+$ offspring which were still Hfrs carried no free $\text{Flac}^+$, instead the $\text{lac}^+$ gene was integrated into the chromosome. Dubnau and Maas (1968) also studied Hfr $\times \text{Flac}^+$ matings using starved Hfr recipients. After starvation male strains behave like females phenotypically. They made similar observations to those of Scaife and Gross and showed that in Hfr recipients the superinfecting $\text{Flac}^+$ factor does not replicate and is diluted out in the growing cell population. In Hfr $\times \text{Flac}^+$ matings, Hfr $\text{Flac}^+$ double males are formed at a low frequency (Maas and Goldschmidt, 1969). In these strains the $\text{Flac}^+$ factor is also integrated into the chromosome. When an Hfr strain mutant at $\text{recA}$ (a locus for recombination proficiency) is used as the recipient, no such double male strains were obtained. Thus two $F$ factors can normally replicate in the same cell only if both of these are integrated into the chromosome. However, Maas and
Goldschmidt have isolated a mutant Hfr strain which allows replication of an autonomous F factor (Maas and Goldschmidt, 1969).
III Regulation of DNA synthesis

The capacity of the chromosome and episomes like the F factor to replicate as separate units within the same cell, suggests that each replicating unit may have its own regulatory apparatus. Jacob, Brenner and Cuzin (1963) named such units replicons and suggested a model to explain the regulation of DNA synthesis of all replicons. They postulated that a structural gene of the replicon controls the synthesis of a specific initiator protein. The initiator acts on a corresponding structural element, the replicator, at which the cycle of replication begins. The model also assumes that different replicons are attached independently to specific sites in the bacterial membrane along the equatorial perimeter of the cell. At a certain stage of the bacterial life cycle, the cell membrane transmits a signal permitting the initiator to start the replication, which starts at the replicator and proceeds along the circular structure. From each replicon two daughter structures are formed which are assumed to be attached independently to the bacterial membrane. The growth of the membrane between the two planes of attachment of the daughter replicons results in their progressive separation.

This model, however, does not explain an important part of the regulation, namely the mechanism by which the cell times the signals leading to the series of events involved in the initiation
of replication. Satisfying this need, Maaløe and Kjeldgaard (1966) suggested a mechanism involving: (a) constant derepressed production of initiator, (b) a high affinity of this product for double stranded DNA and (c) initiation supervening when the DNA is saturated with the hypothetical, presumably basic, protein. This hypothesis is based on the assumption that a fixed quantity of initiator is produced per generation time irrespective of the growth rate.

The replicon model gives a possible explanation for the failure to obtain cells carrying two distinct F factors in the autonomous state. If the F factors are attached to specific sites in the membrane, all of which are occupied, a second superinfecting F factor will fail to replicate unless it replaces a resident particle.

Elaborating on the idea of a specific attachment site to a regulatory mechanism, Marvin (1968) suggested a "site territory" hypothesis. A complex of enzyme and structural proteins distinguishes a site from other parts of the membrane. The presence of a site alters the membrane structure so that new site proteins can no longer bind to the membrane in the neighbourhood of the existing site. The magnitude of the change would decrease with increasing distance from the site until, at a certain distance, a new site could be formed. The initiation would require two
factors, sufficient membrane area with proper structure and specific site proteins. The frequency of initiation would depend on the number of site territories permitted on the available membrane and therefore on the rate of increase of surface area of the cell.

However, some other features of episome replication are not readily understood in terms of the replicon model or the site territory hypothesis. In Hfr cells the autonomous replication of the F system seems to be suppressed. It is replicated passively as part of the chromosome. If the F factor of an Hfr cell remains attached to its site in the membrane it should be able to replicate autonomously unless the event of integration brings about a genetical or structural change affecting the autonomous replication of F factor. If, on the other hand, the F factor in an Hfr cell is detached from its site, the F specific sites of the membrane should be vacant for incoming F factors. Therefore Hfr cells should not show superinfection immunity. However, Hfr cells do show superinfection immunity like F\(^+\) cells.

Pritchard, Barth and Collins (1969) suggested a negative control mechanism for the initiation of DNA synthesis. They assumed, as Maaløe and Kjeldgaard have done, that the initiator is produced constitutively. In addition, an inhibitor of replication is produced discontinuously soon after each act of
initiation of replication. The gene coding for the inhibitor is located adjacent to the origin or is part of the origin itself. Transcription of the inhibitor gene takes place only at the time of its replication and results in the production of a fixed amount of inhibitor independent of the growth rate. The inhibitor prevents a second round of initiation immediately following the previous round. However, the concentration of the inhibitor in the cell is progressively diluted by the increase in cell volume as the cell grows, until a critical concentration is reached when the next initiation takes place.

Such a mechanism is self regulating with a twofold increase in cell mass between two successive rounds of initiation. The model accounts for the superinfection immunity of both F\textsuperscript{*} and Hfr strains. Replication of two F factors (resident and newly infected) in the same cell results in more than a two-fold increase in inhibitor concentration. Therefore, the next round of replication of F factors is prevented until the number of F particles per cell settles down to the equilibrium number again.

Based on the assumption that the F particle of an F\textsuperscript{*} strain replicates late in the chromosome replication cycle, the model also gives a possible mechanism to explain how the autonomous replication of the F particle is suppressed in an Hfr strain. When the F particle is integrated into the bacterial chromosome,
passive replication of the F particle occurs earlier in the cell cycle than it would in autonomous state. This results in an increase of the concentration of the F specific inhibitor to more than double its critical concentration and as a result, the concentration of F inhibitor never falls below that level which permits the initiation of the F specific replication.
IV Mutants defective in DNA replication

One method of investigating the control of DNA synthesis is to isolate and study mutants defective in DNA synthesis. Several thermosensitive mutants for functions involved in DNA synthesis have been studied quite extensively.

Jacob, Brenner and Cuzin (1963) have described two types of thermosensitive mutants of F replication, one type with the mutation located on the F factor itself and a second type in which the mutation is located on the host chromosome. The presence of chromosomal mutations preventing the replication of the F factor is compatible with the replicon model if a chromosomal gene codes for at least a part of the attachment site protein of the F factor. Jacob, Brenner and Cuzin have found that some F factors carrying thermosensitive mutations can integrate into the chromosome to make Hfr cells and these Hfrs are not thermosensitive. This observation supports the view that in Hfr cells the replication of the F factor is mediated by the host's replication system.

A large number of thermosensitive mutants with defective chromosomal DNA synthesis also have been studied (Bonhoeffer and Schaller, 1965; Fangman, 1966; Kohiyama, 1968; Hirota, Ryter and Jacob, 1968; Gross, Karamata and Hampstead, 1968; Kuempel, 1968; Monk and Gross, 1971; Carl, 1970). Most of the temperature sensitive mutants can synthesise DNA normally at 30°C but not at
40°C, although protein and RNA synthesis seems to be normal at both these temperatures.

These mutants have been classified according to the map position of the mutations and several distinct groups have been identified (Gross, 1971). Some mutants stop DNA synthesis immediately after a shift to high temperature, while others show some residual synthesis after the temperature shift. Some of the mutants which belong to the latter class behave as if they can only complete the DNA replication rounds which were in progress at the time of temperature shift and cannot initiate new rounds of DNA replication at high temperature. Much of the evidence that these mutants are defective in initiation rests upon the comparable behaviour of these strains after a shift to high temperature and following inhibition of protein synthesis at low temperature, which is known to inhibit initiation of DNA synthesis (Maaløe and Hanawalt, 1961). Two such mutants, namely CRT83 and CRT46, isolated and characterised by Kohiyama, Lanfran, Brenner and Jacob (1963) and Hirota, Ryter and Jacob (1968), have been quite extensively studied.

Hirota, Mordo and Jacob (1970) have shown that in the case of CRT46 the residual increase of DNA after a shift to high temperature depends on the rate of previous growth at low temperature. This is to be expected in an initiation defective mutant since the
average number of replication forks per chromosome increases with increasing growth rate (Helmstetter and Cooper, 1968). Furthermore, they have demonstrated that the mutant does not synthesise any DNA at high temperature if the cells are previously starved of amino acids at low temperature until DNA synthesis comes to a halt. Also, if a culture of CRT46 is shifted back to 30°C from 41°C after residual synthesis of DNA, the cells initiate DNA replication again after a short lag. Experiments involving density labelling of DNA synthesised under these conditions have shown that this initiation always occurs in a specific region of the chromosome. Such a result is also expected if the mutant is defective in initiation at high temperature.

The nature of the aberrations caused by high temperature in all of the temperature-sensitive mutants is not clearly understood in molecular terms. In the case of mutant CRT46, when the cells are returned to the permissive temperature in the presence of chloramphenicol after a period at high temperature, DNA synthesis does not resume (Hirota, Mordoh, and Jacob, 1970). The product affected by this mutant is therefore irreversibly denatured at high temperature.

Kogama and Lark (1970) have reported that, after a period of thymine starvation, *E. coli* cells initiate and replicate DNA for several hours in the presence of 150 μg/ml of chloramphenicol. As
a possible explanation for this phenomenon, they suggested that a stable protein complex is made under the conditions of inhibition of DNA synthesis. When DNA synthesis is resumed, this protein complex mediates repeated rounds of chromosome replication. The protein complex which is made under normal conditions dissociates after a single round of replication. When the above experiment was done with some temperature-sensitive mutants which cannot synthesise DNA at the high temperature, they found widely different results. In the case of some mutants, if the cells were starved for thymine at a non-permissive temperature and then transferred to a permissive temperature in the presence of chloramphenicol, the DNA synthesis did not continue as found in the wild type. In case of another mutant tested, when the cells were held at high temperature in complete medium or in the absence of thymine and returned to low temperature in the presence of chloramphenicol, DNA replication occurred to the extent observed following thymine starvation at low temperature. Thus in this mutant, the interruption of DNA synthesis which occurs at high temperature can substitute for thymine starvation. Some other mutants gave results different from both these patterns. Results of such experiments indicate that, although these mutants have the common defect of inability to synthesise DNA at high temperature, they have quite different forms of aberrations.

Another abnormal type of initiation of DNA synthesis has
been studied by Worcel (1969) with another temperature-sensitive mutant. When this mutant is transferred to 42°C, DNA synthesis stops. Upon return to low temperature after a short time, DNA synthesis resumes not only from existing replication forks but also from newly induced replication forks. These new forks apparently are established only on one of the two partially replicated daughter chromosome. Such patterns of DNA synthesis may indicate intrinsic differences between the two origins of a partially replicated chromosome.

Widely different types of behavior observed with many mutants defective in DNA synthesis, shows the complexity of the machinery of DNA synthesis and its regulation. It is quite clear that several different genes are involved which play essential roles in this process.
V Present Work

Although a massive amount of experimental work has been done in an attempt to understand the process controlling DNA synthesis, none of the experiments gives direct evidence that would distinguish between suggested positive and negative control mechanisms. In the first part of the following work an attempt has been made to isolate a specific type of an F factor mutant predicted by negative control mechanisms of DNA synthesis. Although I was unable to isolate such a mutant, a mutant was found which, on preliminary investigation, was found to have properties similar to those of temperature-sensitive initiation mutants. Further study of this mutant gave evidence indicating that the temperature-sensitive defect in the initiation of chromosome replication might be suppressed by the integration of an F factor into the chromosome.

This aspect of suppression of temperature-sensitivity of initiation was more thoroughly investigated with the better studied initiation mutants, CRT83 and CRT46.

The final part of the work describes some experiments designed to investigate an apparent recombination defect in CRT83 and CRT46 found in the course of the above experiments.
Pritchard, Barth and Collins (1969) have proposed that initiation of DNA synthesis is controlled negatively, through the mediation of an inhibitor. One way of testing this hypothesis is to look for mutants of the gene specifying the postulated inhibitor. Such mutants would synthesise DNA in an uncontrolled fashion and they would be expected to be non-viable. Thus, only conditional mutants could be isolated.

A direct selective method to isolate such mutants is very difficult to devise but an indirect selective technique could be used on the basis of the following hypothesis. A strain which carries a temperature-sensitive mutation of the postulated inhibitor gene of an Flac\(^+\) factor may have the following properties. Assuming that the mutation is expressed at 40°C but not at 30°C, when a culture of the strain is shifted to 40°C from 30°C, the Flac\(^+\) factor would begin to initiate and replicate its DNA in an uncontrolled manner, which would ultimately cause the death of the host cells. However, segregant cells which have lost the Flac\(^+\) factor before the temperature shift would be unaffected and would multiply as phenotypic revertants. Therefore, such mutants would have an abnormally high reversion frequency since the frequency of occurrence of F\(^-\) segregants in an Flac\(^+\) culture is much greater than normal mutation frequencies. Thus the majority of revertants would be Lac\(^-\).
Mutants with these properties were looked for using N-methyl-N'-nitro-N-nitrosoguanidine as the mutagenic agent, and replica plating at 30°C and 42°C as the screening technique.

Three mutants were found which, although in the primary analysis showed the required characteristics, were found to be different classes of mutants. One of these mutants, on detailed studies, proved to carry a temperature-sensitive defect in DNA synthesis.
MATERIALS AND METHODS

Bacterial strains The strains used, their source and genotype are as follows:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Sex</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co600/Flac+</td>
<td>J. Collins</td>
<td>F'</td>
<td>thr⁻ leu⁻ thi⁻ lac⁻/Flac⁺</td>
</tr>
<tr>
<td>RV113</td>
<td>R.H. Pritchard</td>
<td>F⁻</td>
<td>thi⁻ lacΔ⁻</td>
</tr>
<tr>
<td>X8003</td>
<td>R.H. Pritchard</td>
<td>F⁻</td>
<td>pro⁻ trp⁻ lacZ⁻ strR</td>
</tr>
<tr>
<td>108</td>
<td>R.H. Pritchard</td>
<td>F⁻</td>
<td>pro⁻ met⁻ thy⁻ lacZ⁻ strR</td>
</tr>
<tr>
<td>P4X</td>
<td>B.M. Wilkins</td>
<td>Hfr</td>
<td>thi⁻ metB⁻</td>
</tr>
</tbody>
</table>

The origin and direction of transfer of P4X DNA is shown in Figure 5.

Media The glucose minimal medium (pH 7.0-7.2) contained Na₂HPO₄, 0.7%; KH₂PO₄, 0.3%; NaCl, 0.5%; NH₄Cl, 0.1%; CaCl₂, 0.02%; MgSO₄, 0.02% and Glucose, 0.4%. This medium was supplemented with 60 µg/ml of required amino acids or 10 µg/ml of thiamine.

For minimal agar plates, the above medium was solidified with 1.5% Davis agar.

Nutrient agar plates contained 2.5% Oxoid nutrient broth and 1.25% Davis agar. This was supplemented with 40 µg/ml thymine, when necessary.
Nutrient agar tetrazolium lactose plates contained 0.8\% lactose and 0.005\% tetrazolium chloride, in addition to nutrient agar.

Nutrient broth liquid medium contained 2.5\% Oxoid No. 2 nutrient broth.

Mutagenesis and replica plating of the strain C600/Flac\textsuperscript{+} Strain C600/Flac\textsuperscript{+} was grown in nutrient broth at 37\degree C with shaking, to a concentration of about $1 \times 10^8$ cells/ml. 10 ml of this culture was filtered onto a membrane filter (4.7 cm Oxoid) and the cells were resuspended in 10 ml of phosphate buffer (pH 7). 5 ml of this suspension was treated with 5 ml of phosphate buffer containing 200 $\mu$g/ml NTG (N-methyl-N'-nitro-N-nitrosoguanidine, final concentration 100 $\mu$g/ml), and incubated at 37\degree C for 30 min. The cells were refiltered, washed with phosphate buffer, and resuspended in 10 ml of phosphate buffer. The whole procedure was done at 37\degree C using prewarmed buffer. The final suspension was diluted $10^{-3}$ with phosphate buffer and 0.1 ml samples were spread on tetrazolium lactose nutrient agar plates. The plates were incubated at 30\degree C for 2 days. After this treatment about 200 colonies appeared per plate indicating a survival of 2\% of cells treated with NTG.

The colonies were replica plated on tetrazolium lactose nutrient agar plates using sterile velvet cloths and the replica
plates were incubated at 30°C and 42°C (on tetrazolium lactose
nutrient agar plates, Lac+ colonies appear pink and Lac- colonies
appear dark red).

**Bacterial matings** For mating experiments, strains of male and
female bacteria were grown in nutrient broth at 30°C with aeration.
When the concentration reached about 1 x 10^6 cells/ml, they were
mixed in equal volumes and incubated at 30°C for 1 hr. The culture
was diluted appropriately with phosphate buffer and 0.1 ml samples
were plated on minimal plates selective for conjugants.

**Interrupted mating experiments** For interrupted mating experiments,
the above procedure was used except that, after the male and female
cultures were mixed, 1 ml samples of the mating mixture were diluted
at given times into 4 ml of phosphate buffer and blended (Low and
Wood, 1963) for 7 seconds. 0.1 ml of this was then spread on test
plates.

**Optical density measurements** The optical density of cultures was
measured with a Gilford microsample spectrophotometer at a wavelength
of 450 nm.

**Determination of cell number** Cell numbers in cultures were determined
with a Coulter electronic particle counter model B. Samples from
the culture were diluted to 4/5th with 2% formaldehyde before counting the particle numbers.

**Viable counts** Viability of cells was determined by spreading samples of the culture, appropriately diluted with phosphate buffer, on nutrient agar plates and counting the number of colonies formed.

**Isolation of thymine-requiring mutants** Bacteria were grown in nutrient broth and filtered onto a membrane filter, washed with phosphate buffer and suspended in supplemented minimal medium containing 30 µg/ml of thymine and 10 µg/ml of trimethoprim. The culture was shaken for 48 hours, and diluted 1/5th with the same medium. After another 24 hours shaking, diluted samples were plated on supplemented minimal glucose plates containing 40 µg/ml of thymine. Colonies which grew on the plates were replica plated on similar medium with or without thymine. After this treatment, about 90% of the colonies proved to be thymine-requiring.

**Incorporation of $^{14}$C-thymine into DNA** Samples of cultures grown in the presence of $^{14}$C-thymine were added to an equal volume of 10% TCA (trichloro acetic acid) at 0°C. After one hour they were filtered through a membrane filter (Sartorius, 0.45 µm, 27 mm) and washed with six 5 ml volumes of hot (95°C) distilled water. The filters were dried (using infra red lamps) and placed with a constant
orientation in glass vials (\( \frac{1}{2}" \times \frac{1}{2}" \)). 3.5 ml of non-aqueous scintillation fluid was added to each vial (PPO 5 gms, POPOP 0.3 g, 1000 ml toluene, Pritchard and Lark, 1964). The vials were then placed in standard scintillation vials and radioactivity was counted in a Packard liquid scinillation counter.

Bacteriophages

\( \lambda \)  A virulent strain of \( \lambda \) (\( \lambda \text{vir} \)) was obtained from B.M. Wilkins. This was grown and assayed on bacterial strain C600 before each experiment. The detailed procedure used in experiments is described in the legend to Fig. 3.

\( \text{P1} \)  The P1 strain used was obtained from S.I. Ahmed. The transduction procedure used was that described by Glover (1962).
RESULTS

Isolation of T316/Flac^+

About 4 x 10^4 colonies were tested by replica plating after the mutagenic treatment described in Materials and Methods and 3 mutants showing the expected features were found. All the three mutants, T35/Flac^+, T310/Flac^+ and T316/Flac^+ grew normally to form Lac^+ colonies on nutrient agar tetrazolium lactose plates incubated at 30°C. The strains did not grow on plates incubated at 42°C but gave rise to large numbers of revertant colonies, many of which appeared to be Lac^- . Samples from purified cultures of these strains grown in nutrient broth at 30°C were spread on nutrient agar tetrazolium lactose plates and incubated either at 30°C or 42°C, to determine the reversion frequencies to temperature-resistance. All the three strains had reversion frequencies between 10^-2 - 10^-4 per cell.

The first important question was to determine whether the mutation is carried in the Flac^+ particle or in the chromosome. For this purpose the mutant strains were mated with a female strain, RV113, at 30°C to obtain RV113 Flac^+ conjugants. Mating mixtures were plated on minimal lactose plates supplemented with thiamine. Lac^+ conjugants appeared with appreciable frequencies with all the three strains. The conjugant colonies were replated on similar plates and then tested for temperature-sensitivity (Ts^- ) on
tetrazolium lactose nutrient agar plates. All the conjugants tested were temperature-resistant. This experiment was repeated using strain X8003 as the recipient and the same observations were made. These experiments indicated that the Flac\(^+\) factor of the 3 mutant strains did not carry the Ts\(^-\) mutation and the mutations were probably located in the bacterial chromosome.

When the mutant strains were grown in nutrient broth at 30\(^\circ\)C in the presence of 40 \(\mu\)g/ml of acridine orange for several hours and then plated on tetrazolium lactose nutrient agar plates, Lac\(^-\) colonies appeared at a frequency of about 50\%. All such Lac\(^-\) colonies, when tested, were still found to be temperature-sensitive. These observations confirmed the view that, in all the three strains, the thermosensitive mutations were located in the chromosome and not in the Flac\(^+\) factor. Furthermore, it is also clear that the presence of Flac\(^+\) factor is not necessary for the expression of the mutations.

TS10 and TS16 did not grow on minimal glucose medium supplemented with threonine, leucine and thiamine, although TS5 and the parent strain of the mutants grow on this medium. Subsequent tests for their additional requirements showed that both TS10 and TS16 required isoleucine and valine for growth in minimal media. The temperature-resistant revertants of these mutants also required isoleucine and valine which suggested that the requirements for isoleucine and valine may be a result of additional mutations.
independent of that conferring temperature-sensitivity.

Preliminary experiments on growth characteristics of TS16 at high temperature indicated the possibility that the mutant is defective in DNA synthesis at high temperature. To facilitate the studies of DNA synthesis, a thymine-requiring mutant of TS16 was isolated by the procedure described in Materials and Methods. This mutant required about 20 μg/ml of thymine to form normal colonies on supplemented minimal glucose plates. A low thymine-requiring derivative of this strain was isolated by plating the strain on minimal glucose plates supplemented with 2 μg/ml thymine in addition to the required amino acids. Low thymine-requiring mutants are known to carry secondary mutations enabling the cells to incorporate external thymine more efficiently than the parent thy- strain (Alikanian et al., 1966).

Growth properties

Figure 1 shows the growth characteristics of TS16. When a culture growing exponentially at 30°C in nutrient broth is transferred to 42°C, cell division slows down and stops in about 2½ hours. There is an 8-fold increase in particle number during this period. The optical density, however, continues to increase at a faster rate than at 30°C for about 5 hours at 42°C, and results in about a 40-fold increase. The viability of cells, as determined by their ability to form colonies on nutrient agar plates, begins to
Figure 1  Growth characteristics of TSI at 42°C in nutrient broth

TSI was grown in nutrient broth at 30°C with shaking for several hours. At 0 time, the culture was diluted 10-fold into a flask containing nutrient broth prewarmed at 42°C. The flask was maintained at 42°C in a shaking water bath. 2 ml samples were removed into 0.5 ml of 2% formaldehyde for O.D. and particle count measurements. 0.1 ml samples were removed, diluted with phosphate buffer and spread on nutrient agar plates for viable counts.
TS16 was grown for several hours at 30°C in minimal glucose medium supplemented with 60 μg/ml threonine, leucine, valine and isoleucine, 10 μg/ml of thiamine, 80 μg/ml of deoxyadenosine and 5 μg/ml of 14C-thymine at a specific activity of 0.06 μCi/μg. At 0 time, the culture was diluted 10-fold into the same medium prewarmed at 30°C or 42°C, and grown in flasks with shaking. 1 ml samples were removed at given times into 1 ml of ice cold 10% TCA. Radioactivity in the samples was determined as described in Materials and Methods.
fall after cell division has stopped. The unbalanced increase in optical density over particle number reflects a large increase in cell size. When the cells at this stage were examined under the microscope, filaments of varying lengths were seen. These features of growth of TS16 could be understood if the cells abruptly or gradually stop synthesising DNA when shifted to 42°C whereas protein synthesis continues for a longer time. The doubling time of the strain at 30°C before the temperature-shift was about 55 minutes. The increase in particle number observed in this experiment after the temperature-shift goes on for at least 2 hours, and results in an 8-fold increase. Therefore, the increment of particle number observed is more than expected under a hypothesis of immediate cessation of DNA synthesis at high temperature.

The pattern of DNA synthesis by TS16 at 30°C and at 42°C was followed by studying the incorporation of ¹⁴C-thymine by cells growing in minimal medium and the results of such an experiment are shown in Figure 2. When a culture is shifted to 42°C from 30°C, DNA synthesis initially occurs at a faster rate but gradually slows down and stops completely after about 2 hours. The increment in DNA observed under these conditions is about 160%. This residual amount of DNA synthesised at 42°C was found to be even greater when the cells were grown in minimal glucose medium supplemented with 2% casamino acids (data not shown). Although the results indicate that
DNA synthesis is affected by high temperature in this mutant, the effects are not immediate. The cells continue to synthesise DNA for some time after the temperature-shift. Since the cells were growing at a rate of about one generation per 130 minutes at 30°C, the cells synthesising DNA at the time of temperature-shift cannot be expected to have more than one fork per replicating chromosome. However, the increment of DNA represents more than one doubling. Therefore, at least some cells are probably capable of initiating new round of DNA synthesis after the temperature-shift. The nature of the DNA synthesised at high temperature is not known.

Episome replication

Different types of temperature-sensitive mutants defective in DNA synthesis vary in their ability to replicate other replicons at high temperature (Hirota, Ryter and Jacob, 1968). The ability of a virulent λ particle (λvir) to grow in TS16 was therefore studied to provide a comparison with the behaviour of the previously studied strains. The details of the procedure are described in the legend to Figure 3. After a period of adsorption of λ particles at 30°C in phosphate buffer, the cells were incubated in nutrient broth either at 30°C or 42°C. The change in the concentration of λ particles in the culture was followed by 2 methods.
The bacterial strain was grown in nutrient broth supplemented with thymine, at 30°C to a concentration of about $5 \times 10^8$ cells/ml. The culture was then centrifuged and the pellet resuspended in phosphate buffer, at about $5 \times 10^9$ cells/ml. 3 ml of this suspension was treated with 0.5 ml of 0.1 M MgCl$_2$ and 0.5 ml of 0.1 M NaCN and incubated at 30°C for 5 minutes. 1 ml of a λvir phage preparation was then added ($5 \times 10^8$ λ/ml) and mixed. After a further 25 minutes of incubation at 30°C, 1 ml of the suspension was removed, filtered through a membrane filter, washed several times with phosphate buffer and resuspended in 10 ml of phosphate buffer. This was diluted $10^{-4}$ into nutrient broth + thymine, prewarmed either at 30°C or at 42°C, and incubated at the respective temperatures. 0.1 ml samples were removed at given times, added to 0.9 ml of buffer saturated with CHCl$_3$ for free phage determination, or mixed with 4 ml of soft agar and 1 ml of a culture of C600 ($10^8$ cells/ml) and over-layered on nutrient agar plates. After about 2 hours, 0.1 ml volumes of the samples collected into CHCl$_3$/buffer were also mixed with 1 ml of C600 culture and 4 ml of soft agar. They were then over-layered on nutrient agar plates. Plates were incubated at 37°C overnight and the plaques were then counted.
(a) After different times of incubation, samples from the culture were mixed with excess of indicator cells (a culture of C600) and overlayed with soft agar on nutrient agar plates. The numbers of plaques appearing on the plates after overnight incubation of the plates at 37°C were counted. This gives the concentration of infective centres in the culture.

(b) Samples were removed at different times from the culture and mixed with phosphate buffer saturated with chloroform. After about 2 hours, the mixture was assayed by plating with the indicator strain used in (a). This gives the concentration of mature λ particles in the culture. The results are shown in Figure 3.

In the culture incubated at 30°C, the concentration of infective centres increases suddenly after about 70 min to give a burst size of about 50. In the chloroform-treated samples, a similar increase in phage particles is detected at about 60 min. The time between 60 and 70 min, presumably indicates the period between the maturation and liberation of λ particles. In cultures incubated at 42°C, there is very similar increase in λ particles after about 45 min of incubation. These results indicate clearly that λ phage can replicate and grow in TS16 cells at 42°C as well as at 30°C.

Furthermore, comparable experiments done with the parent strain of the mutant (C600) gave very similar results, although
the efficiency of adsorption of λ phage by TS16 appeared to be much lower than that of C600.

Whether an F particle can replicate at high temperature in TS16 is not known. When the strain carries an Flac⁺ particle, the cells appear to be capable of synthesizing DNA for at least 4 hours (data not shown), although an F⁻ culture under these conditions stops DNA synthesis after about 2½ hours. This difference might be interpreted as replication of Flac⁺ at high temperature, as partial complementation of the temperature-sensitive defect of the bacterium by the Flac⁺ factor or as suppression of the temperature-sensitive defect in some cells by the Flac⁺ factor.

**Mapping of the Ts⁻ mutation**

Interrupted mating experiments of TS16 with Hfr P4X showed that the Ts⁻ mutation of this mutant maps close to ilv.

The results of such an experiment are shown in Figure 4. The matings were done at 30°C. The time of entry of the leu⁺ and ilv⁺ markers is about double the time expected at 37°C according to the Taylor (1970) map of E. coli chromosome. The Ts⁺ marker appears to enter closely following the ilv⁺ marker.

In a transduction experiment it was found that the Ts⁺ marker is cotransducible with ilv. Ilv⁺ transductants were selected from TS16 using C600 as the donor strain. Out of 15
Figure 4. **Interrupted mating between P4X and TS16 to map the Ts⁻ mutation**

P4X and TS16 were grown with aeration in nutrient broth at 30°C. At about 1 x 10^8 cells/ml, equal volumes of the two cultures were mixed and maintained at 30°C with slow shaking. At given times, 1 ml of the mixture were removed into 4 ml of phosphate buffer, blended for 7 seconds and 0.1 ml samples spread on plates selective for either Leu⁺, Met⁺ or Ily⁺,Met⁺ recombinants. These plates were incubated at 30°C. Ts⁺ conjugants were selected by plating the cells on complete minimal medium followed by incubation at 42°C.
transductants obtained, 7 were temperature-resistant while 8 were temperature-sensitive. This confirmed that the Ts mutation of TS16 is closely linked to ily.

Revertants of TS16

When a culture of TS16 grown in nutrient broth at 30°C is spread on nutrient agar plates and incubated at 42°C, revertant colonies appear at a frequency approximating to $2 \times 10^{-4}$/cell. These revertants when restreaked grow normally at 42°C. This reversion frequency appears to be much higher than the normal mutation frequency for single mutants of E. coli. However, the absolute reversion frequency cannot be determined in such experiments, because, as seen in the experiments done in liquid media, the cell number can increase as much as 10-fold after a shift from 30°C to 42°C.

The apparent reversion frequency of TS16 carrying an Flac+ factor, as determined by similar experiments, is about $2 \times 10^{-3}$/cell. This is about 10-fold higher than that of the F- strain. About half or more of the revertant colonies obtained with TS16 Flac+ had the Lac- phenotype on tetrazolium lactose nutrient agar plates. 20 revertant colonies, including Lac+ and Lac- ones, were tested for fertility in the marker transfer experiments described below and all of them were found to be males.

The relative frequencies of transfer of the lac+,
Relative yields of different types of recombinants from matings involving either the Ts" strain or its revertants and strain 108F"

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>Selected markers (Conjugants per 0.1 ml mating mixture)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lac^+ Str^R</td>
</tr>
<tr>
<td>Revertant 1 (Lac^+)</td>
<td>142</td>
</tr>
<tr>
<td>Revertant 2 (Lac^-)</td>
<td>127</td>
</tr>
<tr>
<td>Revertant 3 (Lac^-)</td>
<td>172</td>
</tr>
<tr>
<td>Revertant 4 (Lac^-)</td>
<td>138</td>
</tr>
<tr>
<td>Ts16/Plac^+ (Lac^+)</td>
<td>14</td>
</tr>
</tbody>
</table>

All the strains were grown in nutrient broth supplemented with thymine at 30°C to a concentration of about $1 \times 10^8$ cells/ml. Recipient and donor strains are mixed in equal volumes and incubated for 90 min at 30°C. 0.1 ml volumes of the mixture were then spread on appropriate minimal plates. All plates contained streptomycin. Plates were incubated at 30°C for 2 days before scoring.
pro* and met* markers of some revertants were determined in mating experiments using 108 as the recipient strain. The results of such an experiment is shown in Table 1. Lac− revertants, as well as Lac+ revertants, are capable of giving Lac+ conjugants with the recipient strain. The population of Pro+ to Lac+ conjugants obtained with all the revertants is higher than that obtained with the parental Flac+ strain. In case of revertant 2 and 4, the frequency of Pro+ conjugants is nearly the same as the frequency of Lac+ conjugants. This tends to indicate that in revertants the Flac+ factor is integrated into the chromosome. The reduced frequency of Met+ conjugants is expected according to the gradient of marker transfer if the site of Flac+ integration is closer to lac and pro than to met.

All Met+ conjugants obtained with revertant 1 and 2 and two conjugants obtained with revertant 2, were temperature-sensitive. All the others were temperature-resistant. The ability of revertant strains to transfer the Ts mutation indicates that the reversion is by suppression.
DISCUSSION

Although the postulated inhibitor-deficient mutants of the Flac\(^+\) factor were not found, further attempts to isolate such mutants are necessary. The mutants which were found were clearly of a different type. All the three mutants, TS5, TS10 and TS16 had Ts\(^-\) mutations in the chromosome and their temperature-sensitivity was independent of the presence of Flac\(^+\) particle. One of the characteristics which led to the isolation of these mutants is their high frequency of reversion. Only TS16 was studied in any detail. It is remarkable that both TS10 and TS16 had other independent mutations causing requirement for isoleucine and valine. Other Ts mutants with defective DNA synthesis, namely CRT83 and CRT46, are also isoleucine and valine-requiring.

The temperature-sensitivity of TS16 could be attributed to a defect in DNA synthesis at high temperature. Although the nature of protein and RNA synthesis of the strain at high temperature was not examined, optical density measurements of cultures shifted to high temperature show that mass increase can continue at the high temperature long after cell division had stopped.

The residual amount of DNA synthesised by TS16 at the high temperature is much more than that which would account for the completion of replication cycles initiated before the temperature-shift. However, the map position of TS16 mutation and its ability
to replicate λ at high temperature are common features with CRT83 and CRT46, which are generally accepted as initiation-defective Ts mutants (Hirota et al., 1968). It is likely that TS16 is a leaky initiation mutant similar to these.

The aspect of TS16 which is more interesting is its reversion to temperature-resistance. The apparently high reversion frequency may be dependent on its leakiness or it is possible that the reversion could take place by suppressor mutations occurring at a different site or sites.

In TS16/Flac+, the reversion frequency is apparently higher than that of TS16. In at least some of the revertants of TS16/Flac+, the chromosome still carries the Ts mutation. Integration of the Flac+ particle into the chromosome may result in suppression of the Ts− mutation. In such strains the F replication system may serve as an alternative to the defective chromosomal initiation system. This is expected according to the model proposed by Pritchard, Barth and Collins (1969) for the regulation of DNA synthesis. This possibility was tested in more detail with the well-studied initiation mutants CRT83 and CRT46 and will be described in the next chapter.

A peculiarity of revertant strains of TS16/Flac+, which is not understood, is that most of the revertants have a Lac− phenotype on tetrazolium lactose nutrient agar plates. However, as seen in the experiments, these revertants are still male strains which carry an Flac+ factor probably integrated into the chromosome.
CHAPTER 3

The experiments done with the mutant TS16 suggested that its temperature-sensitive defect might be suppressible by the integration of an Flac\(^+\) factor into the chromosome.

In certain properties, this strain behaved in a similar way to the temperature-sensitive mutants CRT83 and CRT46 described by Hirota, Ryter and Jacob (1968) and Kohiyama (1968). A large number of experiments have been done with these mutants and they are generally believed to be defective in initiation of chromosome replication at high temperatures. Therefore, these two mutants were selected for detailed study; first, to see if the presence of an Flac\(^+\) factor also increased their reversion frequency and, if so, to attempt to determine whether the enhanced reversion frequency was due to integration. The following section describes some experiments done on this aspect and other properties of these mutants.

MATERIALS AND METHODS

Strains used

Table 3 gives a list of the bacterial strains used. Both CRT83 and CRT46 grow very slowly in supplemented minimal media. It was found in the course of experiments that some recombinant strains isolated from matings between Hfr Cau74 (Table 3) and these mutants grow much faster, although they are still temperature-sensitive. These recombinants, i.e. CRT15 and A3 were used in many experiments.
### Table 3  
List of the strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRT83</td>
<td>F−</td>
<td>thr− leu− thi− ilv− thy− lacY− Ts−</td>
<td>R.H. Pritchard</td>
</tr>
<tr>
<td>CRT83 NaR</td>
<td>F−</td>
<td>thr− leu− thi− ilv− thy− lacY− NaR Ts−</td>
<td>CRT83</td>
</tr>
<tr>
<td>CRT15</td>
<td>F−</td>
<td>thr− leu− thi− ilv− lacY− Ts−</td>
<td>CRT83 x Cav74</td>
</tr>
<tr>
<td>1638T33</td>
<td>F−</td>
<td>thr− leu− try− his− ilv− pur− gal− strR Ts−</td>
<td>CRT83 NaR/ Flac+ x THW1638</td>
</tr>
<tr>
<td>1638T23</td>
<td>F−</td>
<td>try− his− ilv− pur− met− gal− strR Ts−</td>
<td>CRT83 NaR/ Flac+ x THW1638</td>
</tr>
<tr>
<td>CRT46</td>
<td>F−</td>
<td>thr− leu− thi− ilv− thy− lacY− Ts−</td>
<td>R.H. Pritchard</td>
</tr>
<tr>
<td>A3</td>
<td>F−</td>
<td>thr− leu− thy− lacY− Ts−</td>
<td>CRT46 x Cav74</td>
</tr>
<tr>
<td>THW1638</td>
<td>F−</td>
<td>thr− leu− pro− try− his− ilv− metB− arg− pur− gal− mtl− strR</td>
<td>R.H. Pritchard</td>
</tr>
<tr>
<td>108</td>
<td>F−</td>
<td>met− pro− thy− lacZ− strR</td>
<td>R.H. Pritchard</td>
</tr>
<tr>
<td>231</td>
<td>F−</td>
<td>thr− leu− thi− pro− arg− his− ara− lacY− gal− xyl− mtl− strR recA−</td>
<td>R.H. Pritchard</td>
</tr>
<tr>
<td>P4X</td>
<td>Hfr</td>
<td>thi− metB−</td>
<td>B.M. Wilkins</td>
</tr>
<tr>
<td>Cav74</td>
<td>Hfr</td>
<td>pro−</td>
<td>R.H. Pritchard</td>
</tr>
<tr>
<td>H72</td>
<td>Hfr</td>
<td>thi− lacZ−</td>
<td>R.H. Pritchard</td>
</tr>
</tbody>
</table>
Table 3 (contd)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRT15/F⁺</td>
<td>F⁺</td>
<td>thr⁻ leu⁻ thi⁻ ilv⁻ lacY⁻ Ts/F⁺</td>
<td>CRT15 x 166/F⁺</td>
</tr>
<tr>
<td>1638T33/F⁺</td>
<td>F⁺</td>
<td>thr⁻ leu⁻ try⁻ his⁻ ilv⁻ pur⁻ gal⁻ Str⁺ Ts⁻/F⁺</td>
<td>1638T33 x 166/F⁺</td>
</tr>
<tr>
<td>166/F⁺</td>
<td>F⁺</td>
<td>thr⁻ leu⁻ thy⁻ lacY⁻/F⁺</td>
<td>R.H. Pritchard</td>
</tr>
<tr>
<td>A3/F⁺</td>
<td>F⁺</td>
<td>thr⁻ leu⁻ thi⁻ lacY⁻ Ts⁻/F⁺</td>
<td>A3 x 166/F⁺</td>
</tr>
<tr>
<td>CRT83Nal⁺/Flac⁻</td>
<td>F⁻</td>
<td>thr⁻ leu⁻ thi⁻ ilv⁻ thy⁻ lacY⁻/Flac⁻ Na⁺ Ts⁻/Flac⁻</td>
<td>CRT83Nal⁺ x C600/Flac⁻</td>
</tr>
<tr>
<td>CRT15/Flac⁻</td>
<td>F⁻</td>
<td>thr⁻ leu⁻ thi⁻ ilv⁻ lacY⁻ Ts⁻/Flac⁻</td>
<td>CRT15 x 231/Flac⁻</td>
</tr>
<tr>
<td>A3/Flac⁻</td>
<td>F⁻</td>
<td>thr⁻ leu⁻ thi⁻ lacY⁻ Ts⁻/Flac⁻</td>
<td>A3 x 231/Flac⁻</td>
</tr>
<tr>
<td>CRT83Nal⁺/KLF1</td>
<td>F⁻</td>
<td>thr⁻ leu⁻ thi⁻ ilv⁻ thy⁻ lacY⁻/KLF1 Na⁺ Ts⁻/F thr⁻ leu⁺</td>
<td>CRT83Nal⁺ x AB2463</td>
</tr>
<tr>
<td>1638T33/Fgal⁺</td>
<td>F⁻</td>
<td>thr⁻ leu⁻ try⁻ his⁻ pur⁻ gal⁻ Str⁺ Ts⁻/Fgal⁺</td>
<td>1638T33 x 270/Fgal⁺</td>
</tr>
<tr>
<td>CRT83Nal⁺/F13</td>
<td>F⁻</td>
<td>thr⁻ leu⁻ thi⁻ ilv⁻ thy⁻ lacY⁻/F13 Na⁺ Ts⁻/F purE⁺ proC⁺ lac⁺</td>
<td>CRT83Nal⁺ x W3747/F13</td>
</tr>
<tr>
<td>C600/Flac⁺</td>
<td>F⁻</td>
<td>thr⁻ leu⁻ thi⁻ lacY⁻/Flac⁻</td>
<td>C600 x 231/Flac⁻</td>
</tr>
<tr>
<td>270/Fgal⁺</td>
<td>F⁻</td>
<td>gal⁻/Fgal⁻</td>
<td>R.H. Pritchard</td>
</tr>
<tr>
<td>W3747/F13</td>
<td>F⁻</td>
<td>met⁻ A[lac pho tsx purE⁺]/F lac⁺ pho⁺ tsx⁺ purE⁺</td>
<td>J. Collines</td>
</tr>
<tr>
<td>AB2463/KLF1</td>
<td>F⁻</td>
<td>thr⁻ leu⁻ thi⁻ pro⁻ arg⁻ his⁻ lac⁻ gal⁻ rec⁻ Str⁺/F thr⁻ leu⁺</td>
<td>R. Buxton</td>
</tr>
<tr>
<td>Hfr 38</td>
<td>Hfr</td>
<td>ilv⁻ thy⁻ Ts⁻</td>
<td>CRT46 x P4 X</td>
</tr>
<tr>
<td>Hfr 27</td>
<td>Hfr</td>
<td>ilv⁻ thy⁻ Ts⁻</td>
<td>CRT46 x P4 X</td>
</tr>
<tr>
<td>74H</td>
<td>Hfr</td>
<td>ilv⁻ Ts⁻</td>
<td>CRT83 x H72</td>
</tr>
</tbody>
</table>

The origin and direction of transfer of the Hfr strains are shown in Figure 5.
instead of CRT83 and CRT46.

Media The media used were the same as those described in Chapter 2.

Bacterial matings For mating experiments, bacterial strains were grown with shaking in nutrient broth, supplemented with thymine when required, in flasks incubated at 37°C or 30°C. At near stationary phase, equal volumes of male and female cultures were mixed and incubated at 30°C or 37°C. When mentioned in experimental procedures this mixture was diluted 10⁻¹ into prewarmed nutrient broth or phosphate buffer and incubated again. Samples from this were spread on plates selective for the required recombinants. When required, streptomycin (200 µg/ml) or nalidixic acid (20 µg/ml) was added to the plating media to select against the donor bacteria.

Interrupted mating The procedure was the same as that described in Chapter 2.

Other techniques The procedures were the same as those described in Chapter 2 or are described in the legends.

Aqueous scintillation fluid contained POP, 5g; POPOP, 33mg; triton-X, 333 ml and toluene, 666 ml.

TES buffer contained Tris (hydroxymethyl)-aminomethane hydrochloride, 0.05 M (pH 8.2); EDTA (ethylenediaminetetraacetate, 0.05 M and NaCl, 0.1 M.
TABLE 4  Reversion frequencies to temperature-resistance of CRT83, CRT46 and some of their derivative strains

Procedures as described in Materials and Methods (Chapter 2)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Approximate reversion frequency/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRT83</td>
<td>F−</td>
<td>2 x 10^{-5}</td>
</tr>
<tr>
<td>CRT15</td>
<td>F−</td>
<td>5 x 10^{-6}</td>
</tr>
<tr>
<td>1638T33</td>
<td>F−</td>
<td>5 x 10^{-6}</td>
</tr>
<tr>
<td>CRT46</td>
<td>F+</td>
<td>5 x 10^{-6}</td>
</tr>
<tr>
<td>CRT15/F+</td>
<td>F+</td>
<td>1.4 x 10^{-4}</td>
</tr>
<tr>
<td>1638T33/F+</td>
<td>F+</td>
<td>5 x 10^{-5}</td>
</tr>
<tr>
<td>CRT83Nal^{R}/Flac^{+}</td>
<td>F'</td>
<td>2 x 10^{-5}</td>
</tr>
<tr>
<td>CRT15/Flac^{+}</td>
<td>F'</td>
<td>5 x 10^{-5}</td>
</tr>
<tr>
<td>CRT83Nal^{R}/KLF1</td>
<td>F'</td>
<td>1.5 x 10^{-4}</td>
</tr>
<tr>
<td>1638T33/Fgal^{+}</td>
<td>F'</td>
<td>5 x 10^{-6}</td>
</tr>
<tr>
<td>CRT83Nal^{R}/F13</td>
<td>F'</td>
<td>2.3 x 10^{-6}</td>
</tr>
</tbody>
</table>
RESULTS

Reversion frequencies

Revertant strains which can grow normally at high temperatures can be isolated from both CRT83 and CRT46. The reversion frequency of the latter strains can be measured easily since only revertant cells can grow to form colonies on plates incubated at high temperature. Table 4 shows the reversion frequencies of CRT83, CRT46 and some of the derivative strains. Reversion frequencies were measured by spreading samples from cultures of the strains grown in nutrient broth at 30°C on nutrient agar plates and incubating at 30°C or 42°C. Some of the values obtained have been approximated to the nearest whole number and consistently similar values were obtained in repeated experiments. All the female strains which carry /Ts" mutation had very similar reversion frequencies. F+ and Flac+ derivatives of these strains clearly had 10-30 fold higher reversion frequencies. In the strain carrying KLF1, which is a larger F prime factor, the reversion frequency was even greater. Strangely, however, F13 or Fcal+ factors did not increase the reversion frequency of the strains tested. As will be shown in later sections, the increase in reversion frequency caused by F factors is at least partially associated with integration of the F factor into the chromosome. If the increased reversion frequency is indeed due to suppression by integration, it might be expected that an Flac+
strain would have a higher frequency of reversion than an $F^+$ strain. Because the frequency of integration of an Flac$^+$ factor into the chromosome is higher than that of an $F^+$ factor due to the homology of DNA between the chromosome and the Flac$^+$ episome. Furthermore, a strain carrying a long F prime factor would be expected to revert more frequently than one carrying a shorter episome. This relationship apparently holds true only for Flac$^+$ and KLF1. $F^+$ strains seemed to have reversion frequencies similar or higher than Flac$^+$ strains. $F_gal^+$, as well as F13 which is a much longer F prime factor (see Fig. 5), did not increase the reversion frequency. This behaviour indicates that the homology between the F$'$ factor and the chromosome is not an important factor in suppression by integration, quite possibly due to the fact that integration of F prime factors occurs at sites which differ from the regions of principal homology.

Experiments very similar to these have been done by Nishimura, Caro, Berg and Hirota (1971). According to their observations, an F$^+$ factor increases the reversion frequency of strain CR146 about 30-fold while an Flac$^+$ factor increases it by as much as 500-fold. Their observations are quite different from those reported above. Although the reason for such a discrepancy is not understood, it is possible that the growth conditions of the cultures used for determining reversion frequencies might be an important factor.
Figure 5  Chromosome map of *E. coli* showing the relevant markers

(Adapted from Taylor, 1970)
TABLE 5  The relative frequency of conjugants obtained for different markers when CRT1/F+ and its revertant strains are mated with THW1638 F−

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>Phenotype of selected conjugants (numbers/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Met+StrR</td>
</tr>
<tr>
<td>15/F+ Revertant 1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>CRT1/F+</td>
<td>39</td>
</tr>
</tbody>
</table>

Revertant strains were grown at 42°C in nutrient broth for several hours, strain THW1638 was grown at 37°C and CRT1/F+ at 30°C. The numbers of particles in all cultures were determined and the suspensions were diluted to 1 x 10^7 cells/ml using prewarmed nutrient broth. 1 ml of female culture and 1 ml of male culture were mixed and incubated at 37°C for 60 min. Appropriately diluted samples were spread on plates supplemented with streptomycin to select against the donors and all the requirements of THW1638 except the selected marker. Plates were incubated at 30°C for 2 days. The numbers given are conjugant colonies per 1 ml of mating mixture.
were done with stationary phase cultures whereas their studies involved exponentially-growing cultures.

Properties of revertants

Integration of the F factor into the chromosome

Some revertants of strain CRT15/F^+ were tested for their fertility in conjugation experiments. The relative frequency of various classes of conjugants obtained when the revertants and the parent F^+ strain were mated with strain THW1638/F^- are given in Table 3. The details of the procedure are described in the legend to Table 5. The conjugants were scored by plating equal volumes of the mating mixtures on selective plates. The results clearly show that there are two classes of revertants: those with a very high fertility which transfer some of the markers at a higher frequency than others and a second class which have a very low fertility and donate markers without any apparent gradient of transfer. The numbers given against CRT15/F^+ are those determined by spreading a 10-fold higher concentration of a mating mixture on selective plates in order to obtain sufficient recombinants to determine whether there is gradient of transfer from this male. The fertility of the parent strain CRT15/F^+ is very similar to that of its low fertility revertants. Out of 25 revertants tested, 19 had high fertility and 6 had low fertility. The high fertility (about $10^{-2}$ Met^+ conjugants/cell)
TABLE 6 The relative yields of $\text{lac}^+\text{Str}^R$ and $\text{Pro}^+\text{Str}^R$ conjugants when $\text{Flac}^+$ revertants are mated with $\text{recA}^-$ and $\text{recA}^+$ recipients.

The procedure is described in the text.

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>Conjugants/ml of mating mixture</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\text{recA}^+$ recipient</td>
<td>$\text{recA}^-$ recipient</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\text{Lac}^+\text{Str}^R$</td>
<td>$\text{Pro}^+\text{Str}^R$</td>
</tr>
<tr>
<td>CRT15/Flac$^+$</td>
<td></td>
<td>$7.2 \times 10^4$</td>
<td>$1.5 \times 10^3$</td>
</tr>
<tr>
<td>CRT15/Flac$^+$</td>
<td></td>
<td>$1.7 \times 10^4$</td>
<td>$2.1 \times 10^3$</td>
</tr>
<tr>
<td>revertant 2</td>
<td></td>
<td>$1.7 \times 10^4$</td>
<td>$2.0 \times 10^3$</td>
</tr>
<tr>
<td>CRT15/Flac$^+$</td>
<td></td>
<td>$1.7 \times 10^4$</td>
<td>$2.0 \times 10^3$</td>
</tr>
</tbody>
</table>
and the presence of a distinct gradient of transfer of markers indicates that such revertants are Hfrs (see Fig. 5 for the relative positions of the different markers). In these 19 strains the F factor appears to have integrated into the chromosome at a position close to the met and arg markers and the chromosome is transferred in a clockwise direction into the recipient strain. The other 6 revertants may still be F+ strains. Since the majority of revertants seem to be Hfrs, the integration of the F particle into the chromosome is clearly associated with the reversion of these strains to the Ts+ phenotype.

In the case of revertants of a strain carrying an Flac+ factor, it can be determined experimentally whether the Flac+ particle is integrated into the chromosome. A strain which carries an autonomous Flac+ factor can give Lac+ conjugants with a lac- recipient which is either recA- or recA+. On the other hand, when the Flac+ factor is integrated into the chromosome, it can give Lac+ conjugants only with recombination-proficient recipients. Therefore, by mating revertant strains with two lac- strains, one of which carries a mutation at recA, and scoring the relative yields of Lac+ conjugants, one should be able to determine whether the Flac+ factor is integrated within the chromosome of the donor strain.

The results of such an experiment are shown in Table 6. The strain 108 was used as the recA+ recipient and the strain 231 as the
Strain CRT15/Flac$^+$ and two of its revertants were used as donors. Nutrient broth-grown (30°C) cultures of donor and recipient strains were mixed at equal cell concentrations ($1 \times 10^7$ cells/ml) for 1 hour at 30°C and samples were plated on media selecting for either Lac$^+$Str$^R$ or Pro$^+$Str$^R$ conjugants. The table gives the numbers of Lac$^+$ and Pro$^+$ conjugants scored per ml of mating mixture. The pro$^+$ gene is on the chromosome and therefore serves as a control marker. None of the donor strains gave Pro$^+$Str$^R$ conjugants with recA$^-$ recipient while all of them yielded Pro$^+$Str$^R$ conjugants with the recA$^+$ recipient.

The revertants as well as parent CRT15/Flac$^+$ strain gave Lac$^+$Str$^R$ conjugants with both recA$^+$ and recA$^-$ strains, indicating that in at least some cells of the cultures of the revertants, Flac$^+$ particles are present in the autonomous state. The number of Lac$^+$Str$^R$ conjugants obtained with the two recipient strains were, however, not similar. The frequency of Lac$^+$Str$^R$ conjugants obtained from CRT15/Flac$^+$ mated with the recA$^-$ female was about $1/3$rd of the frequency obtained with the recA$^+$F$^-$ strain, while the two revertant strains show a greater reduction in the number of Lac$^+$ conjugants obtained with the recA$^-$ recipient when compared with the recA$^+$ recipient. The proportion of Lac$^+$ to Pro$^+$ conjugants obtained when the CRT15/Flac$^+$ strain was mated with Rec$^+$ recipient is about 50 : 1, which agrees with the ratio expected for such a mating (Wilkins, 1969). In the case of the two revertants, however, this proportion was less
TABLE 7  Analysis of Met$^+$ Str$^R$ conjugants, obtained in matings between CR$^+$/F$^+$ revertants and TH$^+$, for temperature-sensitivity

<table>
<thead>
<tr>
<th>Revertant strain</th>
<th>Met$^+$ Str$^R$ conjugants</th>
<th>number tested</th>
<th>number temperature-sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR$^+$/F$^+$ revertant 1</td>
<td>37</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&quot;                &quot; 2</td>
<td>36</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>&quot;                &quot; 3</td>
<td>42</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>&quot;                &quot; 4</td>
<td>43</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>&quot;                &quot; 5</td>
<td>38</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>&quot;                &quot; 6</td>
<td>41</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&quot;                &quot; 7</td>
<td>30</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&quot;                &quot; 8</td>
<td>38</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&quot;                &quot; 9</td>
<td>39</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>&quot;                &quot; 10</td>
<td>41</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

The procedure was the same as that described in the legend to Table 5. A number of the Met$^+$ Str$^R$ conjugants were picked at random, and purified by regrowing on selective plates and then tested for temperature-sensitivity on nutrient agar plates.
than 10:1. Clearly the results are ambiguous but suggest that the revertants are Hfrs containing a significant proportion of F' cells. Several other revertants in similar experiments gave comparable results. It is likely that the culture of Flac revertants are mixed cell populations containing cells in which the Flac factor is integrated into the chromosome (true revertant cells) and cells in which the Flac factor may have dissociated from the chromosome to form F' cells (presumably due to the instability of integration or a selective disadvantage of revertant cells). The hypothesis that the Flac revertant cultures are mixed predicts that an Flac revertant strain may produce temperature-sensitive cells. However, such cells could not be identified in an experiment where samples from a culture of a revertant strain were plated on nutrient agar plates at 30°C and the resulting colonies tested for temperature-sensitivity.

Revertants carry the Ts' mutation

To determine whether CRT15/F' revertants still carried the Ts' mutation, some of the Met'StrR recombinants obtained from matings of the revertant strains with THW1638 were tested for temperature-sensitivity and the results are given in Table 7. All the revertant strains which were Hfrs (2-5, 9, 10, Table 5) gave temperature-sensitive Met' conjugants, showing that these revertants
still carry the Ts" mutation although they can grow at high temperature. None of the tested Met^+Str^R recombinants derived from other revertants (1, 6, 7, 8) was temperature-sensitive. The number of colonies tested may be too small, however, to exclude the possibility that at least some of these revertants also carry the Ts" mutation. The fact that in the revertants which still carry Ts" mutation there is clear evidence that the F particle had integrated into the chromosome suggests that integration of the F factor into the chromosome may phenotypically suppress the temperature-sensitive defect in these cells. The simplest hypothesis to explain these results is that the F replication system is not temperature-sensitive while the initiation system of chromosome is temperature-sensitive and that, when the F particle is integrated into the chromosome, the F system acts as an origin for replication. Thus, the chromosome effectively becomes an F prime factor containing the whole bacterial chromosome.

In order to see whether the Flac^+ revertants also carried the Ts" mutation, some revertants of CRT83Nal^R/Flac^+ were made into F^- phenocopies and were mated with Hfr strain Cav74 to obtain Thr^+Leu^+Nal^R conjugants (see Fig. 5 for the direction of chromosome transfer by Cav74). Male cells from stationary cultures are known to behave phenotypically as females (Lederberg, Cavalli and Lederberg, 1952; Dubnau and Maas, 1968). In the experiments described here, large colonies from Flac^+ revertant strains grown on
TABLE 8 Properties of Thr$^+$Leu$^+$Nal$^R$ conjugants obtained by mating Hfr Cav74 and CRT83 Nal$^R$/Flac$^+$ revertants.

Procedure as described in the text.

<table>
<thead>
<tr>
<th>Revertant strain (recipient)</th>
<th>No. of conjugants analysed</th>
<th>Description of conjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRT83Nal$^R$/Flac$^+$</td>
<td></td>
<td>Ts$^-$ $\varphi$</td>
</tr>
<tr>
<td>Revertant 1</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>&quot;</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>&quot;</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>&quot;</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>&quot;</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>&quot;</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>&quot;</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>
nutrient agar plates were suspended in nutrient broth and were used as recipients (large colonies were assumed to have a high proportion of stationary phase cells). The Hfr Cav74 was grown in nutrient broth with aeration until the concentration reached about $1 \times 10^8$ cells/ml. Equal volumes of Hfr and Flac$^+$ revertant cultures were mixed and incubated at $30^\circ C$ for 40 minutes, after which a $10^{-1}$ dilution of this was made into phosphate buffer and incubated for a further 50 minutes. Diluted samples of this were spread on minimal glucose plates to select for Thr$^+$Leu$^+$Nal$^R$ recombinants of the revertants.

A random sample of the recombinant colonies obtained with each of six revertant strains were characterised by testing for temperature-sensitivity on nutrient agar plates incubated at $42^\circ C$, and for fertility, by testing their ability to transfer the lac$^+$ marker to female strain 108. Table 8 shows some of the results of such an analysis. All the revertant strains gave temperature-sensitive conjugants, showing clearly that they still carried the temperature-sensitive mutation. Furthermore, the results show that the Ts$^-$ recombinants were all females. Nearly half of the conjugants tested were females which are temperature-sensitive. No male conjugants were found which are temperature-sensitive. These features agree with the idea that, in these revertants, the Flac$^+$ factor is integrated into the chromosome and it causes a
phenotypic reversion of the Ts\(^{-}\) character and that loss of the integrated Flac\(^{+}\) factor, as a result of recombination between the transferred Hfr DNA and the resident chromosome, results in the restoration of the Ts\(^{-}\) phenotype. The male temperature-resistant conjugants would be those with the Flac\(^{+}\) factor still integrated into the chromosome.

The majority of the conjugants obtained were temperature-sensitive, which suggests that the integrated Flac\(^{+}\) factor has been lost as a result of recombination. Some of the female conjugants obtained in the above experiment were temperature-resistant. This can be explained if such conjugants inherit the Ts\(^{+}\) gene from the donor in addition to the loss of the Flac\(^{+}\) factor. The proportion of such conjugants found in the experiment is much higher than expected since the map position of the Ts marker is quite distant from the thr leu markers. The possible reason for this abnormal gradient is a recombination-defect in these strains. This property of the temperature-sensitive strains is investigated further in the next Chapter.

It could be argued, however, that the suppression of the Ts\(^{-}\) phenotype may not be a result of an Flac\(^{+}\) integration, but is due to a suppressor mutation occurring between the pro-ilv region of the chromosome and that recombination may result in the loss of this mutation. The absence of any male conjugants which are temperature-sensitive in the above experiments makes such a possibility unlikely,
although it cannot be excluded.

Furthermore, if the Flac\(^+\) revertants carry such suppressor mutations, these revertants should be able to transfer such mutations to other temperature-sensitive strains and give temperature-resistant conjugants. In order to test this possibility, the strain CRT83\(R^R/Flac^+\) revertant 1 (see Table 8) was mated with a temperature-sensitive female strain (pro\(^-\) thr\(^+\) leu\(^+\) ilv\(^+\) Ts\(^-\)), obtained in the experiment shown in Table 8), and Pro\(^+\) Ilv\(^+\) recombinants were selected at 30\(^0\)C. 100 of the recombinant colonies obtained were tested for temperature-sensitivity and for threonine- and leucine-requirement. All the recombinants were temperature-sensitive while 70 of them were Thr\(^-\) Leu\(^-\). This result indicates that Flac\(^+\) revertants do not carry suppressor mutations which could be transferred as early markers.

**The site of F integration**

The site of integration of the F factor in two of the CRM15/F\(^+\) revertants was determined with greater accuracy by interrupted mating experiments. The female strain THW1638 was used as the recipient. The time of entry of the met\(^+\) and arg\(^+\) markers at 37\(^0\)C was determined. The results of the experiments are given in Figures 6a and 6b. The details of the procedure are given in the legend to the figures. Both of the revertants tested began to
CRT15/F+ revertant 2 and THW1638 were grown in nutrient broth at 37°C. At about $1 \times 10^8$ cells/ml, equal volumes of the two cultures were mixed and maintained at 37°C with slow shaking. At given times 1 ml of the mixture was removed into 4 ml of phosphate buffer, blended for 7 seconds and 0.1 ml samples were spread on plates selective for Met$^+$Str$^R$ or Arg$^+$Str$^R$ recombinants. The plates were incubated at 30°C for 2 days before the colonies were counted.
transfer \text{met}^+ \text{ and } \text{arg}^+ \text{ to the recipient at about 12 and 14 minutes respectively. The close proximity of } \text{met}^+ \text{ and } \text{arg}^+ \text{ agrees with the known positions of these markers. The results suggest that both of the tested Hfrs have an origin at 60-65 minutes on the } \text{E. coli} \text{ chromosome map (Taylor, 1970). The fact that both the revertants have an apparently similar site of F integration is quite remarkable. Since the gradient of transfer obtained with these two revertants (see Table 5) was very similar to that of all the other revertant Hfrs, it is quite probable that all these Hfrs had the F particle integrated at a very narrow region of the chromosome. Since strain CRT15/F^+ \text{ and its non-Hfr revertants exhibited no gradient of transfer, it would appear that F integration can occur at random in the chromosome and that the preference of a narrow region of integration in revertants is not a peculiarity of the parent strain. It is quite likely that the site of integration plays an important part in suppression of the } \text{Ts}^- \text{ mutation.}

These results agree very much with similar observations made by Nishimura, Caro, Berg and Hirota (1971). According to their observations, out of 79 tested revertants of strain CRT46/F^+, 76 were Hfrs, with 42 of them having the F factor integrated into the chromosome within the 60-65 minutes region. 23 of the others had F integrated into the chromosome between 55-60 minutes. All of the others had the F particle integration in apparently scattered positions
within the left half of the *E. coli* chromosome map. All of the Hfrs except four transferred DNA in a clockwise direction.

**Effects of acridine orange on revertant strains**

When *E. coli* strains harbouring autonomous F factors are grown in the presence of acridine orange for several generations, a large proportion of cells in the culture become F^- cells. The mechanism of this sex factor-elimination is obscure. However, the experimental evidence indicates that this effect of acridine orange on F^+ cells is not due to abnormal segregation of F factors in cell division after the treatment, but is due to a direct effect of acridine orange on the multiplication of the F factor (Stouthamer, de Haan and Bulten, 1963; Yamagata and Uchida, 1969). It is generally believed that the dye inhibits the replication of F particles at concentrations which allow the replication of the chromosome.

If the F^+ revertants, which have been described above, are Hfrs in which the chromosome is replicated at high temperature by the F replication system, the possibility arises that these strains might be sensitive to acridine orange at high temperature. When a culture of an Hfr revertant of CRT15/F^+ growing in nutrient broth at 42°C was treated with 20 μg/ml of acridine orange, no significant change in growth rate was observed for several generations. At a concentration of 30 μg/ml or more, the growth rate of the culture,
as measured by particle counts, continued for several generations before it stopped. Such an effect was also observed with F⁻ revertant strains as well. Thus acridine orange apparently has no differential effect on chromosome replication of revertant Hfrs.

This result is apparently contradictory to that of Nishimura, Caro, Berg and Hirota. They have observed that Hfr revertants of CRT46/F⁺ do not grow on plates containing 20 μg/ml acridine orange at 42°C, although they grow at 30°C, while F⁻ revertants can grow both at 30°C and 42°C. However, they have also obtained ambiguous results in liquid media.

The response of different CRT88NalR/Flac⁺ revertants to treatment with acridine orange varied. When the revertant strains were grown in nutrient broth at 30°C in the presence of 50 μg/ml of acridine orange for about 20 hours at 30°C and the samples were plated on nutrient agar tetrazolium lactose plates at 30°C, most of the revertant strains gave highly mucoid colonies which could not be identified as Lac⁻ or Lac⁺. However, 5 out of 20 revertants tested gave non-mucoid colonies, 5-30% were Lac⁻. All such Lac⁻ colonies proved to be temperature-sensitive in subsequent tests, while all the Lac⁺ colonies were still temperature-resistant. The Lac⁻ colonies were also found to be females when tested for fertility in mating experiments. This observation shows that the temperature-resistance of these revertants is directly dependent on the presence
of the Flac\(^+\) factor. As described in previous experiments, Flac\(^+\) revertants can give Lac\(^+\) conjugants with recA\(^-\)lac\(^-\) recipients. When the Flac\(^+\) factor derived from an original revertant was reintroduced into these cured Lac\(^-\) temperature-sensitive cells by mating with an appropriate recA\(^-\)/Flac\(^+\) donor, the Lac\(^+\) conjugants remained temperature-sensitive. This indicates that the Flac\(^+\) particle of the revertants probably did not carry a mutation conferring temperature-resistance on the cell.

The dependence of the Ts\(^+\) phenotype on the presence of the Flac\(^+\) factor and the observation that the rescued Flac\(^+\) factor appears not to carry a suppressor mutation supports the view that the Flac\(^+\) factor must integrate into the chromosome for reversion. On the other hand, if the Flac\(^+\) factor is integrated into the chromosome, it is not understood why it should segregate out in at least some revertants after acridine orange treatment. This again leads to the possibility that the Flac\(^+\) factor of the revertants, although integrated into the chromosome, is unstable and dissociates to form F prime cells.

**Hfr strains with the Ts\(^-\) mutation**

The experiments done with F\(^+\) revertants showed that the majority are Hfrs and that the integration of the F factor into the chromosome can cause a phenotypic suppression of the Ts\(^-\) mutation.
### TABLE 9a

**CROSS 1 P4X x CRT83**  
Phenotype selected, Met\(^{+}\)Lac\(^{+}\)  
Total number of conjugants analysed: 94

<table>
<thead>
<tr>
<th>Description of the conjugants:</th>
<th>Ts(^{-}) Ilv(^{-})</th>
<th>Ts(^{+}) Ilv(^{+})</th>
<th>Ts(^{-}) Ilv(^{+})</th>
<th>Ts(^{+}) Ilv(^{-})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>♀</strong></td>
<td>22</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>♂</strong></td>
<td>19</td>
<td>5</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

### TABLE 9b

**CROSS 2 P4X x CRT46**  
Phenotype selected: Met\(^{+}\)Lac\(^{+}\)  
Total number of conjugants analysed: 93

<table>
<thead>
<tr>
<th>Description of the conjugants:</th>
<th>Ts(^{-}) Ilv(^{-})</th>
<th>Ts(^{+}) Ilv(^{+})</th>
<th>Ts(^{-}) Ilv(^{+})</th>
<th>Ts(^{+}) Ilv(^{-})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>♀</strong></td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td><strong>♂</strong></td>
<td>35</td>
<td>31</td>
<td>7</td>
<td>10</td>
</tr>
</tbody>
</table>

### TABLE 9c

**CROSS 3 Hfr H72 x 1638T23**  
Phenotype selected: Met\(^{+}\)Str\(^{R}\)  
Total number of conjugants analysed: 62

<table>
<thead>
<tr>
<th>Description of the conjugants:</th>
<th>Ts(^{-}) Ilv(^{-})</th>
<th>Ts(^{+}) Ilv(^{+})</th>
<th>Ts(^{-}) Ilv(^{+})</th>
<th>Ts(^{+}) Ilv(^{-})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>♀</strong></td>
<td>10</td>
<td>5</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td><strong>♂</strong></td>
<td>19</td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>
The experiments done with Flac\textsuperscript{+} revertants, although they do not give such clear evidence, agree with this interpretation. The next important aspect of suppression to be investigated was whether the Ts\textsuperscript{-} mutation is suppressed when it is introduced into a known Hfr.

For this purpose, strains carrying the Ts\textsuperscript{-} mutation were constructed as follows. Hfr P4X was mated with strain CRT83 and Met\textsuperscript{+}Lac\textsuperscript{+} recombinants were selected. Since the lac\textsuperscript{+} marker is located near the tail end of the P4X chromosome, some of the conjugants obtained inherit the integrated F particle of the P4X chromosome. The Met\textsuperscript{+}Lac\textsuperscript{+} conjugants obtained were scored for the requirement of isoleucine and valine and for temperature-sensitivity. Their fertility was scored by testing the ability of the conjugants to give Met\textsuperscript{+}Str\textsuperscript{R} recombinants in mating with strain 108. The results of this experiment are shown in Table 9a. The ilv marker is close to the Ts marker and therefore association of the Ilv\textsuperscript{-}Ts\textsuperscript{-} and Ilv\textsuperscript{+}Ts\textsuperscript{+} phenotypes is to be expected. A great majority of conjugants tested seemed to inherit the female parental markers Ts\textsuperscript{-}, ilv\textsuperscript{-}. However, nearly 50\% of these seemed to be Hfrs, indicating that there is no correlation between temperature-resistance and the Hfr character. Out of all the Hfr conjugants tested, two thirds were temperature-sensitive, although the majority of the Ts\textsuperscript{+} conjugants were Hfrs. This result clearly shows that Hfr P4X cannot suppress the Ts\textsuperscript{-} mutation. When the above experiment was repeated with CRT46 and P4X, the similar observations were made (Table 9b). In this case,
majority of the conjugants obtained were Hfrs and nearly 50% of them were temperature-sensitive.

There is a possibility that the inability of Hfr P4X to suppress the Ts− mutation may be due to a defect in the integrated F particle in this Hfr, since according to the hypothesis presented above, a functional F replication system is necessary for suppression. Although chromosome transfer by this Hfr in conjugation is normal, the possibility that the autonomous replication system of F may be defective cannot be excluded.

An alternative possibility is that the site and orientation of integration of the F particle may be important factors determining whether or not Hfrs can suppress the Ts− mutation. This aspect is made more likely by the fact that in all tested CRT15/F+ revertants which were Hfrs, the F particle had integrated in the same region of the chromosome with the same orientation. The orientation of F integration in Hfr P4X is the opposite of that found in the revertants.

An Hfr strain with a different site of F integration and different orientation, namely Hfr H (see Figure 5) was used in an experiment similar to the above. Hfr H72 was mated with 1638T23 (a recombinant of CRT3, see Table 3) and Met+StrR conjugants were selected. The fertility and the temperature-sensitivity of the conjugants were scored as in the above experiments. The results of this experiment are given in Table 9c. About half of the conjugants
obtained were Hfrs; but the majority of the Hfrs seemed to be
temperature-sensitive and more than half of the temperature-sensitive
conjugants were classed as Hfrs. These observations are similar to
those obtained above with Hfr F4X and indicate that this Hfr also
cannot suppress the Ts" mutation.

Three other Hfr H strains gave similar results when the
above experiment was repeated; 2 of these strains have been found to
be capable of producing F primes in matings with recA− female strains
at a frequency of about 2-6 x 10^{-5} per Hfr cell (Roger Buxton,
personal communication). This strongly suggests that the autonomous
F replication system of the integrated F particle is not defective.
Thus the inability of both Hfr F4X and Hfr H to suppress the Ts−
mutation is not likely to be due to a defect in the replication
system of the integrated F particle.

Nishimura, Caro, Berg and Hirota (1971) have isolated 5 Hfr
strains from CRT46/F' at 30°C by fluctuation techniques. Four of them
were found to be still temperature-sensitive while one was temperature-
resistant. The temperature-resistant Hfr had the F particle
integrated at a position similar to the majority group of revertant
Hfrs. But one of the temperature-sensitive Hfrs also had similar
transfer properties. Their observations also agree with those described
above and indicate that integration of the F particle is not sufficient
for the suppression of the Ts− mutation.

It is likely that two steps are necessary for reversion by
suppression. Integration of F into the chromosome is only one of these. Nishimura et al. (1971) postulate that the second step may involve a derepression of the F replication system. This view is based on the general belief that in normal Hfr strains autonomous replication of the F particle is repressed. For the F system to be functional in the Hfr state, a mutational event is necessary which derepresses the F particle.

A second possibility is that the F replication system of the Hfr is not temperature-sensitive while the chromosomal replication system is temperature-sensitive. At high temperature, the F particle can initiate and replicate the whole chromosome as an F prime factor. However, the cells are not viable and cannot form colonies. The second step involves a mutational event which brings about a change necessary to make the cells viable. This possibility is based on the Pritchard, Barth and Collins' (1969) model for the control of DNA replication. The model predicts that in normal Hfr strains the F replication system is repressed by the F specific inhibitor which is produced by passive replication of the F particle (see Chapter 1). When the chromosome cannot replicate at high temperature, the inhibitor concentration is reduced to the critical level by growth of the cell, thus enabling the F system to initiate.

According to these explanations for the temperature-sensitivity of Hfrs it might be expected that the frequency of reversion of a temperature-sensitive Hfr strain may be much higher than that of
an $F^+$ strain. Nishimura et al. (1971) have measured the reversion frequency of some temperature-sensitive Hfr strains and have observed that such strains have 2 to 10-fold higher reversion frequency than the parent $F^+$ strain. They have also isolated temperature-sensitive strains from Hfr revertants of CRT46/$F^+$. These temperature-sensitive strains are still Hfrs and they have a reversion frequency to $Ts^+$ about 50-fold higher than an $F^+$ strain. These observations have been taken by these authors as evidence that two steps are necessary for reversion by suppression.

I have measured the reversion frequency of 6 temperature-sensitive Hfr strains obtained in the CRT83 x P4X mating experiment described above. Their reversion frequencies were similar to the reversion frequency of an $F^+$ strain. This observation is contradictory to those of Nishimura et al. (1971) and indicates that a temperature-sensitive Hfr cannot revert to temperature-resistance at a higher rate. It is possible that the reversion frequency of a Hfr varies with different Hfrs.

If the integration and the second postulated step are two independent events, the temperature-sensitive Hfrs should have a much higher reversion frequency than $F^+$ strains. The 2 to 10-fold increase in reversion frequency which Nishimura et al. (1971) have observed in the case of Hfrs isolated from the fluctuation technique is really not as much as one would expect. On the other hand, the temperature-sensitive derivatives of revertant Hfrs had a much higher
reversion frequency. Furthermore, the total reversion frequency of strain CRT46/Flac\(^+\) (about 1 x 10\(^{-3}\)/cell) which Nishimura et al. (1971) have observed, is much higher than one would expect on the basis of a requirement for two independent events for reversion.

An altogether different possibility is that it is the manner of integration of the F particle which determines whether the Hfr will be temperature-sensitive. Only certain types of integration may give a revertant phenotype. If the integration event itself normally suppresses the F replication system, as is generally believed (through a mechanism which is not understood), it would be necessary to assume that certain types of integrations may not result in such suppression.
DNA synthesis in temperature-sensitive strains

Many experiments have been done to investigate the nature of DNA synthesis in CRT46 and CRT83 at high temperature (Hirota, Ryter and Jacob, 1968; Hirota, Mordoh and Jacob, 1970). Most of these give indirect evidence that these mutants are defective in the initiation of chromosome replication at high temperature.

When an exponentially growing culture of CRT83 or CRT46 at 30°C is transferred to 40°C, DNA synthesis in the culture decreases progressively for about 60 minutes and then stops. The increase in DNA content at 40°C is dependent on the previous growth rate at 30°C and is in the range of what would be predicted if the cells do not initiate new rounds of replication at high temperature but only complete those cycles already initiated. When cells are grown at 30°C in a "heavy" medium and transferred to a "light" medium at 40°C, all the DNA which is synthesised at high temperature is of intermediate density (Hirota, Ryter and Jacob, 1968). This observation is consistent with the hypothesis that new cycles of DNA synthesis are not initiated at high temperature.

Furthermore, the kinetics of DNA synthesis observed after a shift to high temperature is similar to that observed when the cells are starved for required amino acids at 30°C. The latter condition is known to stop initiation of chromosome replication (Lark, Repko and Hoffman, 1963).

When a culture of CRT46 is maintained at 41°C for 60 minutes
and then transferred back to 30°C, DNA synthesis is resumed at an initially increased rate which then decreases. After about one generation time at 30°C, the rate of cell division also sharply increases (Hirota, Mordoh and Jacob, 1970). Resumption of DNA synthesis does not take place if chloramphenicol is present in the medium at 30°C. This behaviour is expected if the cells stop initiation of replication at high temperature but can re-initiate when transferred back to 30°C.

Labelling experiments have indicated that when a culture is submitted to two successive treatments at 41°C, with intervening overnight growth at 30°C, resumption of DNA synthesis takes place in the same area of the chromosome (Hirota, Mordoh and Jacob, 1970). All such observations suggest strongly that CRT46 and CRT83 are defective in initiation of chromosome replication at high temperature.

Hirota, Ryter and Jacob (1968) have found that a culture of CRT46/Flac+ can incorporate 14C-thymine into DNA at high temperature long after incorporation has ceased in an F− culture. This observation has been interpreted to mean that the Flac+ particle can continue to replicate at high temperature even after the chromosome replication has stopped and that the extra DNA synthesised is Flac+ DNA. However, there is no experimental evidence showing that this DNA is in fact Flac+ DNA. Furthermore, the amount of extra DNA synthesised by an Flac+ strain over the F− strain appears
to be too great to be accounted for by the replication of the Flac particle alone.

The experiments which were described in the previous section suggested that, although an F or Flac particle can suppress the Ts mutation by integration into the chromosome, integration itself is not sufficient. Thus, when a Ts mutation is introduced into a known Hfr the strain remains temperature-sensitive. However, the possibility was raised that the temperature-sensitive Hfr strain might be capable of initiating DNA synthesis at high temperature although they cannot form colonies.

The following section describes some experiments designed to investigate the nature of DNA synthesis by several temperature-sensitive strains and to answer the following questions:

1. Do Flac or F strains synthesise significantly more DNA at high temperature than F strains and, if so, is the extra DNA episomal?
2. Are the temperature-sensitive Hfrs capable of initiating DNA synthesis at high temperature?

When an exponentially growing culture of a temperature-sensitive strain is starved for amino acids at 30°C, DNA replication cycles presumably go to completion and no new initiations take place (Maaløe and Hanawalt, 1961; Lark, Repko and Hoffman, 1963). If the culture is now shifted to 42°C in the presence of amino acids, no DNA synthesis should be observed if the strain is defective in initiation at high temperature. However, if the F and Flac
factors can replicate at high temperature, strains carrying these factors should synthesis some DNA after transfer to high temperature, although the amount might be expected to be too small to be detectable in uniformly labelled cultures. If temperature-sensitive Hfr strains can initiate replication at high temperature, such strains should synthesise significant amounts of DNA at high temperature following amino acids-starvation at 30°C.

Figure 7 shows the pattern of DNA synthesis of the strain CRT15 observed after amino acids-starvation. A culture of the strain growing exponentially at 30°C in minimal glucose medium containing 0.5% casamino acids and 14C-thymine, was divided into two parts. One part was shifted to 42°C in the presence of amino acids while the other part was filtered and the cells were washed and resuspended at 30°C in a medium lacking casamino acids at 30°C. After 3½ hours the amino acids-starved culture was shifted to 42°C. After 6 minutes, one portion of this culture was supplemented with casamino acids. The pattern of DNA synthesis was followed by measuring the acid-precipitable radioactivity in samples removed at regular intervals.

It is seen from the results shown in Fig. 7 that when the cells were starved for amino acids about 80% more DNA was synthesised in about 2 hours and DNA synthesis then stopped. The pattern of DNA synthesis observed in the culture which was shifted
Figure 7  Incorporation of $^{14}$C-thymine into cold TCA-precipitable material in CRT15 after amino acids-starvation

The strain was grown for several hours at 30°C in minimal glucose medium supplemented with 1.0 µg/ml of $^{14}$C-thymine at a specific activity of 0.05 µCi/µg, 100 µg/ml of deoxyadenosine* and 0.5% casamino acids. When the culture was growing exponentially, 20 ml was removed, filtered through a membrane filter, washed with phosphate buffer and resuspended in 4 ml of phosphate buffer. 1.0 ml of the suspension was transferred into 10 ml of medium prewarmed at 42°C. 3 ml was transferred to 30 ml of the medium lacking amino acids, prewarmed at 30°C. Samples were removed into cold TCA (0°C) at given times. After 3½ hours, 15 ml of the amino acids-starved culture was shifted to 42°C. 6 minutes later, 5 ml of this was transferred into 5 ml of medium with 1.0% casamino acids prewarmed at 42°C. Samples from both the cultures were removed at given times into cold TCA. The radioactivity in the acid precipitable material of the samples was determined as described in the Materials and Methods.

* Since CRT15 is a Thy$^+$ strain, external thymine will not be incorporated efficiently into DNA unless the strain is grown in the presence of deoxyadenosine (Kammen, 1967).
to 42°C in the presence of amino acids, is very similar. When amino acids were added to the starved culture there was slow synthesis of DNA. This would not be expected if the cells complete cycles of replication at 30°C in the absence of amino acids and if they cannot initiate new rounds of replication at high temperature. The total amount of DNA synthesised in 3 hours at high temperature after the amino acids-starvation was, however, very small compared with the amount of DNA synthesised at 30°C in the absence of amino acids. One possible explanation of this unexpected resumption of DNA synthesis at high temperature may be that not all of the cells in the culture have terminated replication cycles during amino acids-starvation by the time DNA synthesis comes to a halt and, in the presence of amino acids at high temperature, these cells terminate the replication cycles even though new cycles are not initiated.

In the above experiment, amino acids were not added to the amino acids-starved cells until 6 minutes after the cells were shifted to 42°C. This was done as a precaution against the possibility that if amino acids are added at the same time as the temperature-shift, cells might be able to initiate DNA synthesis. In a preliminary experiment to test this possibility, a pre-labelled culture of CRT15 was amino acids-starved as in the experiment described previously and then shifted to 42°C in the absence of amino acids. Samples from this culture were removed at short intervals into aliquots of prewarmed medium containing casamino acids. After incubation for one hour at
TABLE 10  Incorporation of $^{14}$C-thymine into cold TCA-precipitable material when CRT15F$^{-}$ is grown for one hour at 42°C in the presence of amino acids after a previous amino acids-starvation period at 30°C

<table>
<thead>
<tr>
<th>Time in-aa at 42°C minutes</th>
<th>CPM - background</th>
<th>Time in-aa at 42°C minutes</th>
<th>CPM - background</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>410</td>
<td>8</td>
<td>445</td>
</tr>
<tr>
<td>1</td>
<td>407</td>
<td>10</td>
<td>432</td>
</tr>
<tr>
<td>2</td>
<td>466</td>
<td>12</td>
<td>450</td>
</tr>
<tr>
<td>3</td>
<td>477</td>
<td>14</td>
<td>467</td>
</tr>
<tr>
<td>4</td>
<td>532</td>
<td>16</td>
<td>444</td>
</tr>
<tr>
<td>5</td>
<td>416</td>
<td>20</td>
<td>437</td>
</tr>
<tr>
<td>6</td>
<td>416</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
42°C, the radioactivity in the acid-precipitable material of each sample was determined. The results of this experiment, given in Table 10, suggest that if amino acids are added in the first 4 minutes after the shift, the cells may be capable of synthesising some DNA.

Transfer of growing cells from a casamino acids-containing medium to one lacking amino acids may have more extreme effects on DNA synthesis than occurs after a simple amino acids-starvation and this may result in incomplete termination of replication cycles. However, in experiments very similar to those described above, done by Hirota, Mordoh and Jacob (1970) on CRT46 in which starvation for threonine and leucine alone was used, a similar continuation of DNA synthesis at high temperature can be observed from their published data. Therefore, the resumption of DNA synthesis observed in the culture at high temperature after amino acids-starvation, is probably not totally dependent on the use of casamino acids.

When the above experiment was repeated with strain CRT46, very similar results were obtained (data not shown) and these agree with the data of Hirota et al. (1970).

In the case of strain A3, a derivative of CRT46, (Figure 8) the DNA synthesis observed at 42°C after a previous amino acids-starvation period, is even more striking. This strain synthesised only about 36% more DNA when starved for amino acids at 30°C. When shifted to 42°C in the presence of amino acids, it synthesised about 25% more DNA in the ensuing 3 hours.
Figure 8

Incorporation of $^{14}C$-thymine into cold TCA-precipitable material in A3 after amino acids-starvation. Procedure as described for Fig. 7.
Figure 9 shows the pattern of DNA synthesis in CR6/Flac". When amino acids-starved at 30°C, this strain synthesised about 50% more DNA in the course of 3.5 hours. When shifted to 42°C in the presence of amino acids after this initial treatment, it synthesised about 25% more DNA in 1.5 hours.

The amounts and rates of DNA synthesised at 42°C after amino acids-starvation are probably not comparable because different strain appear to have different patterns of DNA synthesis under the same conditions.

Figures 10, 11 and 12 show the patterns of DNA synthesis observed with 3 temperature-sensitive Hfr strains. Hfr38 and 27 are recombinants of CR6 and P4X, while Hfr 14H is a recombinant of CRT33 and Hfr H72. All the temperature-sensitive Hfr strains synthesised some DNA when shifted to high temperature as did F" strains. However, after about one hour's growth at 42°C, degradation of DNA could be detected. Degradation was not observed when the cells were starved for amino acids at 30°C which resulted in an increment in DNA content of about 60-75%. When amino acids-starved cultures were transferred to high temperature and treated with amino acids, there was clear evidence that, in case of Hfr 27, DNA synthesis was resumed and proceeded for several hours at a slow rate. The behaviour of the other strains under similar conditions suggests that resumption of DNA synthesis is followed by degradation, although the results are
The strain was grown for several hours at 30°C in minimal glucose medium supplemented with 1.0 μg/ml of \(^{14}\)C-thymine at a specific activity of 0.05 μCi/μg, 100 μg/ml of deoxyadenosine and 0.5% casamino acids; when the culture was growing exponentially, 10 ml was removed, filtered through a membrane filter, washed with phosphate buffer and suspended in 30 ml of the medium lacking amino acids prewarmed at 30°C. Samples were removed at given times into cold TCA (0°C). After \(3\frac{1}{2}\) hours, the rest of the culture was shifted to 42°C. 6 minutes later, 10 ml of the culture was transferred into 10 ml of medium with 1.0% casamino acids prewarmed at 42°C. Samples were removed at given times into cold TCA. The radioactivity in the acid precipitable material of the samples was determined as described in Materials and Methods.
Figure 10  Incorporation of $^{14}$C-thymine into cold TCA-precipitable material in Hfr38 after amino acids-starvation.

Procedure as described for Figure 7.
Figure 11  Incorporation of $^{14}$C-thymine into cold TCA-precipitable material in Hfr27 after amino acids-starvation.

Procedure as described for Figure 7.
Incorporation of $^{14}$C-thymine into cold TCA-precipitable material in Hfr 14H after amino acids-starvation.

Procedure as described for Figure 7.
not unambiguous. A characteristic feature of the studies of DNA synthesis in Hfr strains is the high degree of scatter in the measurements of the incorporated radioactivity, especially if the cells were growing at high temperature after a prior amino acids-starvation period. This abnormality may be connected in some way with the degradation of DNA.

The degradation of DNA by temperature-sensitive Hfr strains is not understood at present. This phenomenon has been observed in all the Hfr strains tested. The degradation was observed only when the cells were grown at 42°C in the presence of amino acids. Amino acids-starved cultures did not degrade DNA even after a shift to high temperature. It is possible that in Hfr strains replication of the inserted F particle occurs when the culture is transferred to high temperature and that this DNA is not stable and is subsequently degraded by the cells.

The possibility that degradation and resynthesis of DNA takes place simultaneously when the Ts Hfr strains grow at high temperature was confirmed in the case of Ts Hfr 38. A culture of the strain growing at 30°C was divided into 2 parts. One part was prelabelled with 14C-thymine. After several hours of growth at 30°C, a portion of the prelabelled culture was filtered, washed and transferred to a "cold" medium prewarmed at 42°C. Similarly, a portion of the unlabelled culture was transferred to prewarmed "hot" medium at 42°C. The rest of the cultures were amino acids-starved
at 30°C (in the presence of the label in the case of the labelled culture and in the absence of the label in the case of the unlabelled culture). After 3½ hours the labelled culture was filtered, washed and resuspended in a "cold" medium with amino acids prewarmed at 42°C and the unlabelled culture was transferred to a "hot" prewarmed medium with amino acids at 42°C. A schematic representation of the experimental procedure is given in Figure 13a. DNA synthesis in the cultures was followed by removing samples at regular intervals and counting the radioactivity. Figure 13b gives the results of this experiment. When the prelabelled culture was transferred to 42°C in the absence of the label, the amount of cold TCA precipitable radioactivity in the cells began to fall after about 1 hour and results in a loss of about 30% of the label in the course of 3½ hours (left panel). On the other hand, when the unlabelled cells were transferred to 42°C in the presence of the label there was rapid incorporation of the label into the DNA for about 2 hours. The results indicate that the synthesis and degradation of DNA takes place simultaneously in this strain at 42°C. The loss of prelabelled DNA indicates that the DNA which is being degraded is not that which is synthesised immediately before the degradation but the pre-existing DNA of the cell.

When the prelabelled cells were amino acids-starved at 30°C and then transferred to high temperature in the presence of amino
Figure 13a  Scheme for the experiment shown in Fig. 13b. Procedure based on that given in the legend to Figure 7.
Figure 13b  Plot of radioactivity in cold TCA-precipitable material versus duration of incubation in the experiment outlined in Figure 13a
acids, there appears to be some slight loss of the label although it is not as pronounced as before (right panel). However, there was also rapid incorporation of label into unlabelled cells during this time. As observed in a previously described experiment on this strain (Figure 10), there was apparently no net increase in DNA during this period. All these observations taken together indicate that even after a period of amino acids-starvation the DNA is degraded at high temperature and that which is degraded in this case must have been synthesised after the shift to high temperature.

All the tested temperature-sensitive strains showed some incorporation of label when amino acids were restored at high temperature after a period of amino acids-starvation at 30°C (Figures 7-12). The amount and the rate of this DNA synthesis varied with different strains. Strain CRT46/Flac\(^+\) synthesised a significantly higher amount of DNA after the shift. Although this could be dependent on the presence of the Flac\(^+\) particle, no clear conclusion can be gained since CRT46 and CRT15 also synthesised some DNA at high temperature. Qualitatively all the strains appear to behave similarly. Quantitative comparisons also cannot be made because non-isogenic strains appear to differ in the amounts of DNA synthesised at high temperature. Strain A3 which is an F\(^-\) strain appears to synthesise as much DNA as CRT46/Flac\(^+\) at high temperature. Furthermore, in Hfr strains degradation of the DNA makes interpretation more complicated. For these reasons treatment of cells with chloramphenicol
was tried as an alternative technique to amino acids-starvation in the following experiments. Treatment of cells with chloramphenicol is known to inhibit protein synthesis and therefore to prevent initiation of DNA replication (Maaløe and Hanawalt, 1961). However, as in the case of amino acids-starvation, the cells are capable of completing initiated cycles of DNA replication.

Figures 14, 15, 16 and 17, show four experiments involving chloramphenicol treatment. A portion of an exponentially growing culture of the strain was transferred to high temperature, another portion of the same culture was treated with 200 μg/ml of chloramphenicol at 30°C. After 3½ hours, the chloramphenicol-treated cells were filtered, washed and resuspended in medium prewarmed at 42°C in the presence or absence of chloramphenicol. DNA synthesis in all the cultures was followed as described before.

Figure 14 shows the amount of 14C-thymine incorporation obtained in A3 under these conditions. When the culture was shifted to 42°C directly, the cells synthesised 40% more DNA than was observed in previous experiments. When the cells were treated with chloramphenicol at 30°C, however, they synthesised well over 100% more DNA. The amount of DNA synthesised under these two conditions should be similar if shifting the cells to high temperature or treatment with chloramphenicol at 30°C both stop initiation of DNA synthesis. The large amount of residual synthesis observed in the presence of chloramphenicol is probably due to initiation of at least
Incorporation of $^{14}$C-thymine into cold TCA-precipitable material in A3 after treatment with chloramphenicol

The strain was grown for several hours at 30°C in minimal glucose medium supplemented with 1.0 µg/ml of $^{14}$C-thymine at a specific activity of 0.05 µCi/µg, 100 µg/ml of deoxyadenosine and 0.5% casaminoacids. When the culture was growing exponentially, 10 ml was removed, filtered through a membrane filter, washed with phosphate buffer and resuspended in 4 ml of phosphate buffer. 1.0 ml of the suspension was transferred into 10 ml of medium prewarmed at 42°C. 3 ml was transferred to 30 ml of medium with 200 µg/ml chloramphenicol prewarmed at 30°C. Samples were removed into cold TCA (0°C) at given times. After 3.5 hours, 15 ml of the CAP-treated culture was removed, filtered, washed with buffer and resuspended in 2 ml of buffer. 1.0 ml of the suspension was transferred into 10 ml of medium without CAP, prewarmed at 42°C. 1.0 ml was transferred into 10 ml of medium with CAP prewarmed at 42°C. Samples were removed at given times into cold TCA. The acid-precipitable radioactivity in the samples was determined as explained in Materials and Methods.
one new replication cycle by this strain in the presence of chloramphenicol. However, DNA synthesis came to a halt in about \(2\frac{3}{4}\) hours when the cells were treated with chloramphenicol. When the cells were washed and resuspended at \(42^\circ C\) in a medium without chloramphenicol, no more DNA was synthesised. Apart from the anomalously high amount of DNA synthesised in the presence of chloramphenicol, the results of the experiment agrees with what is expected for an initiation defective mutant.

Figure 15 shows the results of the same experiment repeated with strain A3/Flac\(^+\). In this strain direct transfer of a culture to \(42^\circ C\) from \(30^\circ C\) resulted in residual DNA synthesis of about 85%. This increment was much more than that observed with A3. However, DNA synthesis came to a halt after about 1\(\frac{3}{4}\) hours. When the culture was treated with chloramphenicol this strain also synthesised an abnormally high amount of DNA. However, this increment is very similar to that observed with strain A3. When the cells were washed and resuspended at \(42^\circ C\) free of chloramphenicol, DNA synthesis recommenced and continued for over 3 hours at a slow rate, resulting in at least a doubling of the total DNA in the course of this period. This observation is quite different from that made with the isogenic strain A3 and indicates that the presence of an Flac\(^+\) particle in some way permits cells which have previously been treated with chloramphenicol to synthesise substantial amounts of DNA at \(42^\circ C\).

The pattern of DNA synthesis at \(42^\circ C\) after the treatment of
Figure 15  Incorporation of $^{14}$C-thymine into cold TCA-precipitable material in A3/Flac$^+$ after treatment with chloramphenicol

Procedure as described for Figure 14.
chloramphenicol is quite different from that observed when the culture was directly shifted to $42^\circ\text{C}$. Although in the latter conditions DNA synthesis came to a halt in about 90 minutes after an initial period of rapid synthesis, after chloramphenicol treatment the DNA synthesis did not seem to stop even after 3 hours and the rate of synthesis appeared to be constant. The total amount of DNA synthesis under these conditions seems to be too great to be accounted for simply by the replication of the Flac$^+$ particle itself.

To check this possibility more directly, the DNA which is made under these conditions was examined by a DNA-DNA hybridization procedure which will distinguish between chromosomal and episomal DNA.

For this experiment the above procedure was used starting with unlabelled cultures. After $3 \frac{3}{4}$ hours' treatment of the culture with chloramphenicol the cells were filtered, washed and resuspended at $42^\circ\text{C}$ in a medium containing $^{14}\text{C}$-thymine. Only the DNA synthesised after the removal of chloramphenicol is labelled under these conditions. After 3 hours of growth at $42^\circ\text{C}$, the DNA was extracted from the culture and purified on a CsCl gradient. The labelled DNA was then hybridised at a concentration of about 0.3 μg/ml with a 2000-fold excess of DNA preparations from the following strains: E. coli Flac$^+$, Proteus mirabilis Flac$^+$, Proteus mirabilis. The method of preparation and hybridization of DNA is that described by Collins (1971) in collaboration with whom this experiment was performed.
The details of the hybridization procedure are as follows.

After 3 hours' growth of strain A3/Flac\(^+\) at 42\(^\circ\)C in the presence of \(^{14}\)C-thymine (7.5 \(\mu\)Ci/\(\mu\)g) following a period of chloramphenicol treatment, the cells were pelleted by centrifugation and resuspended in 10 ml of TES buffer. After centrifuging down twice through TES buffer at 0\(^\circ\)C, the final resuspension was made in 2 ml of TES buffer. The suspension was frozen and thawed rapidly three times in a solid CO\(_2/\)ethanol mixture and 0.1 ml of a cold freshly prepared lysozyme solution (500 \(\mu\)g/ml) in TES was added. After one minute at room temperature, 0.08 ml of a 10\% solution of sodium-N-lauroyl sarcosinate and 0.1 ml of a 10 mg/ml pronase solution was added. After 4 hours' incubation at 42\(^\circ\)C, CsCl was added and the refractive index adjusted to 1.4018 at a total volume of 4.5 ml. The CsCl gradient was centrifuged for 36 hours at 36,000 rpm. Cellulose nitrate tubes were used and fractions were collected by syringing 0.2 ml aliquots from the top of the tube. The DNA peak fractions were determined by counting/radioactivity in 0.05 ml samples on filter paper after TCA washing and these were then pooled.

This DNA was then mixed separately with unlabelled DNA preparations derived from \textit{E. coli} Flac\(^+\), \textit{P. mirabilis} Flac\(^+\) and \textit{P. mirabilis}, with the unlabelled DNA at a concentration of 400 to 500 \(\mu\)g/ml. The mixture was dialysed against 0.12 M phosphate buffer for 16 hours and then sonicated to a M.W. of about 3 \(\times\) 10\(^5\) daltons.
TABLE 11  Annealing characteristics of the DNA synthesised at high temperature by A5/Flac\(^+\) after CAP treatment

<table>
<thead>
<tr>
<th>Labelled DNA hybridized with</th>
<th>Time of hybridization</th>
<th>Percentage of annealing</th>
<th>Percentage of annealing</th>
<th>Estimated homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>11.75h.</td>
<td>24hr.</td>
<td></td>
</tr>
<tr>
<td>\textit{E. coli Flac(^+)}</td>
<td>3.4</td>
<td>83.7</td>
<td>82.3</td>
<td>79.6</td>
</tr>
<tr>
<td>\textit{P. mirabilis Flac(^+)}</td>
<td>2.3</td>
<td>11.8</td>
<td>12.6</td>
<td>9.9</td>
</tr>
<tr>
<td>\textit{P. mirabilis}</td>
<td>1.3</td>
<td>4.8</td>
<td>2.9</td>
<td>2.6</td>
</tr>
</tbody>
</table>
The hybridization mixtures were distributed in 0.9 ml aliquots into small glass ampoules which were sealed and dropped into boiling water and kept at 100°C for 10 minutes. Zero time samples were immediately removed into CO₂/ethanol mixture at -70°C. Other samples were incubated in a water bath at 75°C for 11.75 hours or 24 hours before freezing them in CO₂/ethanol mixture. Samples were kept frozen until they were to be fractionated using hydroxyapatite columns. All samples were taken in duplicate. For fractionation, the samples were thawed, pipetted onto hydroxyapatite columns and washed through it with 13 ml of 0.12 M phosphate buffer at 75°C. The effluent from the column was collected in 13 ml aliquots in glass scintillation vials. The columns were washed at 75°C, followed by 3 more washes at 95°C. A final wash with 0.4 M phosphate buffer was made at 100°C. Radioactivity in the effluent samples were counted after mixing 6.5 ml aliquots with 16 ml of an aqueous scintillation fluid. The results shown in Table 11 give the aggregate percentage of radioactivity counted in the effluent collected at 95°C and 100°C as a percentage of the total.

The annealing reaction between the labelled and unlabelled DNA was expected to be 90% complete after 12 hours annealing. Little additional annealing was expected in a further 12 hours and a second sample was taken at 24 hours to check this. To obtain the "percentage annealing", the zero time value was subtracted from the mean of the
11.75 and 24 hour values for each annealing reaction. The 79.6% annealing between \( \text{E. coli Flac}^+ \) DNA and labelled DNA is similar to the maximum amount of annealing previously obtained between homologous DNA preparations under these conditions (Collins, 1971). Therefore, the homology between labelled DNA and \( \text{E. coli Flac}^+ \) DNA is assumed to be 100%. The percentage of annealing with other DNA species were expressed in terms of the "estimated homology" of the labelled DNA with these species by dividing by 0.796, thus correcting them in accordance with the maximum annealability control.

The amount of labelled DNA annealing with \( \text{P. mirabilis Flac}^+ \) DNA in excess of that annealing with \( \text{P. mirabilis} \) (12.4 - 3.3 = 9.1) is due to the annealing of the Flac\(^+\) DNA. Two components of the labelled DNA will contribute to this additional hybridization. One is the episomal DNA and the other is the chromosomal DNA from the region of homology between Flac\(^+\) and the chromosome. The amount of hybridization between Flac\(^+\) and the homologous chromosomal region was found to be approximately 2.6% at all growth rates. Therefore, the estimated percentage of Flac\(^+\) episomal DNA in the labelled DNA is (9.1 - 2.6) 6.5%. The errors inherent in this estimate allow the figures to be taken as 6.5 ± 3. The conclusion gained from this experiment is that at least 90% of the DNA synthesised at high temperature after removal of chloramphenicol is chromosomal DNA.

The extra synthesis of DNA found in the Flac\(^+\) strain in contrast to the Flac\(^-\) strain might be due to the integration of the Flac\(^+\)
into the chromosome in some of the cells. In this case an $F^+$ culture would be expected to synthesise less DNA than an $Flac^+$ culture under the same conditions since the frequency of integration of an $Flac^+$ factor into the chromosome will be higher than that of an $F^+$ factor owing to the homology between chromosomal and episomal DNA in the $lac$ region. DNA synthesis of $A3/F^+$ was therefore studied in a similar experiment.

Figure 16 shows the pattern of DNA synthesis observed with strain $A3/F^+$ under conditions similar to those described for Figures 14 and 15. The behaviour of this strain is very similar to that of $A3/Flac^+$. The increment of DNA synthesis observed when the culture was shifted directly to high temperature and the nature and rate of DNA synthesis observed at $42^\circ$C after a previous treatment of cells with chloramphenicol are very similar to those observed with strain $A3/Flac^+$. Therefore, it is likely that the extra synthesis of DNA observed with $Flac^+$ and $F^+$ strains is not due to the integration of the $F$ particle into the chromosome.

It is possible that $Flac^+$ and $F^+$ cultures are contaminated with a sufficient number of revertants (revertants of the $Ts^-$ mutant and integratively suppressed cells) to account for the DNA synthesis observed at high temperature. Such cells if present in the cultures should be able to form colonies on plates incubated at $42^\circ$C. When samples of the $Flac^+$ cultures used in the above experiments were spread on nutrient agar plates and incubated at $42^\circ$C, no colonies
Figure 16  Incorporation of $^{14}$C-thymine into cold TCA-precipitable material in A3/F+ after treatment with chloramphenicol

Procedure as described for Figure 14.
were found at a notable frequency. Furthermore, if the DNA synthesis observed after the chloramphenicol treatment is due to the presence of revertant cells, these cells should be able to synthesise DNA continuously even after the cultures were directly shifted from 30°C to 42°C. Since the residual DNA synthesis of the culture stops in about 90 minutes, this is made an unlikely explanation.

Figure 17 shows the pattern of DNA synthesis observed in strain Hfr 38 under conditions similar to those detailed above. As was observed before (Figure 10), a direct transfer of a culture of the strain from 30°C to 42°C resulted in synthesis and degradation of DNA. Treatment of the culture with chloramphenicol at 30°C in this strain resulted in an increment in DNA of about 66%. This increment, although much less than that observed with strains A3, A3/F+ or A3/Flac+, may be associated with the fact that this strain grows at 30°C much more slowly than any of the above strains. After the removal of chloramphenicol, strain Hfr 38 also synthesised DNA at high temperature. Quite remarkably, however, this extra DNA synthesised at high temperature is apparently not degraded. This observation also suggests strongly that the nature of DNA synthesis after the chloramphenicol-treatment is different from that observed after direct transfer of a culture to high temperature. The rate of DNA synthesis observed following chloramphenicol treatment in the case of Hfr 38 is not more than that observed with A3/F+ or A3/Flac+. 
Incorporation of $^{14} \text{C}$-thymine into cold TCA-precipitable material in Hfr 38 after treatment with chloramphenicol

Procedure as described for Figure 14.
This observation might suggest that the induced DNA synthesis at high temperature is not facilitated by the integration of the F factor into the chromosome. However, the interpretation of this result may be complicated by the fact that Hfr 38 is a slow-growing strain at 30°C.

The very similar patterns of DNA synthesis observed with F\textsuperscript{+}, Flac\textsuperscript{+} and Hfr strains at high temperature following chloramphenicol treatment suggests that this DNA synthesis is a common feature of strains carrying an F factor. It is very likely that integration of the F factor is not necessary for such induction of DNA synthesis by an F factor. The most likely explanation is that a product of the F factor can complement or substitute for a protein essential for the initiation of chromosome replication which is defective in these strains at high temperature. Since the F\textsuperscript{+} strains as well as F\textsuperscript{-} strains cannot form colonies at high temperature, it has to be assumed that this complementation is a partial or weak one and cannot result in full suppression of the temperature-sensitive defect. The rate of DNA synthesis which is apparently induced by the F factor is very slow compared to the growth rates of the strains and cannot result in balanced growth necessary for cell multiplication. It is possible that for an unknown reason the complementing product of the F particle is more effective after a period of chloramphenicol treatment of the cells. However, the fact that, after a direct shift to high temperature, F\textsuperscript{+} and Flac\textsuperscript{+} cultures synthesise DNA for a longer time
(2 hours) and to a greater extent than \( F^- \) cultures, suggests that the \( F \) particle can complement the \( Ts^- \) defect to some extent even if there is no treatment with chloramphenicol.

The unexpectedly high amount of DNA synthesised by strain \( A3 \) and its derivatives in the presence of chloramphenicol may be peculiar to this strain. The amount of DNA synthesised by \( A3 \) under amino acids-starvation was much less, although the two treatments are expected to have similar effects on DNA synthesis. In contrast, the amount of DNA synthesised by \( Hfr 38 \) under these two conditions looks very similar. It is possible that this discrepancy is due to the fact that \( Hfr 38 \) is a thymine-requiring strain while \( A3 \) is not.

Amino acids-starvation and treatment with chloramphenicol may have different effects on the endogenous dTTP precursor pools of the cells and therefore may have different effects on the efficiency of incorporation of external \(^{14}C\)-thymine into DNA. It is known that thymine-requiring strains have low internal pools of dTTP compared to wild type strains (Beacham et al., 1971).

The effects of amino acid starvation and treatment with chloramphenicol on DNA synthesis of two non-temperature-sensitive strains were studied under the conditions used in the experiments described above. Figure 18 shows the pattern of DNA synthesis observed in \( C600 \) and \( 1628 \) (a thymine-requiring derivative of \( C600 \)) after such treatments. Both the strains synthesised more DNA in the presence of chloramphenicol compared to the amount synthesised
Figure 18  Incorporation of $^{14}$C-thymine into cold TCA-precipitable material in strains 1628 and C600 after amino acids-starvation and treatment with chloramphenicol.

The strains were grown for several hours at 30°C in minimal glucose medium supplemented with 1.0 μg/ml of $^{14}$C-thymine at a specific activity of 0.05 μCi/μg, 100 μg/ml of deoxyadenosine and 0.5% casamino acids. When the cultures were growing exponentially, 10 ml of each culture were removed, filtered, washed with phosphate buffer and resuspended in 2 ml of phosphate buffer. 1.0 ml of the suspension was transferred into 10 ml of the medium lacking amino acids prewarmed at 30°C. 1.0 ml was transferred into 10 ml of the medium with 200 μg/ml of chloramphenicol prewarmed at 30°C. Samples were removed at given times into cold TCA. The acid-precipitable radioactivity of the samples were determined as described in Materials and Methods.
The graph shows the relationship between CPM (counts per minute) and time in hours for two different conditions: with and without CAP (1628) and with and without AA (C600).

- **1628**
  - **+ CAP**
  - **- AA**

- **C600**
  - **+ CAP**
  - **- AA**

The CPM is expressed as $10^3$. The graph indicates a decrease in CPM with time for both conditions when CAP and AA are present.
under amino acids-starvation and, in both strains, the magnitude
of the difference was nearly the same. Thus the apparent discrepancy
between A3 and Hfr 38 is not dependent on the requirement for thymine
of the latter strain.
DISCUSSION

Integrative suppression

The experiments done with the revertants of CRT15/F⁺ and CRT83/Flac⁺ strains showed clearly that the temperature-sensitivity of these strains can be suppressed, and that integration of the F particle into the chromosome is involved in this suppression. The simplest hypothesis to explain this observation is that the strains CRT83 and CRT46 carry a mutation preventing the initiation of DNA replication at high temperature which does not affect the replication of the F factor, and that when the F factor is integrated into the chromosome, the whole genome can be replicated at high temperature like an F prime under the control of the F replication system. Similar observations have been made by Nishimura et al. (1971), who refer to this phenomenon as "integrative suppression". However, many other observations described in this thesis indicate that integrative suppression is a more complex process.

The increased reversion frequencies of F⁺ and Flac⁺ derivatives of temperature-sensitive strains can be explained in terms of integrative suppression. Different F primes, however, had quite different reversion frequencies. Some, e.g. Fgal⁺, F13, did not have reversion frequencies significantly greater than F⁻ control cultures. Others, like F⁺ and KLF1, apparently had a greater effect than Flac⁺. Thus, the extent of homology between
the F prime and the chromosome, although expected to increase the
frequency of integration, did not have the parallel effect of
increasing the reversion frequency. Apparently, the homology
between the F prime and the chromosome is relatively unimportant
for integrative suppression. It may be interesting to note that
the F prime KLF1, which had the highest effect of increasing the
reversion frequency, integrates into the chromosome in a clockwise
orientation while the other tested F primes integrate in the
opposite direction.

The site of F integration clearly is an important factor
in suppression. All the F\(^+\) revertants tested which were
integratively suppressed probably had the same position and
orientation of integration. The observations of Nishimura et al.
(1971) show that although this region is not unique, a great
majority of the suppressed strains contain the F particle integrated
within this segment of the chromosome (between the 55 and 65 minutes
positions of the E. coli map). This preferential site of integration
may explain the apparent unimportance of the extent of homology of
the F prime with the chromosome in determining the frequency of
suppression. The experiments of Nishimura et al. (1971) have
indicated that, in many strains which are suppressed by the Flac\(^+\)
factor, the episome had integrated between the 60-65 positions and
not in the lac region of the chromosome. Although the position of
the vegetative origin of the chromosome has not been mapped accurately,
some experiments have shown that it is located at about 63 minutes position on the chromosome (Millicent Masters and P. Broda, personal communication). This tends to suggest that in most cases of suppression the F particle integrates at a position close to the origin of the chromosome. Why this should be so is not clear. On the other hand, some other experiments have indicated that the origin of the chromosome may be located near 74 minutes position of the E. coli map (Bird R.E., Louarn J. and Caro L.G., personal communication). If this is the case, however, the above argument does not hold.

The most intriguing aspect of integrative suppression is the fact that integration of the F factor alone is not a sufficient condition for suppression. Hfrs Hayes and P4X cannot suppress the Ts* mutation. Nishimura et al. (1971) have shown that many of the Hfrs isolated by the fluctuation method also cannot suppress the mutation. One of the Hfrs isolated by this method, however, was found to suppress the mutation. The inability of Hfrs to suppress the temperature-sensitivity is apparently not dependent on the position of integration because the position of F integration in one of the non-suppressed Hfrs has been found to be similar to that of the suppressed strains (Nishimura et al., 1971). The simplest explanation for the fact that integration does not directly result in suppression is to imagine that 2 steps may be involved in the
process of suppression. One is the integration of the F particle and the second, a mutational event.

On the hypothesis that the normal integration of the F particle into the chromosome results in an automatic repression of the autonomous replication of the F system, the second step postulated above may have the function of derepressing the F replication system.

A different explanation for the need of a second step in suppression may be that in temperature-sensitive Hfr strains, the F particle can replicate autonomously as in the F prime state, but the cells are not viable at high temperature. The second step in suppression involves a mutation which makes the cells viable. This hypothesis predicts that a temperature-sensitive Hfr strain would synthesise a much greater amount of DNA after a shift to a non-permissive temperature than an F\(^+\) strain. Studies of DNA synthesis in Hfr 38 have shown that this is probably not the case. This Hfr strain when treated with chloramphenicol at 30°C for 3\(\frac{1}{2}\) hours and then transferred to 42°C in the absence of chloramphenicol, synthesised a considerable amount of DNA, but this phenomenon was also observed to the same extent with F\(^+\) and Flac\(^+\) strains. Thus it is likely that the autonomous replication system of the F particle is not functional in temperature-sensitive Hfr strains.

A different hypothesis which agrees with many of the observations is that, while integration is an essential part of the
suppression, all integrations do not form suppressed Hfrs. Only some kind of integrations are capable of suppressing the Ts\textsuperscript{−} mutation, while others are not. One out of 5 Hfr strains isolated from CRT15/F\textsuperscript{+} (by Nishimura et al., 1971) using the fluctuation technique was found to be temperature-resistant. This argument also explains the relatively low reversion frequencies of Hfr, F\textsuperscript{+} and Flac\textsuperscript{+} strains. The frequency of episome integration in an Flac\textsuperscript{+} strain, as judged by chromosome mobilization experiments, is normally between \(10^{-2} - 10^{-4}\) per cell and is between \(10^{-4} - 10^{-5}\) in an F\textsuperscript{+} strain. The overall reversion frequencies of Flac\textsuperscript{+} and F\textsuperscript{+} derivatives of temperature-sensitive strains are considerably less than these figures would suggest. However, at the same time, the reversion frequencies of such strains appear to be too great to be explained in terms of integration and a mutation.

**Complementation between the F particle and the chromosome**

The experiments involving chloramphenicol-treatment gave some evidence that an F particle can complement, to some extent, the temperature-sensitive defect of the initiation mutants.

Strain A3/F\textsuperscript{−}, when treated with chloramphenicol at 30°C for \(\frac{3}{2}\) hours and then shifted to high temperature in the absence of chloramphenicol, synthesised no more DNA, agreeing with what is expected from an initiation mutant. On the other hand, A3/F\textsuperscript{+} and
A3/Flac\(^+\) strains synthesised a considerable amount of DNA at high temperature following chloramphenicol-treatment, indicating that these strains, in contrast to F\(^-\) strain, can initiate and replicate DNA at high temperature under these conditions. The amount of DNA synthesised by these male strains at high temperature is far too great to account for the replication of the F particle alone. The DNA-DNA hybridization experiments showed clearly that nearly all the DNA which is synthesised at high temperature in the case of A3/Flac\(^+\) is chromosomal. The most reasonable explanation to account for these observations is that a product of the F particle can complement or substitute for a function which is defective at high temperature in these strains.

The chromosomal DNA synthesis found at high temperature in A3/F\(^+\) and A3/Flac\(^+\) is probably not due to the integration of the F particle into the chromosome, because if this were the case, the Flac\(^+\) strain would be expected to synthesise more DNA than the F\(^+\) strain, since the frequency of integration of an Flac\(^+\) particle is greater than that of an F\(^+\) factor. On the contrary, the amounts and the rates of synthesis found in Flac\(^+\) and F\(^+\) strains are remarkably similar. This DNA synthesis is also not probably due to a few revertant cells present in the cultures, because the frequency of reversion in these strains is too small to account for the amount of DNA synthesis found at high temperature.

The complementation between the chromosome and the F
particle, however, is not complete since the $F^+$ or $Flac^+$ strains are still temperature-sensitive. $F^-$ strains stop DNA synthesis within one hour after a shift to $42^\circ C$. On the other hand, $F^+$ and $Flac^+$ strains synthesize DNA for about 2 hours after a similar temperature-shift resulting in an amount of residual synthesis much larger than that found in $F^-$ strains. Thus, although the $F^+$ or $Flac^+$ factors cannot fully suppress the $Ts^-$ defect, the complementation between these factors and the chromosome allows the synthesis of more chromosomal DNA after a temperature-shift. The observation that $Flac^+$ cultures synthesize DNA for a longer time than $F^-$ cultures after a temperature-shift, agrees with similar observations made by Hirota, Rytter and Jacob (1966) using CRT46. However, the explanation given here for this phenomenon is different from theirs.

The remarkably different behaviour of $F^+$ and $Flac^+$ strains after a direct shift to high temperature and after a temperature-shift following chloramphenicol treatment cannot be understood at present. Treatment of cells with chloramphenicol and the shifting of a culture to a high temperature are expected to have similar effects on DNA synthesis in the case of temperature-sensitive initiation mutants, namely the completion of replication cycles without further initiation of new cycles. However, the complementation between $F$ and the chromosome is apparently more effective if the replication cycles were completed in the presence of chloramphenicol.

It is quite possible that treatment with chloramphenicol may have
different effects on the replication of the F particle and that of chromosome. Bazaral and Helinski (1970) have found that the F particle can initiate and replicate even after the removal of amino acids from an $F^+$ culture, suggesting that limited initiation of F factor synthesis can occur in the absence of protein synthesis. If the F particle in $F^+$ and $Flac^+$ cultures can initiate and replicate in the presence of chloramphenicol, after such treatment $F^+$ and $Flac^+$ cultures would have more copies of the F particle per cell than untreated cultures. If this is true, the complementation between the F particle and the chromosome can be expected to be more apparent after a period of chloramphenicol treatment since increased dosage of F particles would result in increased complementation. Certainly very little is known about this process and more experiments have to be done before any hypothesis can be put forward.

The behaviour of Hfr 38 is very similar to that of $A3/Flac^+$ and $A3/F^+$ after chloramphenicol treatment. The rate and the amount of DNA synthesis by this strain is not greater than that of $A3/Flac^+$ and $A3/F^+$. However, quantitative comparison between the two strains cannot be made since the strains are not isogenic and because Hfr 38 grows slowly at $30^\circ C$ compared to $A3/Flac^+$ and $A3/F^+$. It seems possible that the nature of DNA synthesis in Hfr 38 after chloramphenicol is the same as that of $Flac^+$ and $F^+$ strains, and that the integrated F particle can complement the $T_{s^{-}}$ defect, just as
autonomous F factors do.

If the hypothesis that there is some complementation between the F particle and the chromosome is correct, the extent of this complementation should be enhanced if the dosage of the F particle is increased. This suggests the following alternative mechanism for the phenomenon of integrative suppression. In a growing culture of *E. coli* there are more copies of genes located near the origin of the chromosome than there are copies of the more distal genes. If it is assumed that in growing cultures the number of autonomous F factors per cell is less than the number of chromosome origin; integration of the F particle into the chromosome near the origin would increase the number of copies of the F factor per unit mass of cell and the increased complementation would result in complete suppression. If this explanation for integrative suppression is correct, it is expected that the replication of the chromosome in the integratively-suppressed strains would commence at the chromosomal origin and not at the site of the integrated F particle. The origin of replication in integratively suppressed strains has not been mapped.

The results in relation to the Pritchard, Barth and Collins' model of control of DNA synthesis

As described in the introduction to this thesis, several
models have been put forward to explain the possible mechanism of the control of DNA synthesis. Certainly the best defined is that of Pritchard, Barth and Collins (1969). Not only does the model explain a self-regulatory control mechanism but it also accounts for many of the observations which are difficult to explain in terms of other suggested models. The most attractive features of the Pritchard, Barth and Collins' (1969) model are that it explains the immunity properties of both F' and Hfr strains and the possible mechanism by which autonomous replication of the F particle may be suppressed in Hfr strains (see Introduction).

In short, Pritchard, Barth and Collins (1969) assume that the replication of the F particle normally occurs late in the cell cycle. When the F particle is integrated into the chromosome, passive replication of the F factor occurs earlier in the cell cycle than it would in the autonomous state. Thus, the F factor is replicated before the critical concentration of the F specific inhibitor is reached and this results in an increase of the F inhibitor concentration to more than double its critical concentration. As the concentration of the inhibitor never falls to the critical concentration, the autonomous replication of the integrated F factor is always suppressed.

It is an implicit prediction of this hypothesis that if the initiation of chromosome replication fails in growing cells, the
F inhibitor will be diluted to the critical concentration and autonomous replication of the F factor will occur. Therefore, it can be expected that an integrated F particle should suppress the temperature-sensitivity of initiation mutants.

However, as described previously, all integrations do not result in suppression. When the Ts" mutation is introduced into an Hfr, the strain becomes temperature-sensitive. It could be argued, on the basis of the Pritchard, Barth and Collins' model, that F-controlled replication of the chromosome occurs at the high temperature but the cells are not viable. However, studies of DNA synthesis in Hfr strains did not give conclusive evidence for this type of replication and they make this argument unlikely. The pattern of DNA synthesis in Hfr 38 at high temperature after chloramphenicol-treatment was similar to that of A3/F' and A3/Flac', suggesting that autonomous replication of the integrated F particle remains suppressed at the non-permissive temperature.

All the suggested models for the control of DNA synthesis assume that the replication of the chromosome and the F particle are controlled by similar mechanisms. However, there is growing evidence that this is perhaps not true. Collins (1971) has studied the time of replication of an Flac' particle in E. coli in relation to chromosome replication by determining the amount of Flac' DNA as a percentage of the total, at different growth rates. His data
suggests that, in slow-growing cultures, the F particle replicates early in the chromosome replication cycle whereas in fast-growing cultures, the F particle replicates relatively late. Thus, clearly the F replication is not linked to the initiation of chromosome replication. Zeuthen (personal communication) has drawn similar conclusions from his studies of the time of Flac\textsuperscript{+} replication, as determined by comparative assays of β-galactosidase produced by F\textsuperscript{-} and Flac\textsuperscript{+} cultures at different growth rates. Other experiments (M. G. Chandler - personal communication) involving measurements of a β-galactosidase synthesis by Flac\textsuperscript{+} and F\textsuperscript{-} cultures of/thymine-requiring E. coli strain after a step-up of the thymine concentration, have suggested that the F replication is not coupled to termination of the chromosome replication cycle. Stepping up the thymine concentration in a culture of a thymine-requiring strain is known to increase the chromosome replication velocity (Pritchard and Zaritsky, 1970). This would result in an increase of the relative dosage of the late replicating genes in a growing culture. These observations suggest that the replication of the F particle is governed by different rules from those involved in chromosome replication. Furthermore, Bazaral and Helinski (1970) have shown that the initiation of the F factor replication can continue at a decreasing rate after a culture is deprived of amino acids, indicating that protein synthesis may not be necessary for the initiation of the F replication. These results also suggest the possibility that
the replication of the F factor may be controlled differently from that of the chromosome.

If the replication of the F particle and the chromosome are, in fact, regulated by different mechanisms, the understanding of the phenomenon of integrative suppression may prove even more difficult. The kinetics of DNA replication in integratively suppressed strains may be a rewarding aspect for further study of this complex process.
### TABLE 12
A list of some of the strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>C600 thr&lt;sup&gt;+&lt;/sup&gt; leu&lt;sup&gt;+&lt;/sup&gt;</td>
<td>F&lt;sup&gt;-&lt;/sup&gt;</td>
<td>thi&lt;sup&gt;-&lt;/sup&gt; lacY&lt;sup&gt;-&lt;/sup&gt;</td>
<td>C600 x F4X</td>
</tr>
<tr>
<td>C600 thr&lt;sup&gt;+&lt;/sup&gt; leu&lt;sup&gt;+&lt;/sup&gt;/lac&lt;sup&gt;+&lt;/sup&gt;</td>
<td>F'&lt;sup&gt;-&lt;/sup&gt;</td>
<td>thi&lt;sup&gt;-&lt;/sup&gt; lacY&lt;sup&gt;-/+&lt;/sup&gt;/lac&lt;sup&gt;+&lt;/sup&gt;</td>
<td>C600 thr&lt;sup&gt;+&lt;/sup&gt; leu&lt;sup&gt;+&lt;/sup&gt;/F4X</td>
</tr>
<tr>
<td>CRT15 Str&lt;sup&gt;R&lt;/sup&gt;</td>
<td>F&lt;sup&gt;-&lt;/sup&gt;</td>
<td>thr&lt;sup&gt;-&lt;/sup&gt; leu&lt;sup&gt;-&lt;/sup&gt; thi&lt;sup&gt;-&lt;/sup&gt; Str&lt;sup&gt;R&lt;/sup&gt; lacY&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CRT15</td>
</tr>
<tr>
<td>TS27</td>
<td>Hfr</td>
<td>thr&lt;sup&gt;-&lt;/sup&gt; ilv&lt;sup&gt;-&lt;/sup&gt; To&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CRT23 x F4X</td>
</tr>
</tbody>
</table>
CHAPTER 4

As indicated in the previous chapter of this thesis, strains CRT83, CRT46 and some of their derivative strains, exhibit low frequencies of recombination and abnormal recombination gradients. The experiments described in this section were designed to investigate this aspect of these strains in more detail.

Materials and Methods

Bacterial strains

Table 12 shows some of the bacterial strains used. Other strains are the same as those described in previous chapters.

Media

AB Sal medium (Schaefer and Maas, 1967) contained

$$K_2HPO_4, 1.4g; \quad KH_2PO_4, 0.6g; \quad (NH_4)_2SO_4, 0.02g; \quad \text{salicin, 1g; yeast extract (Oxoid), 0.15g; bromothymol blue, 0.04g; and 200 ml of water solidified with 3g of agar (Oxoid).}$$

AarbSal Medium contained $$K_2HPO_4, 1.4g; \quad KH_2PO_4, 0.6g; \quad (NH_4)_2SO_4, 0.02g; \quad \text{arbutin, 0.5g; salicin, 0.5g; and 200 ml of water solidified with 3g of agar. Other media are the same as those described previously.}$$
Bacterial matings

The techniques as described previously were used; all matings were done at 30°C.

uv irradiation

Bacteria grown to stationary phase in nutrient broth were diluted to about $10^7$ cells/ml, into phosphate buffer. 10 ml samples of this suspension were irradiated with uv from a 15 watt General Electric germicidal lamp in glass petri dishes (9 cm diameter), on a rotary shaker. Dose rate was adjusted to 10 ergs/mm²/sec using a dosimeter constructed and calibrated by Dr. R. Latas (1953).

Treatment with NMS (methyl methane sulfonate)

A nutrient broth-grown culture of the strain was diluted into a solution of NMS (obtained from Eastman Organic Chemicals) in phosphate buffer at a concentration of 0.05 M, at about $10^7$/cells/ml.

Results

Abnormal gradient of recombination

A more detailed analysis of conjugants obtained in the experiment described in Table 8 (Chapter 3) is given in Table 13. Six temperature-resistant revertants of the CRT03 NalR/Fia+ strains
TABLE 13  Analysis of Thr<sup>+</sup>Leu<sup>+</sup>Nal<sup>r</sup> conjugants obtained in matings between Hfr Cav74 and CRI83 Nal<sup>r</sup>/Flac<sup>+</sup> revertants

<table>
<thead>
<tr>
<th>Recipient strain</th>
<th>number tested</th>
<th>phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ilv&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rev. 1</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Rev. 2</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Rev. 3</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Rev. 4</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Rev. 5</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Rev. 6</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>1-6 inclusive</td>
<td>62</td>
<td>29</td>
</tr>
</tbody>
</table>

- Ilv<sup>+</sup>Ts<sup>+</sup>
- Ilv<sup>+</sup>Ts<sup>-</sup>
- Ilv<sup>-</sup>Ts<sup>-</sup>
- Ilv<sup>-</sup>Ts<sup>+</sup>
were made into F− phenocopies and mated with Hfr Cav74 and
Thr+Leu+NaI− recombinants were selected. Some of the conjugants
obtained (selected at random) were scored for the Pro, Ilv, Ts
and Thy phenotypes. The pro marker is located between the
origin of Hfr Cav74 and the thr and leu markers. About half of
the conjugants were Pro+ as expected. However, the great majority
of the conjugants were Ilv+ and about half of them were Ts+.

This result is quite unexpected since the ilv and the
Ts mutation are further from the transfer origin than the thr and leu
markers and only a proportion of conjugants, much less than half,
can be expected to be Ilv+Ts+ according to the normal recombination
gradient (Verholf and De Haan, 1966). Only one of the conjugants
is Thy+ as expected from the recombination gradient normally found
in E. coli strains.

The Ts− mutation and ilv are closely linked and the two markers
are cotransducible (Hirot a, Jacob, Buttin and Nakai, 1968; Hirot a,
Mordoh and Jacob, 1970). In crosses between CRT46 and Hfr strains,
where Ilv+ recombinants have been selected, about 90% consegregation
of Ilv+ and the Ts+ phenotype has been found (Hirot a, et al., 1968).
In the experiment described above, when the numbers of conjugants
tested from all the six revertants are added together (see Table 13),
the majority class of recombinants has the Ilv+Ts+ donor parental
phenotype. The $Ilv^{-}Ts^{-}$ phenotype of the recipient is one of the less frequent classes. $Ilv^{+}Ts^{-}$ conjugants form the second most frequent class. The recombination frequency between $Ilv$ and the $Ts$ marker seems to be about 40% which is much higher than expected on the basis of the data of Hirota et al. (1970).

These observations might be accounted for if the recipient strains grow very poorly in minimal media as a result of a mutation located near $Ilv$, and those recombinants which inherit the wild-type allele from the donor can grow faster to form colonies. However, as will be shown in the following experiments, the abnormal gradient of recombinants is not observed when recombinants carrying a male marker distal to $Ilv$ are selected. These results do not agree with the above hypothesis.

A more likely explanation is that the recipient strains are defective in recombination, a mutation being located close to the $Ilv$ marker. In this case, recombination would occur only when the corresponding wild-type marker from the donor strain has entered the recipient. This hypothesis, although accounting for the presence of a higher proportion of $Ilv^{+}$ conjugants than expected, does not explain why the great majority of these are $Ilv^{+}$. If normal recombination takes place after the entry of the wild-type marker into the recipient strain, only about half of the conjugants
TABLE 14  Analysis of Lac^+Met^+ conjugants obtained in a mating between CRTΩ and Hfr P^+X

<table>
<thead>
<tr>
<th>Number tested</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>((\text{Lac}^+\text{Met}^+))</td>
<td>(\text{Thr}^+\text{Leu}^+)</td>
</tr>
<tr>
<td>54</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>(\text{Ilv}^+\text{Ts}^+)</td>
</tr>
<tr>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>
TABLE 15  Analysis of various classes of recombinants obtained in matings between Hfr F'X and CRT46

<table>
<thead>
<tr>
<th>Selected markers</th>
<th>Ilv&lt;sup&gt;+&lt;/sup&gt;Te&lt;sup&gt;-&lt;/sup&gt;</th>
<th>Ilv&lt;sup&gt;-&lt;/sup&gt;Te&lt;sup&gt;-&lt;/sup&gt;</th>
<th>Ilv&lt;sup&gt;-&lt;/sup&gt;Te&lt;sup&gt;-&lt;/sup&gt;</th>
<th>Ilv&lt;sup&gt;-&lt;/sup&gt;Te&lt;sup&gt;-&lt;/sup&gt;</th>
<th>Total No. of conjugants</th>
<th>r.f. between ilv and Te</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr&lt;sup&gt;+&lt;/sup&gt;Leu&lt;sup&gt;+&lt;/sup&gt;Met&lt;sup&gt;+&lt;/sup&gt;</td>
<td>64</td>
<td>7</td>
<td>31</td>
<td>3</td>
<td>105</td>
<td>32%</td>
</tr>
<tr>
<td>Thy&lt;sup&gt;+&lt;/sup&gt;Met&lt;sup&gt;+&lt;/sup&gt;</td>
<td>29</td>
<td>42</td>
<td>6</td>
<td>13</td>
<td>91</td>
<td>20%</td>
</tr>
<tr>
<td>Lac&lt;sup&gt;+&lt;/sup&gt;Met&lt;sup&gt;+&lt;/sup&gt;</td>
<td>37</td>
<td>39</td>
<td>10</td>
<td>10</td>
<td>93</td>
<td>20%</td>
</tr>
</tbody>
</table>
would ultimately inherit the ilv$^+$ marker from the donor. Furthermore, the hypothesis does not account for the high recombination frequency between ilv and the Ts marker without further assumptions. Table 14 gives the results of an experiment in which selection was made for recombinants carrying a distal donor marker. Strain CR183 was mated with Hfr F4X and Met$^+$ Lac$^+$ recombinants were selected. 54 of the recombinants were scored for other markers. Only 7 out of the 54 recombinants were Ilv$^+$ and the recombination frequency between ilv and the Ts marker is only 12%. These results do not show an abnormal marker transfer gradient or high recombination frequency between ilv and the Ts marker as seen in the previous experiment. However, the results agree with the hypothesis that strain CR183 carries a Rec$^-$ mutation close to ilv. Since a distal marker was selected, all the isolated recombinants should have received the wild-type marker from the donor strain and therefore should not show any defect in recombination.

The results of three similar experiments done with the strain CR166 and Hfr F4X are described in Table 15. Thr$^+$ Leu$^+$ Met$^+$, Thy$^+$ Met$^+$, or Lac$^+$ Met$^+$ recombinants were selected. When Thy$^+$ Leu$^+$ Met$^+$ conjugants were selected, the great majority of them were Ilv$^+$. The proportion of the conjugants containing ilv$^-$ and Ts$^-$ female parental markers was low, the majority group being Ilv$^+$ Ts$^-$. The recombination frequency between ilv and the Ts marker was 32%.
TABLE 16  Analysis of Thr+Leu+strR conjugants obtained in matings between 1638T33 and either Hfr P4X or Hfr H72

<table>
<thead>
<tr>
<th>Parent strains</th>
<th>Ilv+Ts+</th>
<th>Ilv-Ts-</th>
<th>Ilv+Ts-</th>
<th>Ilv-Ts+</th>
<th>Total tested</th>
<th>r.f. between Ilv and Ts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1638T33 x Hfr P4X</td>
<td>29</td>
<td>2</td>
<td>16</td>
<td>0</td>
<td>47</td>
<td>30%</td>
</tr>
<tr>
<td>1638T33 x Hfr H72</td>
<td>35</td>
<td>3</td>
<td>10</td>
<td>1</td>
<td>49</td>
<td>25%</td>
</tr>
</tbody>
</table>
On the other hand, when Thy'Met' or Lac'Met' markers were selected, only about half of the conjugants were Ilv' and the majority group carried the Ilv'Ts' markers of the female parent. The recombination frequency between ilv and the Ts marker is lower (20%) in these conjugants. These observations are very similar to those made with strain CRT83 and suggest that strain CRT46 as well as CRT83 carries a recombination-defect.

The experiments described in Table 16 show two other examples of abnormal recombination gradients. Strain 1638T33 (a recombinant strain carrying the Ts' mutation of CRT83) was mated with two Hfr strains, F4X and Hayes Hfr H72, and Thr'Leu'Str R recombinants were selected in each case. Conjugation of 1638T33 with F4X gives similar results to those observed with CRT83 and CRT46. In the conjugation between 1638T33 and H72, the selected thr' and leu' markers are located close to the origin and are transferred early, while the ilv' marker is located near the tail end. Still, however, the great majority of the conjugants are Ilv'. This observation confirms those made above and suggests that viable recombinants can be obtained only when the region of the chromosome adjacent to ilv is transferred to the recipient strain from the Hfr strain.

Recombinant frequencies

Many experiments done with strains CRT83 and CRT46 and their derivative strains during the course of these studies,
TABLE 17a  Recombinant frequencies in matings between F^- strains 1638 and 1638TS33 and Hfr strains P4X and TS27

<table>
<thead>
<tr>
<th>Female strain</th>
<th>Hfr strain</th>
<th>P4X</th>
<th>TS27</th>
</tr>
</thead>
<tbody>
<tr>
<td>1638</td>
<td></td>
<td>4 x 10^{-3}</td>
<td>2.6 x 10^{-4}</td>
</tr>
<tr>
<td>1638TS33</td>
<td></td>
<td>1.4 x 10^{-5}</td>
<td>0 (&lt; 1 x 10^{-3})</td>
</tr>
</tbody>
</table>

(The frequencies are the numbers of Thr^+Leu^+Str^R conjugants per female cell added to the mating mixture)

TABLE 17b  Analysis of the Thr^+Leu^+Str^R conjugants obtained in the experiment described in Table 17a

<table>
<thead>
<tr>
<th>Parent strains</th>
<th>Total number of Thr^+Leu^+Str^R conjugants tested</th>
<th>Ade^+</th>
<th>Ilv^+</th>
<th>Ts^+</th>
<th>His^+</th>
<th>Trp^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4X x 1638</td>
<td>78</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P4X x 1638TS33</td>
<td>74</td>
<td>36</td>
<td>62</td>
<td>47</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>TS27 x 1638</td>
<td>78</td>
<td>4</td>
<td>0</td>
<td>78</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TS27 x 1638TS33</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
suggested that these strains give low yields of recombinants when used as females in mating experiments. To demonstrate the low frequency of recombination in these strains more quantitatively, the following experiments were performed. Strain 1638 and its derivative strain, 1638T33, were mated with two Hfr strains P4X and Ts27, the latter being a temperature-sensitive recombinant derived from CRI35 and Hfr P4X. Mating conditions were identical and the frequency of Thr<sup>+</sup>Leu<sup>+</sup>Str<sup>R</sup> conjugants obtained in each case was determined. The results of the experiments are shown in Table 17a. Strain 1638 gives Thr<sup>+</sup>Leu<sup>+</sup> recombinants with Hfr P4X at a frequency of about $0.4\%$ of input number of female cells in the mating mixture. The frequency obtained with 1638T33 is, however, nearly 300-times less. Although the temperature-sensitive Hfr strain Ts27 gave Thr<sup>+</sup>Leu<sup>+</sup> conjugants in matings with 1638 at a considerable frequency, it did not give any conjugants with strain 1638T33. The absence of recombinants in matings between these two strains shows that both carry a mutation leading to a defect in recombination and that recombinants cannot be obtained unless at least one of the parents carries the wild-type marker.

Some of the recombinants obtained in the above experiments were scored for the presence of the male markers, wmr<sup>+</sup>, ily<sup>+</sup>, Te<sup>+</sup> and try<sup>+</sup>. The results of such an analysis are given in Table 17b. The recombinants obtained with Hfr P4X x 1638 and Ts27 x 1638 showed
### TABLE 18

Recombination frequencies of some of the conjugants obtained from 1638233 x Hfr P4X mating, as determined by remating with strain Hfr P4X

<table>
<thead>
<tr>
<th>Conjugant</th>
<th>Phenotype</th>
<th>yield of Ade&lt;sup&gt;+&lt;/sup&gt; conjugants when remated</th>
<th>No. of conjugants tested for IIv&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Ilv&lt;sup&gt;+&lt;/sup&gt; conjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Ilv&lt;sup&gt;+&lt;/sup&gt;Ts&lt;sup&gt;-&lt;/sup&gt;</td>
<td>$2 \times 10^{-3}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>Ilv&lt;sup&gt;+&lt;/sup&gt;Ts&lt;sup&gt;-&lt;/sup&gt;</td>
<td>$2 \times 10^{-3}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>36</td>
<td>Ilv&lt;sup&gt;+&lt;/sup&gt;Ts&lt;sup&gt;-&lt;/sup&gt;</td>
<td>$2 \times 10^{-3}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>51</td>
<td>Ilv&lt;sup&gt;+&lt;/sup&gt;Ts&lt;sup&gt;-&lt;/sup&gt;</td>
<td>$2 \times 10^{-3}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>65</td>
<td>Ilv&lt;sup&gt;+&lt;/sup&gt;Ts&lt;sup&gt;-&lt;/sup&gt;</td>
<td>$2 \times 10^{-3}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>Ilv&lt;sup&gt;-&lt;/sup&gt;Ts&lt;sup&gt;+&lt;/sup&gt;</td>
<td>$1 \times 10^{-3}$</td>
<td>49</td>
<td>8</td>
</tr>
<tr>
<td>33</td>
<td>Ilv&lt;sup&gt;-&lt;/sup&gt;Ts&lt;sup&gt;+&lt;/sup&gt;</td>
<td>$1 \times 10^{-3}$</td>
<td>49</td>
<td>8</td>
</tr>
<tr>
<td>42</td>
<td>Ilv&lt;sup&gt;-&lt;/sup&gt;Ts&lt;sup&gt;+&lt;/sup&gt;</td>
<td>$4 \times 10^{-3}$</td>
<td>49</td>
<td>16</td>
</tr>
<tr>
<td>60</td>
<td>Ilv&lt;sup&gt;-&lt;/sup&gt;Ts&lt;sup&gt;+&lt;/sup&gt;</td>
<td>$2 \times 10^{-3}$</td>
<td>49</td>
<td>3</td>
</tr>
</tbody>
</table>
very similar normal gradients of recombination marker transfer. However, those obtained with Hfr P4X x 1638T33 gave an abnormal gradient, the great majority of the conjugants being Ilv\textsuperscript{+}. The recombination-deficiency and the abnormal gradient appear to be expressed only when the recipient strain carries the mutation. CRT83, CRT96 and all the derivative strains of these strains described above, show defective recombination. Therefore, there is a possibility that the recombination-deficiency may be a secondary result of the Ts\textsuperscript{−} mutation itself. However, the gradient data suggest that a marker more closely linked with ilv may be involved.

To test whether the recombination defect is a property of all strains carrying the Ts\textsuperscript{−} mutation, the following experiment was done. If the recombination-deficiency is expressed by a mutant gene in the region of the chromosome carrying ilv and the Ts\textsuperscript{−} mutation, many of the recombinants obtained in the 1638T33 x Hfr P4X mating described above should now be no longer recombination-defective. This could be determined by testing the recombination frequency of some of the conjugants. 9 recombinant strains from the 1638T33 x Hfr P4X cross were selected for examination. 5 of them were Ilv\textsuperscript{+}Ts\textsuperscript{−} while the others had the Ilv\textsuperscript{−}Ts\textsuperscript{+} phenotype. All of them also carried pur\textsuperscript{−} (A or D), Str\textsuperscript{R} and tro\textsuperscript{−} markers. They were mated with Hfr P4X under conditions similar to those in the above experiments and Ade\textsuperscript{+}Str\textsuperscript{R} recombinants were selected. Table 18 gives
### TABLE 19  
Recombinant frequencies in matings between 1638 and 1638T33 and Hfr strains P4X and TS27

<table>
<thead>
<tr>
<th>Parent strains</th>
<th>frequency of conjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thr$^+$Leu$^+$Str$^R$</td>
</tr>
<tr>
<td>1638 x P4X</td>
<td>5 x $10^{-3}$</td>
</tr>
<tr>
<td>1638T33 x P4X</td>
<td>1 x $10^{-4}$</td>
</tr>
<tr>
<td>1638 x TS27</td>
<td>3 x $10^{-4}$</td>
</tr>
<tr>
<td>1638T33 x TS27</td>
<td>0 ($&lt; 1 x 10^{-8}$)</td>
</tr>
</tbody>
</table>

(The frequencies are the numbers of conjugants per female cell added to the mating mixture).
the frequencies of recombinants obtained with these strains. All the Ilv$^{+}$Ts$^{-}$ strains/tested and 3 Ilv$^{-}$Ts$^{+}$ strains do not show any recombination-deficiency. The frequency of recombination in these strains is not much different from that of 1638 described above. However, one Ilv$^{-}$Ts$^{+}$ conjugant still shows the recombination-deficiency characteristic of the parent strain. Some of the Ade$^{+}$ conjugants obtained in these crosses were scored for the male ilv$^{+}$ marker. The strain which appears to be still recombination-defective gives an abnormal recombination gradient, while the other strains do not. These observations clearly indicate that the recombination-deficiency is separable from the Ts$^{-}$ and the ilv$^{-}$ mutation, and is presumably due to another mutation close to ilv$^{+}$.

If it is correct to view the low recombination frequencies of such strains as being due to a mutation close to the ilv$^{+}$ marker, the deficiency should be expressed only when proximal markers are selected in conjugation. When ilv$^{+}$ or distal markers are selected, these strains should not show any deficiency in recombination compared to wild-type. A comparison of the recombination-proficiency of strains 1638 and 1638T33, scored by selecting Thr$^{+}$Leu$^{+}$Str$^{R}$ or Ilv$^{+}$Str$^{R}$ recombinants in matings with Hfr P4X or Hfr TS27, is given in Table 19.

The difference in the frequency of Thr$^{+}$Leu$^{+}$ recombinants in crosses involving 1638 and 1638T33, was about 50-fold, while
when Ilv^+ recombinants were selected, the difference was only 8-fold. This result is in qualitative agreement with the hypothesis that the recombination-deficient mutation is located near ilv and that when ilv or distal markers are selected, the recombination-deficiency is not expressed. However, even when Ilv^+ recombinants are selected, the recombinant frequency of strain 1638T38 appears to be less than that of 1638.

One possible explanation for the low recombinant frequency in such strains could be that they degrade the transferred donor DNA at a slow rate. When the wild-type allele of the rec gene is transferred to the recipient the degradation stops. Such an explanation, although it would account for the reduced frequency of recombinants obtained in the above experiment, even when recombinants for ilv are selected, is made unlikely by the fact that recombinants carrying male markers, which are transferred considerably earlier than the rec^+ allele (such as thr^+ and leu^+ in the case of Hfr H72), are obtained with a substantial frequency (Table 17a).

Whether the transfer of donor DNA into the Rec^- strains is normal or not can be studied in experiments involving F' donors since a transferred F' factor can be established in a Rec^- cell (Clark and Margulies, 1965; Wilkins, 1969). If the donor DNA is degraded in Rec^- recipient strains, the frequency of F prime conjugants obtained with these strains may be much less than that
TABLE 20  Conjugant frequencies of strain CRT15 compared with wild-type strains

<table>
<thead>
<tr>
<th>Parent strains</th>
<th>Frequency of conjugants</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{Lac}^+\text{Str}^R$</td>
<td>$\text{Thr}^+\text{Leu}^+\text{Str}^R$</td>
<td></td>
</tr>
<tr>
<td>G600 $\text{thr}^+\text{leu}^+/\text{Flac}^+$ x G600 Str$^R$</td>
<td>$4.0 \times 10^{-2}$</td>
<td>$4.7 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>G600 $\text{thr}^+\text{leu}^+/\text{Flac}^+$ x CRT15 Str$^R$</td>
<td>$4.4 \times 10^{-2}$</td>
<td>$4.5 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>G600/Flac$^+$ x 108</td>
<td>$1.1 \times 10^{-2}$</td>
<td>$1.0 \times 10^{-4}$</td>
<td>$1.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>CRT15/Flac$^+$ x 108</td>
<td>$7.0 \times 10^{-3}$</td>
<td>$5.0 \times 10^{-5}$</td>
<td>$1.8 \times 10^{-6}$</td>
</tr>
</tbody>
</table>
obtained with wild-type strains. The following experiment was
performed in order to investigate this possibility. Strain
C600 thr-leu-Flac was mated with C600 Str and CRT15 Str strains
and the frequency of Lac+Str and Thr+Leu+Str conjugants was scored.
Similarly, strain C600/Flac and CRT15/Flac strains were mated
with T08 and Lac+Str, Pro+Str and Met+Str conjugants were scored
in each case. The results of such an experiment are given in Table
20. Strain CRT15 is a recombinant strain of CRT83 and it shows
the Rec- property characteristic of the parent strain. CRT15 Str is
a streptomycin-resistant derivative of this strain. All the
other strains used in the experiment are recombinant-proficient.
It is seen from the results given in Table 20 that when CRT15 Str
was the recipient strain, the frequency of Lac+ conjugants was very
similar to that obtained with C600 Str. However, when Thr-Leu+
recombinants were selected, the frequency obtained with this Rec-
strain was about 10-fold less than that obtained with C600 Str.
This indicates clearly that the transfer of DNA into the recombination-
defective strains is normal. It does not, however, rule out the
possibility of breakdown of donor DNA by the recipient, since it is
possible that F prime DNA is protected from degradation (by
immediate circularization perhaps), while chromosome DNA is not.

When strain CRT15/Flac was the donor, the frequency of
Flac\(^+\) transfer was comparable to that from C600/Flac\(^+\), whereas the frequency of mat\(^+\) transfer was reduced more than that from C600/Flac\(^+\). However, there was only a slight reduction in pro\(^+\) transfer when the donor strain was Rec\(^-\). It appears that the recombination-deficiency of these strains has little effect on the frequency of integration of the Flac\(^+\) particle into the chromosome. An independent piece of evidence for this is that the increase in reversion frequency of temperature-sensitive strains to temperature-resistance observed with Flac\(^+\) is similar in CRT83, CRT46, CRT15 and in A5, which does not show any recombination-deficiency. Since the increase in reversion frequency is partly due to the integration of Flac\(^+\) into the chromosome, it would be expected that the Rec\(^-\) strains would have low frequencies of integration and therefore low frequencies of reversion. Apparently normal frequencies of integration of the F primes into the chromosome in these Rec\(^-\) strains gives more weight to the argument that their recombination-deficiency may be due to degradation of transferred DNA while other observations make it a less likely explanation. A definite conclusion about this cannot be achieved without further experiments.

Sensitivity of the recombination-deficient strains to uv-irradiation and treatment with MMS

Recombination-defective mutants of E. coli are abnormally
sensitive to ultraviolet light, ionizing radiations and alkylating agents such as EMS (Clark and Margulies, 1965; Howard-Flanders, Boyce and Theriot, 1966; Howard-Flanders and Boyce, 1966). These agents are known to induce several forms of damage in DNA including dimerization of adjacent pyrimidine bases (Beukers, Ijlstra and Berends, 1960), single strand breaks (Freifelder, 1966; McGrath and Williams, 1966) and alkylation of the bases (Brookes and Lawley, 1961). Wild-type cells can efficiently repair these forms of damage. Although several genes are clearly involved, functions of the rec genes play an important part in these repair processes (Howard-Flanders et al., 1963). All known recombination-deficient mutants are more sensitive to uv than wild-type strains. Strains mutant at recA are very sensitive whereas those mutant at recB or recC have intermediate sensitivity (Willets and Mount, 1969).

The sensitivity of strains CRT83 and CRT46 to uv irradiation was examined in parallel with 3 other strains: A3, a temperature-sensitive (thy^+ ilv^+) recombinant of CRT46 which does not show the Rec^- property, 231 which is known to carry a mutation at recA and CS00 as the parental control. The results of such an experiment are given in Figure 19. The strains CRT83, CRT46 and A3 appear to be more sensitive to uv than CS00. However, their sensitivity is much less than that of the recA^- strain. Their sensitivity appears to be less than that of recB and recC mutants.
Figure 19  uv-sensitivity of the Ts strains

Overnight-grown cultures of the bacterial strains were diluted with phosphate buffer to an OD$_{450}$ of 0.3 and exposed to uv in glass petri dishes on a rotary shaker. Samples from the cultures were removed after each dose, diluted with phosphate buffer and plated on nutrient agar plates for viable counts. The plates were incubated at $30^\circ$C.
Willets and Mount, 1959). CRT83 and CRT46 are apparently no more sensitive to uv than the recombination-proficient strain A3. Thus, the recombination-deficiency of CRT83 and CRT46 does not appear to make these strains sensitive to uv.

Figure 20 shows the sensitivity of the 4 strains to MMS. While A3 is no more sensitive to MMS than C600, strain CRT83 and CRT46 are much more sensitive. Their sensitivity approaches that of the recA– strain. Thus, the recombination-deficiency of CRT83 and CRT46 is apparently associated with sensitivity of these strains to MMS.

Location of the rec gene

Since many experiments indicated that the rec marker is located close to ilv, an attempt was made to map it by phage P1 transduction. The process of transduction involves recombination and therefore transduction frequencies obtained with Rec– recipient strains can be expected to be low. However, in preliminary experiments, transductants could not be obtained if the Rec– strains were used as donors, while apparently normal transduction frequencies were obtained for the markers selected in the following experiment if wild-type strains were used as donors and Rec– strains were used as recipients. The bgl marker, which determines the ability of
Overnight-grown cultures of the bacterial strains were diluted into MMS/phosphate buffer mixture to give a final concentration of about 10^7 cells/ml and 0.05 M MMS. At given times, samples were removed, diluted with phosphate buffer and spread on nutrient agar plates. The plates were incubated at 30°C for 2 days before counting the colonies.
E. coli cells to ferment β-glucosides such as arbutin and salicin, is also located near ilv (Schaefler and Maas, 1967). Strains of E. coli K-12 are Bgl but spontaneous revertant strains which are Bgl\(^+\) can be isolated easily. Both Ilv\(^+\) and Bgl\(^+\) transductants of strain 1638T33 were used to map the Rec and Ts markers.

A strain of C600 carrying Bgl\(^+\) was isolated by spreading samples from a culture of C600 on AB sal medium and the yellow colonies which appeared on these plates were purified by restreaking on AarbSal medium. One such mutant was used as the donor for transduction of strain 1638T33. Ilv\(^+\) and Bgl\(^+\) transductants were selected independently on appropriate plates incubated at 30\(^{\circ}\)C. Transductants appeared at a frequency of about 7.5 x 10\(^{-7}\) per recipient cell, with either selection. 100 Bgl\(^+\) transductants and 100 Ilv\(^+\) transductants were scored for temperature-sensitivity and recombination-deficiency as well as for the Ilv and Bgl phenotype. Recombination-deficiency was tested by mating transductants with Hfr P4X and scoring the frequency of Thr\(^+\)Leu\(^+\)Str\(^R\) recombinants. Nutrient broth-grown cultures of transductants and P4X were mixed in equal volumes and incubated at 30\(^{\circ}\)C for 30 minutes when 0.03 ml of each mating mixture was spotted on plates selecting for Thr\(^+\)Leu\(^+\)Str\(^R\) recombinants. After incubation of the plates at 30\(^{\circ}\)C, the colonies which appeared in the spots were counted. Some of the transductants were clearly identifiable as Rec\(^+\). They gave abundant colonies (> 100 per spot), while others were clearly Rec\(^-\) (< 10 colonies per spot).
### TABLE 21a  Analysis of Ilv$^+$ and Bgl$^+$ transductants

<table>
<thead>
<tr>
<th>Selected marker</th>
<th>unselected markers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ilv$^+$</td>
</tr>
<tr>
<td>ilv$^+$</td>
<td>-</td>
</tr>
<tr>
<td>bgl$^+$</td>
<td>43</td>
</tr>
</tbody>
</table>

### TABLE 21b  Cotransduction frequencies of the bgl ilv and rec markers

<table>
<thead>
<tr>
<th>Selection for Ilv$^+$ transductants</th>
<th>Selection for Bgl$^+$ transductants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bgl$^+$ Rec$^+$</td>
<td>Ilv$^+$ Rec$^+$</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Bgl$^+$ Rec$^-$</td>
<td>Ilv$^+$ Rec$^-$</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Bgl$^-$ Rec$^+$</td>
<td>Ilv$^-$ Rec$^+$</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Bgl$^-$ Rec$^-$</td>
<td>Ilv$^-$ Rec$^-$</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[\begin{array}{ccc}
\text{bgl} & \text{rec} & \text{ilv} \\
+ & + & + \\
- & - & - \\
\end{array}\]

\[\begin{array}{ccc}
\text{rec} & \text{bgl} & \text{ilv} \\
+ & + & + \\
- & - & - \\
\end{array}\]

\[\begin{array}{ccc}
\text{bgl} & \text{ilv} & \text{rec} \\
+ & + & + \\
- & - & - \\
\end{array}\]

I  II  III
TABLE 21c  cotransduction frequencies of \textit{ilv bgl} and the Ts marker

<table>
<thead>
<tr>
<th>Selection for \textit{Ilv}^+ transductants</th>
<th>Selection for \textit{Bgl}^+ transductants</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Bgl}^+ Ts^+</td>
<td>\textit{Ilv}^+ Ts^+</td>
</tr>
<tr>
<td>67</td>
<td>21</td>
</tr>
<tr>
<td>\textit{Bgl}^+ Ts^-</td>
<td>\textit{Ilv}^+ Ts^-</td>
</tr>
<tr>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>\textit{Bgl}^- Ts^+</td>
<td>\textit{Ilv}^- Ts^+</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>\textit{Bgl}^- Ts^-</td>
<td>\textit{Ilv}^- Ts^-</td>
</tr>
<tr>
<td>21</td>
<td>53</td>
</tr>
</tbody>
</table>

\begin{tabular}{ccc}
Ts & bgl & ilv \\
+ & + & + \\
- & - & - \\
\end{tabular} 

\begin{tabular}{ccc}
bgl & Ts & ilv \\
+ & + & + \\
- & - & - \\
\end{tabular} 

\begin{tabular}{ccc}
bgl & ilv & Ts \\
+ & + & + \\
- & - & - \\
\end{tabular}
A few transductants, however, gave ambiguous results.

An analysis of the transductants is given in Tables 21a, 21b, and 21c. Cotransduction frequencies between bgl, ilv and rec are given in Table 21b. On the assumption that the least frequent class of transductants resulted from multiple cross over events between the transduced DNA and the recipient chromosome, the order of markers which agrees most with the data in Table 21b is rec bgl ilv (Sketch II). The position of bgl is known to be to the left of ilv in the E. coli chromosome map (Taylor, 1970). Therefore, the rec marker is also located towards the left of ilv.

The data in Table 21c for the cotransduction frequencies between ilv bgl and the Ts marker, agree with both the orders Ts bgl ilv and bgl ilv Ts. Since the Ts marker has been mapped to the left of ilv by several other experiments (Hirota et al., 1970), the correct order is probably Ts bgl ilv.

Discussion

The low frequency of Thr+Leu+ recombinants and the normal yields of F' conjugants obtained from CRT35, CRT46 and 1638T33 in mating experiments, indicate that these strains are defective in one of the processes necessary for recombination. However, when
recombinants carrying ilv\textsuperscript{+} instead of thr\textsuperscript{+}leu\textsuperscript{+} are selected the
deficiency of recombinants is much less marked compared to the
wild-type strains. These observations tend to indicate that
transfer of donor DNA into these strains takes place as in wild-type
strains but the recombination process between the donor DNA and
recipient chromosome is defective. This hypothesis agrees with
the abnormal recombination gradients observed with these strains.
When any donor marker proximal to ilv is selected, the great
majority of recombinants inherit the ilv\textsuperscript{+} marker of the donor.
However, when a marker distal of ilv is selected, no abnormality
in the gradient is observed and only a proportion of recombinants
contain the male ilv\textsuperscript{+} marker. The gradient data strongly suggest
that the Rec\textsuperscript{-} mutation is located close to ilv and normal recombination
in these strains is possible only when the corresponding wild-type
marker is transferred from a donor strain. This position of the
rec gene was confirmed by transduction experiments. Ilv\textsuperscript{+} or Bgl\textsuperscript{+}
transductants of the Rec\textsuperscript{-} strains could be obtained, which are no
longer recombination-defective. According to the cotransduction
frequencies, the Rec\textsuperscript{-} mutation is located most probably towards the
left of ilv marker in the E. coli chromosome map.

The abnormally high proportion of Ilv\textsuperscript{+} recombinants
obtained in conjugation experiments involving these strains cannot,
however, be easily explained. There is associated with this
phenomenon, a high recombination frequency between ilv and the Ts\textsuperscript{-}
marker, although they are closely linked. If normal recombinations take place between donor and recipient DNA when the rec\(^+\) (and ilv\(^+\)) marker is transferred to the recipient, there is an equal probability that the product of recombination inherits or does not inherit the ilv\(^+\) marker. The fact that too many recombinants carry ilv\(^+\) is not likely to be due to any selective advantage of the Rec\(^+\) or Ilv\(^+\) recombinants in subsequent growth, because this abnormality is observed only when a donor marker proximal to ilv is selected. It is likely that when a proximal marker is selected a rapid recombination event takes place immediately after the entry of the rec\(^+\) gene from the donor, and this process almost invariably involves a cross over between the terminus of the already transferred DNA and the host chromosome.

The frequencies of Lac\(^+\) conjugants obtained with Rec\(^-\) strains mated with Flac\(^+\) donor strains is similar to those obtained with Rec\(^+\) strains, indicating that transfer of donor DNA into these strains is not defective. Whether or not the donor DNA is degraded after entry into recipient cells cannot be concluded without further experiments. The apparently normal frequencies of Flac\(^+\) conjugants suggests that there is no loss of donor DNA. But it can be argued that Flac\(^+\) DNA may be protected from degradation.

The integration of Flac\(^+\) or F\(^+\) particles into the chromosome is apparently not affected by the recombination defect. Similarly, the recombination between transduced DNA and the host DNA is not
defective. The reason for these observations cannot be easily understood and they tend to indicate that the recombination process itself may not be defective and apparent recombination defects may be due to degradation of donor DNA.

It is remarkable that the Rec^- strains are very sensitive to MMS but not to uv-irradiation. These strains behave quite similarly to strain A3 (which is temperature-sensitive but not recombination-defective) in uv irradiation experiments, but quite differently from it in MMS-treatment experiments. Their MMS-sensitivity approaches that of a known recA^- strain and indicates that they are defective in one or more stages of DNA repair not involved in the repair of uv-damaged DNA. These mutants differ from recA, recB or recC mutants with regard to their uv and MMS-sensitivity and the map position of the mutations. Recombination-deficient mutants similar to these have not been reported before.
BIBLIOGRAPHY


Properties of Male Derivatives of *Escherichia coli* Mutants Defective in Initiation of Chromosome Replication. By H. G. Nandadasa (Department of Genetics, University of Leicester)

On the assumption that replication of plasmids is under negative cytoplasmic control, temperature-sensitive mutants of an *F lac* particle having uncontrolled replication were looked for. It was anticipated that such mutants would be lethal to the host at 42° but give a high frequency of *lac*~ revertants due to loss of the *F* particle. Although unstable temperature-sensitivities were found, none of them were of this type. In one class, on the contrary, the reversion frequency was *enhanced* by the presence of the *F* particle. These mutants proved to have a chromosomal lesion similar to those classified by others as defective in initiation of chromosome replication.

Known initiation-defective mutants (CRT 83 and CRT 46) also show an enhanced reversion frequency when infected by *F* and some *F* prime particles.

On the basis of one model (Pritchard, Barth & Collins (1969), *Symposia of the Society for General Microbiology*, no. xix, *Microbial Growth*, pp. 263-297), for the control of replication of plasmids it would be predicted that insertion of a plasmid into a host chromosome carrying a lesion preventing initiation of a cycle of chromosome replication might suppress that lesion. Evidence will be presented that the enhanced reversion frequency of such mutants carrying *F* is due, at least in part, to such suppression by insertion.
Studies on the control of DNA replication in Escherichia coli

On the assumption that the replication of plasmids is under negative control, an attempt was made to isolate temperature-sensitive mutants of an Flac\(^+\) factor having uncontrolled replication. It was anticipated that such mutants would be lethal to the host at 42\(^\circ\)C but give a high frequency of Lac\(^-\) revertants due to loss of the F particle. Although several temperature-sensitive mutants were found, none were of this type. In some of the isolated mutants, the reversion frequency was enhanced by the presence of the F particle. On detailed study, one of these mutants proved to have a chromosomal lesion similar to those classified as defective in the initiation of chromosome replication.

Known initiation-defective mutants (CRT83 and CRT46) also show an enhanced reversion frequency when infected with F or some F prime factors. In many of the revertants isolated from F\(^+\) and Flac\(^+\) derivatives of CRT83, the F factor is integrated into the chromosome. Furthermore, such revertants still carry the temperature-sensitive mutation, showing that the reversion is by suppression. It is supposed that in CRT83 and CRT46, the F replication is not temperature-sensitive and when the F particle is integrated into the chromosome the whole genome can replicate at non-permissive temperature under the control of the F replication
However, the integration of the F particle into the chromosome alone is apparently not sufficient for the suppression of temperature-sensitivity.

Studies of DNA synthesis of F', Flac' and Hfr derivatives of CRT46 and CRT83 showed that the temperature-sensitive defect of these mutants can be complemented partially by the F factor.

Several mating experiments involving CRT83 and CRT46 showed that these mutants carry an additional mutation, mapping near ilv which renders the cells partially defective in recombinant production.